SPANDIDOS PUBLICATIONS

EXPERIMENTAL AND THERAPEUTIC MEDICINE 14: 495-498, 2017

miR-16-1 expression, heat shock protein 70 and inflammatory reactions in astrocytes of mice with epilepsy induced by encephalitis B virus infection

YUE YAO, YUJIA YANG, XUEHUA HE and XIA WANG

Department of Pediatrics, Xiangya Hospital, Central South University, Changsha, Hunan 410008, P.R. China

Received September 6, 2016; Accepted January 16, 2017

DOI: 10.3892/etm.2017.4513

Abstract. The upregulation of miR-16-1 expression and heat shock protein 70 (HSP70) and inflammatory reaction mechanism in astrocytes of mice with epilepsy induced by encephalitis B virus infection were studied. Six-to-eight-week-old healthy male C57BL/6 mice received intraperitoneal injection of pilocarpine (320-340 mg/kg, 40 mg/ml) to induce status epilepsy. After 7 days, mice were inoculated with 100 μl Dulbecco's modified Eagle's medium (DMEM) in the neck, including 6.25x23 PFU Japanese encephalitis virus P3 wild strain. The experiment was divided into 4 groups, including, the healthy control group, the epilepsy model group, the model group + negative inoculation group and the virus infection group with 10 mice in each group. The healthy control group received intraperitoneal injection of the same amount of normal saline; the model group + negative inoculation group was injected with the same amount of DMEM without P3. One and three days after infection, 5 mice from each group were sacrificed, hippocampus tissues were obtained and astrocytes were isolated. After purification, glial fibrillary acidic protein was identified by immunohistochemical staining. Infected glial cells were detected by P3 antigen of immunofluorescence staining. RT-PCR method was used to detect the expression of miR-16-1 mRNA in astrocytes. Western blot analysis was used to detect the expression of HSP70. ELISA method was used to detect the levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α and nuclear factor-κB (NF-κB) inflammatory factors in tail vein blood. Level of expression of miR-16-1 mRNA, HSP70 as well as IL-6, TNF-α and NF-κB inflammatory factor levels of virus infected mice of 1 and 3 days were significantly higher ($P<0.05$) than those of model group and negative inoculation group and lowest in control group. In conclusion, the level of expression of miR-16-1 and HSP70 can be increased by the infection of Japanese encephalitis virus on the astrocytes of mice with epilepsy, to promote the expression of IL-6, TNF-α and NF-κB of inflammatory factors.

Correspondence to: Dr Xia Wang, Department of Pediatrics, Xiangya Hospital, Central South University, 87 Xiangya Road, Changsha, Hunan 410008, P.R. China
E-mail: slyg035838645@163.com

Key words: epilepsy mice, astrocytes, Japanese encephalitis virus, miR-16-1, heat shock protein 70, inflammatory response

Introduction

Epilepsy in children is mainly related to unsound central nervous system development and the infection of the central nervous system leading to epilepsy incidence rate of ~10-50%, with generalized tonic clonic seizures and partial seizures mainly (1). Chinese epidemic encephalitis B virus infection is common, at the acute phase of the disease virus replication releasing toxins, inflammatory disorders, thrombosis that affects nerve cell membrane stability, and then becomes the main cause of epilepsy. In the recovery period, the remaining cortical scar and meninges become the important condition for the formation of epilepsy (2). The main features of the occurrence of epilepsy are neuronal excitability and synchronized discharge, has been found that (3) astrocytes play an important role in the occurrence and transmission of epilepsy. It may become a new target for the intervention of epilepsy. Astrocytes participate in the expression and activation of a variety of miRNAs and inflammatory factors, and the high expression of miR-16-1 is closely related to the occurrence of epilepsy (4). Heat shock protein 70 (HSP70) had abnormal expression in the period of epilepsy and the intermittent period (5). The aim of this study was to analyze the upregulation of miR-16-1 expression and HSP70 expression and inflammatory response mechanism in astrocytes of mice with epilepsy induced by encephalitis B virus infection.

Materials and methods

Experimental animals. Selected 6-8-week-old healthy male C57BL/6 mice, with body weight of 20-25 g, purchased from Shanghai Biological Engineering Animal Center, with normal feeding, after adapted for 1 week, were obtained for the experiments.

