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microRNA s (9, 138, 181A, 221, AND 222) AND MESIAL TEMPORAL LOBE EPILEPSY IN DEVELOPING BRAINS

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Abstract

Background: Recently, microRNAs (miRNAs) have attracted much attention as novel players in the pathogenesis of mesial temporal lobe epilepsy (MTLE) in mature and developing brains. This study aimed to investigate the expression dynamics of miR-9, miR-138, miR-181a, miR-221, and miR-222 in the hippocampus of an immature rat model during the three stages of MTLE development and in children with MTLE. Methodology: qPCR was used to measure expression levels during the three stages of MTLE development (2 h, 3, and 8 weeks after induction of lithium-pilocarpine status epilepticus, representing the acute, latent, and chronic stages, respectively. Expression levels were also measured in hippocampi obtained from children with MTLE and normal controls. Results: In the rat model, miR-9 was significantly upregulated during the acute and chronic stages relative to controls, but not during the latent stage. MiR-138, miR-221 and miR-222 were all downregulated during all three stages of MTLE development. MiR-181a was downregulated during the acute stage, upregulated during the chronic stage, and unaltered during the latent stage. In children, miR-9 and miR-181a were upregulated, while miR-138, miR-221, and miR-222 were downregulated. Conclusion: Modulation of these miRNAs may be a new strategy in designing antiepileptic and anticonvulsant therapies for the developing brain.

Keywords

• Mesial temporal lobe epilepsy • microRNAs • Developing brains

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Introduction

Epilepsy is a common disorder affecting up to 1% of the population, with one-third of patients having pharmacoresistant seizures. Mesial temporal lobe epilepsy (MTLE) is a common, chronic type of epilepsy, characterized by recurrent spontaneous seizures originating in brain structures such as the hippocampus [1]. Although temporal lobe epilepsy (TLE) is often diagnosed in adolescence or adulthood, the processes leading to epilepsy may begin earlier, possibly during childhood. Molecular changes contributing to the development of epilepsy are under intense study in the hopes of better understanding the pathogenesis and identifying targets for therapies aimed at treating and even preventing epilepsy.

MicroRNAs (miRNAs) are a class of small, noncoding RNAs of ~21–23 nucleotides that regulate gene expression at the post-transcriptional level [2]. They are believed to fine-tune many biological processes, such as developmental timing and tissue differentiation. Moreover, miRNA deregulation has been implicated in various diseases including pediatric chronic, central nervous system (CNS), and cardiovascular diseases, reviewed in [3-5].

A number of miRNAs have been identified as being brain-specific, or at least highly enriched in brain relative to other tissues. Initially identified as a brain specific miRNA, miR-9 plays a major role in brain development and is expressed specifically in neurogenic regions of the brain during neural development [6]. It has been implicated in several aspects of neuronal development and function [7], and has also recently been shown to be a pro-inflammatory miRNA and involved in modulating the nuclear factor Kappa-B (NF-κB)-dependent inflammatory response [8].

MiR-138 is highly enriched in brain, is localized within dendrites, negatively regulates the size of dendritic spines in rat hippocampal neurons, and is involved in the development of several organs [9,10]. MiR-138 also modulates DNA damage and is an important regulator of the genomic stability [11]. Very recently, downregulation of miR-138 has been found to contribute to the constitutive activation of NF-κB [12].

MiR-181a is involved in the development and differentiation of lymphoid and myeloid cells [13], and plays a very important role in the host inflammatory response [14]. In the CNS, it influences the outcome of cerebral ischemia [15].

MiR-221 and miR-222 are two highly homologous miRNAs, dysregulation of which have been described recently in several types of human tumors. These two miRNAs are considered to be hippocampal enriched [16]. *In vitro* experiments have revealed that miR-221 and miR-222 target the 3´UTR of intercellular adhesion molecule 1 (ICAM1) [17], which in turn mediates interactions with immune cells to influence the inflammatory process [18].

Because of their known enriched expressions in the brain and their roles in brain development and the CNS, we hypothesized that miR-9, miR-138, miR-181a, miR-221, and miR-222 expression levels are associated with the development of MTLE. In this study, we tested for the first time whether their expression levels

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are altered during the three stages of MTLE development in an immature rat model, and in the brains of children with MTLE.

Experimental procedures

Experimental animals

We started our experiment with 55 immature male Sprague-Dawley rats at postnatal day 25 (PN25) from the Experimental Animal Center of Xiangya Medical College, Central South University. The animals were housed at 50–60% humidity and were kept on an alternating 12 h light/dark cycle.