Epilepsy model: Intraperitoneal injections of scopolamine methyl nitrate (1 mg/kg, concentration 0.2 mg/ml) was administered, after 30 min intraperitoneal injection of pilocarpine products (320-340 mg/kg, 40 mg/ml) to induce epilepsy persistent state. After 2 h intraperitoneal injection of diazepam

(7.5 mg/kg, the concentration of 2.5 mg/ml), then the seizures were determinated. Routine feeding was continued.

Japanese encephalitis virus infection model: After 7 days mice were inoculated with 100 μl Dulbecco's modified Eagle's medium (DMEM) in the neck, including 6.25x.23 PFU Japanese encephalitis virus P3 wild strain. Great attention is required in handing the P3 wild strain. In P2 lab operation, the operation personnel had a strong protective consciousness, and took vaccine for protection.

Experimental grouping. The experiment was divided into 4 groups, namely, the healthy control group, the epilepsy model group, the model group + negative inoculation group and the virus infection group with 10 mice in each group. The healthy control group received intraperitoneal injection of the same amount of normal saline; the model group + negative inoculation group was injected with the same amount of DMEM without P3. One and three days after infection: Five mice from each group were sacrificed, hippocampus tissues were obtained and astrocytes were isolated. After purification, glial fibrillary acidic protein (GFAP) was identified by immunohistochemical staining. Infected glial cells were detected by P3 antigen of immunofluorescence staining. RT-PCR method was used to detect the expression of miR-16-1 mRNA in glial cells. Western blot analysis method was used to detect the expression of HSP70. Enzyme-linked immunosorbent assay (ELISA) method was used to detect the levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α and nuclear factor-κB (NF-κB) inflammatory factors in tail vein blood.

Research method. Preparation of slices: Mice were injected with 10% hydrate (5 ml/kg) for anesthesia, the chest was exposed; heart perfusion was by physiological saline. Then 4% polyformaldehyde was used for perfusion. The brain was taken the tissue was placed in 4% formalin fixed overnight, with phosphate-buffered saline (PBS) washing, 30% sucrose solution soaking, embeded, fixed, slicing the midbrain containing the hippocampus, at the thickness of 8 μ, and stored at -80˚C.

Identification of astrocytes: Sections after dewaxing were put into water; hydrogen peroxide solution inactivated endogenous peroxidase for antigen repairing. Sheep serum blocked the antigen. Rabbit monoclonal GFAP antibody (dilution, 1:500; cat. no. SAB2702474; Sigma-Aldrich, St. Louis, MO, USA) were used at 4˚C overnight. Followed by rewarming, PBS washing for 10 min x3 times. Secondary goat anti-rabbit (HRP) IgG antibody (dilution, 1:2,000; cat. no. ab6721; Abcam, Cambridge, MA, USA) were used at 27˚C incubation for 1 h. PBS washing for 10 min x3 times, placed in the immune group in the wet box (Invitrogen, Carlsbad, CA, USA) for the reaction for 1 h. PBS washing for 10 min x3 times, DAB coloration and hematoxylin staining were applied, gradient ethanol dehydration with xylene, neutral resin was used for sealing, and photographs were taken under a microscope.

Immunofluorescence staining: The foregoing steps were the same as immunohistochemical staining, adding first anti-mouse anti-Japanese encephalitis virus antigen monoclonal antibody (working concentration 1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight. Second antibody was sheep anti-mouse IgG (H+L, working concentration of 1:200; R&D Systems, Minneapolis, MN, USA), at 27˚C incubation for 1 h; with DAPI (working concentration of 1:500; Beijing Zhong Shan Jinqiao Biological Co., Ltd.). Light staining for 10 min, in the dark at 25˚C. After mounting, fluorescence microscope (Olympus, Tokyo, Japan) was used for observation.

RT-PCR method: RNA was extracted by conventional TRIzol method, the concentration and purity of RNA was determined by UV spectrophotometry, and the synthesis of cDNA was synthesized by reverse transcription kit. The primers were designed and synthesized by Takara Bio, Inc. (Otsu, Japan): miR-16-1 forward, 5'-GCCGTAGCAGCACGTA AATAA-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'; internal reference GAPDH forward, 5'-TGCTTCACCACCTT CTTGA-3' and reverse, 5'-TCACCATCTTCCAGGAGC-3'. Reaction system was 5X buffer 2.5 μl + $MgCl_2$ 1.5 μl + dNTP 0.5 μl + GAP-43 and the internal upstream and downstream primers 1 μl each + Taq enzyme 0.3 μl + cDNA template 2 μl, adding water to the total volume. The reaction condition was 95˚C for 5 min, 95˚C for 30 sec, 62˚C for 30 sec, 72˚C for 30 sec, total 35 cycles at 72˚C for 10 min. Dissolution curves were constructed, and the relative expression of mRNA was calculated by $2^{-\Delta\Delta Cq}$ method.

Western blot analysis: RIPA cracking liquid was used to treat the cells, on the ice reacting for 30 min, every 5 min violent shock for 1 min, samples were centrifuged at 4˚C, at 2,000 x g for 20 min, the supernatant was the sample of the whole protein. Determination of protein concentration by Bradford method (Invitrogen). SDS-PAGE gel electrophoresis (concentrated gel, 50 V; separation gel, 100 V; electrophoresis time, 3 h), PVDF transfer film, add 5% skim milk powder, at 25˚C incubation for 4 h. Diluted sheep anti-mouse HSP70 first antibody (1:2,500) was added (internal reference β-actin 1:500) for incubation at 37˚C for 1 h. TBST washing for 5 min x3 times, and donkey anti-sheep second antibody (1:2,000) was added for incubation at 37˚C for 1 h. TBST washing for 5 min x3 times, then ECL coloring, and scanning. Image analysis software IPP 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was employed for gray analysis.

ELISA method: blood sample of 3 ml from the tail vein was collected, and the upper serum was collected after centrifugation at 2,000 x g for 15 min, stored at -20˚C. Kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and carried out in strict accordance with the manual instructions.

Statistical analysis. Statistical analysis was performed with SPSS 20.0 software (IBM, Armonk, NY, USA) and measurement data were expressed as mean ± standard deviation (SD). Single factor ANOVA analysis was used to compare among groups. Pairwise comparison was tested by LSD-t method, and paired t-test was used in the intergroup comparison. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of expression levels of miR-16-1 mRNA. miR-16-1 mRNA expression levels of virus infected mice of 1 and 3 days were significantly higher ($P<0.05$) than those of the model group and negative inoculation group and lowest in control group (Table I).

Table I. Comparison of miR-16-1 mRNA expression levels.

Group	Control group	Model group	Negative inoculation group	Viral infection group	F-value	P-value
1 day	0.0634±0.0058	0.3527±0.0548	0.3426±0.0847	0.5689±0.1234	15.632	<0.001
3 days	0.0589±0.0063	0.3545±0.0649	0.3521±0.0638	0.6458±0.1326	24.531	<0.001
t-test	0.048	0.063	0.084	4.526		
P-value	0.926	0.869	0.823	0.032		

Table II. Comparison of HSP70 expression levels.

Group	Control group	Model group	Negative inoculation group	Viral infection group	F-value	P-value
1 day	0.12±0.05	0.34±0.12	0.35±0.13	0.67±0.18	12.306	<0.001
3 days	0.13±0.07	0.33±0.14	0.36±0.15	0.82±0.21	18.624	<0.001
t-test	0.123	0.152	0.186	4.628		
P-value	0.765	0.723	0.659	0.028		

HSP70, heat shock protein 70.

Table III. Comparison of levels of inflammatory cytokines of IL-6, TNF-α and NF-κB (μg/ml).

Group	Control group	Model group	Negative inoculation group	Viral infection group	F-value	P-value
IL-6						
1 day	32.5±12.3	65.7±21.4	63.5±18.6	215.7±45.6	15.624	<0.001
3 days	33.6±13.4	67.2±25.6	65.6±21.3	342.6±62.7	23.526	<0.001
TNF-α						
1 day	12.6±5.6	35.6±13.2	34.8±12.4	89.5±21.3	12.548	<0.001
3 days	12.8±5.7	39.8±13.5	37.2±11.6	123.4±35.4	25.619	<0.001
NF-κB						
1 day	6.9±2.3	24.5±10.2	25.5±8.9	56.7±16.5	13.628	<0.001
3 days	6.8±2.5	26.7±11.4	27.3±9.2	78.5±20.3	19.852	<0.001

IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB.

Comparison of HSP70 expression levels. HSP70 expression level of virus infected mice of 1 and 3 days were significantly higher than those of model group and negative inoculation group and lowest in the control group (P<0.05) (Table II).

Comparison of levels of inflammatory cytokines of IL-6, TNF-α and NF-κB. IL-6, TNF-α and NF-κB inflammatory factor levels of virus infected mice of 1 and 3 days were significantly higher (P<0.05) than those of model group and negative inoculation group and lowest in the control group (Table III).

Discussion

Astrocytes play a supporting role in separation of neurons and insulation in the central nervous system. They regulate the intracellular and extracellular ion concentration and maintain the stability of the internal environment, through the cytoplasm and membrane receptor, neural activity amino acid affinity carrier, enzymes involved in the neurotransmitter uptake, inactivation and supply (6). Astrocytes play an important role in the immune response and inflammatory response of epilepsy (7). TNF-α in the central nervous system is mainly composed of astrocytes, microglia and neurons, through the presence of a wide range of TNF-α receptors in the brain to participate in the regulation of neuroimmune endocrine network (8).

Japanese encephalitis is a common disease in humans and animals, targeting the central nervous system, causing inflammation of the central nervous system, which is the main factor leading to the death of humans and animals by Japanese encephalitis virus (9). Astrocytic activation and inflammatory factor release is an important mechanism of Japanese encephalitis virus (10). *In vitro* cultured glial cells were transfected with the virus, the virus titer was not high, and affected the detection of inflammatory markers (11). This investigation of *in vivo* epileptic mice infected with Japanese

encephalitis virus are more suitable for the pathological state of the disease and also detected higher the rate of viral infection of glial cells. The innovation in our study was to analyze the pathogenesis of the infection of the mouse astrocytes infected with B encephalitis virus. The results show the levels of expression of miR-16-1 mRNA, HSP70 as well as IL-6, TNF-α and NF-κB inflammatory factor of virus infected mice of 1 and 3 days were significantly higher than those of the model group and negative inoculation group and lowest in the control group. The differences were statistically significant. Moreover, there was no difference of the indexes of the model group and the negative inoculation group of 1 and 3 days, while on day 3 the virus infection group was significantly higher than that on day 1. It suggests that altogether miR-16-1, HSP70 and IL-6, TNF-α and NF-κB inflammatory factors play an important role in simple seizures and epilepsy JE virus infection. Japanese encephalitis virus can further affect the disease progression through regulating the expression of these indicators.

miR-16-1 as a tumor suppressor gene is studied mainly in lung cancer, glioma, lymphoma and other malignant tumors (12). Using miRNA microarray technology to screen the epilepsy mouse model showed that the level of miR-16-1 was increased up to 3-5 times (13). The serum miR-16-1 mRNA level in epileptic patients were significantly higher than that in healthy persons by RT-PCR method, and that of the period of epilepsy was significantly longer than that of intermission (14). Anti-epileptic drugs can significantly reduce the levels, and that is related to the treatment effect and prognosis (15). miR-16-1 levels in cerebrospinal fluid and serum of patients with herpes simplex encephalitis were significantly increased, and were closely related to the severity of the disease (16). Therefore, the level of miR-16-1 has important applications in epilepsy and Japanese encephalitis. HSP70, as a molecular chaperone, plays an important role in protein damage and repair (17). In normal brain tissue there was slight HSP70 expression, in the systemic administration of kainic acid induced status epilepticus in brain tissue HSP70 expression significantly increased. HSP70 has a certain correlation with epilepsy formation caused by neuronal stress injury, the HSP70 produced can be used as a sensitive and reliable marker of neuronal damage (18). Further study found that HSP70 was involved in the occurrence of epilepsy and that was closely linked to the release of a variety of inflammatory substances such as IL-6, TNF-α and NFκB (19).

In conclusion, the level of expression of miR-16-1 and HSP70 can be increased by the infection of Japanese encephalitis virus on the astrocytes of mice with epilepsy, to promote the expression of IL-6, TNF-α and NF-κB of inflammatory factors, and to intervene in the generation of astrocytes and miR-16-1, HSP70 and related inflammatory substances. This may play a role in simple seizures and epilepsy caused by Japanese encephalitis virus infection.

Acknowledgements

This study was supported by grants from the NSFC 30672240 and 30801246.

References

1. Mirás Veiga A, Moreno DC, Menéndez AI, Siscart IM, Fernández MD, Sánchez EG, González MG and Sáez FG: Effectiveness of electroconvulsive therapy for refractory status epilepticus in febrile infection-related epilepsy syndrome. Neuropediatrics: Jul 19, 2016 (Epub ahead of print).
2. Misra UK, Tan CT and Kalita J: Viral encephalitis and epilepsy. Epilepsia 49: 13-18, 2008.
3. Kielbinski M, Gzielo-Jurek K and Soltys Z: Roles for astrocytes in epilepsy: insights from malformations of cortical development. Neuropathol Appl Neurobiol 3: 1123-1124, 2016.
4. Li Y, Wang J, Jiang C, Zheng G, Lu X and Guo H: Association of the genetic polymorphisms in pre-microRNAs with risk of childhood epilepsy in a Chinese population. Seizure 40: 21-26, 2016.
5. Kandratavicius L, Hallak JE, Carlotti CG Jr, Assirati JA Jr and Leite JP: Hippocampal expression of heat shock proteins in mesial temporal lobe epilepsy with psychiatric comorbidities and their relation to seizure outcome. Epilepsia 55: 1834-1843, 2014.
6. Hubbard JA, Szu JI, Yonan JM and Binder DK: Regulation of astrocyte glutamate transporter-1 (GLT1) and aquaporin-4 (AQP4) expression in a model of epilepsy. Exp Neurol 283: 85-96, 2016.
7. Robel S: Astroglial scarring and seizures: a cell biological perspective on epilepsy. Neuroscientist: Apr 26, 2016 (Epub ahead of print).
8. Yang J, He F, Meng Q, Sun Y, Wang W and Wang C: Inhibiting HIF-1α decreases expression of TNF-α and caspase-3 in specific brain regions exposed kainic acid-induced status epilepticus. Cell Physiol Biochem 38: 75-82, 2016.
9. Yunoki M, Kurosu T, Koketsu RK, Takahashi K, Okuno Y and Ikuta K: Neutralizing activities of human immunoglobulin derived from donors in Japan against mosquito-borne flaviviruses, Japanese encephalitis virus, West Nile virus, and dengue virus. Biologics 10: 99-102, 2016.
10. Takamatsu Y, Uchida L and Morita K: Delayed IFN response differentiates replication of West Nile virus and Japanese encephalitis virus in human neuroblastoma and glioblastoma cells. J Gen Virol 96: 2194-2199, 2015.
11. Shen SC, Shen CI, Lin H, Chen CJ, Chang CY, Chen SM, Lee HC, Lai PS and Su HL: Susceptibility of human embryonic stem cell-derived neural cells to Japanese encephalitis virus infection. PLoS One 9: e114990, 2014.
12. Azimi A, Hagh MF, Yousefi B, Rahnama MA, Khorrami A, Heydarabad MZ, Najafpour M, Hallajzadeh J and Ghahremani A: The effect of prednisolone on miR-15a and miR-16-1 expression levels and apoptosis in acute lymphoblastic leukemia cell line: CCRF-CEM. Drug Res (Stuttg) 66: 432-435, 2016.
13. Reschke CR and Henshall DC: microRNA and epilepsy. Adv Exp Med Biol 888: 41-70, 2015.
14. Alsharafi WA, Xiao B, Abuhamed MM and Luo Z: miRNAs: biological and clinical determinants in epilepsy. Front Mol Neurosci 8: 59, 2015.
15. Srivastava A, Dixit AB, Banerjee J, Tripathi M and Sarat Chandra P: Role of inflammation and its miRNA based regulation in epilepsy: implications for therapy. Clin Chim Acta 452: 1-9, 2016.
16. Goswami S, Banerjee A, Kumari B, Bandopadhyay B, Bhattacharya N, Basu N, Vrati S and Banerjee A: Differential expression and significance of circulating microRNAs in cerebrospinal fluid of acute encephalitis patients infected with Japanese encephalitis virus. Mol Neurobiol 9: 1567-1568, 2016.
17. Rispoli A, Cipollini E, Catania S, Di Giaimo R, Pulice G, van Houte S, Sparla F, Dal Piaz F, Roncarati D, Trost P, *et al*: Insights in progressive myoclonus epilepsy: HSP70 promotes cystatin B polymerization. Biochim Biophys Acta 1834: 2591-2599, 2013.
18. Rejdak K, Kuhle J, Rüegg S, Lindberg RL, Petzold A, Sulejczak D, Papuc E, Rejdak R, Stelmasiak Z and Grieb P: Neurofilament heavy chain and heat shock protein 70 as markers of seizure-related brain injury. Epilepsia 53: 922-927, 2012.
19. Turturici G, Sconzo G and Geraci F: Hsp70 and its molecular role in nervous system diseases. Biochem Res Int 2011: 618127, 2011.