The 55 rats were randomly divided into two groups: experimental group E (n=31) and control group C (n=24). They were allowed to adapt to laboratory conditions for one week before starting the experiments.

Epilepsy induction

On PN25, the E rats (n=31) were injected with lithium chloride (125 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO, USA), followed 18-20 hours later by pilocarpine hydrochloride treatment (30 mg/kg, i.p., Boehringer Mannheim, Indianapolis, IN, USA) to induce status epilepticus (SE). Methylscopolamine (1 mg/kg, i.p.), a muscarinic antagonist that does not cross the blood-brain barrier, was administered 15 minutes prior to pilocarpine treatment to reduce the peripheral effects of the convulsant and thus enhance survival. The severity of convulsions was evaluated by Racine's classification [19]. Only animals classified higher than stage 3 were used in this study. SE was defined as seizure-like activity lasting at least 30 minutes. Intra-peritoneal pilocarpine administration (10 mg/kg) was repeated every 30 minutes if there was no seizure attack or seizure activities were classified lower than Racine's stage 4. The maximum dose for pilocarpine injection was 60 mg/kg. Diazepam (10 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO, USA) was administered to all SE rats 90 minutes after the onset of SE to terminate the seizure activity. The rats were video-monitored for 8 weeks (24 h/day). We observed spontaneous seizures occurring mainly 3 weeks after onset of SE. Chronic seizures occurred at 8 weeks with a frequency of 5–12 seizures per 24 hours. The time between last spontaneous seizure and analysis was usually less than 24 hours. Based on the epilepsy development stages, the sample size was divided randomly into six groups: (1) acute control group, AC (control rats, 2 h after saline administration; n = 8); (2) acute seizure group, AS (induced rats, 2 h after pilocarpine administration; n =8); (3) latent control group, LC (control rats, 3 weeks after saline administration; n = 8); (4) latent seizure group, LS (induced rats, 3 weeks after pilocarpine administration; n = 8); (5) chronic control group CC (control rats, 8 weeks after saline administration; n = 8); and (6) chronic seizure group, CS (induced rats that showed spontaneous seizures 8 weeks after pilocarpine administration; n = 8). Rats that failed to develop SE after pilocarpine treatment were removed from the experiment and euthanized.

MTLE children and controls

Specimens were obtained at surgery from 8 children undergoing unilateral selective amygdalohippocampectomy for drug-resistant MTLE with typical imaging features and pathologic confirmation of hippocampal sclerosis. The decision for surgery was based on convergent evidence of clinical and electroencephalography (EEG) recordings during prolonged video-EEG monitoring, high-resolution magnetic resonance imaging indicating mesial temporal lobe seizure onset, and invasive electroencephalography recordings. Surgical specimens were subjected to routine histopathological examination. As control tissues, 8 normal hippocampal samples were obtained at autopsy from children (postmortem delay: max. 12 h) with no history of brain disease. Neuropathologic studies confirmed that the control tissues were normal. Clinical information on children with MTLE and controls has been described previously [20].

Rat tissue preparation for RNA isolation

The immature rats were sacrificed under deep anesthesia by an intraperitoneal injection of chloral hydrate (10%, 5 ml/kg) at 2 hours and at 3 and 8 weeks after pilocarpine-induced SE or saline administration. After decapitation, the hippocampus was quickly removed using RNase-free instruments. All materials were frozen on dry ice and stored at –80°C until use.

RNA isolation

50 mg of frozen hippocampal tissue was homogenized in 1 ml Trizol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) for each subject. After adding 0.2 ml of chloroform, the aqueous phase was isolated using Phase Lock tubes (Eppendorf, Hamburg, Germany). RNA was precipitated with 0.5 ml isopropyl alcohol, washed twice with 75% ethanol, and dissolved in nuclease-free water. The concentration and purity of RNA were determined at 260/280 nm using a Nanodrop spectrophotometer (Ocean Optics, Dunedin, FL, U.S.A.).

Assessment of miRNAs' (9, 138, 181a, 221, and 222) expression levels by qPCR in the hippocampi of immature rats and children with MTLE

cDNA synthesis was performed using the One Step PrimeScript® miRNA cDNA Synthesis Kit (TAKARA, Dalian, China), which includes three mixes (2×miRNA Reaction Buffer Mix, miRNA PrimeScript® RT Enzyme Mix and 0.1% BSA). A 10-μL reaction contained 5 μL 2× miR reaction buffer mix, 1 μL miR PrimeScript® RT enzyme mix, and 1 μL 0.1 % BSA. The amount of RNA was 100 pg and DEPC treated water was up to 10 μL. The tube was incubated at 37°C for 60 minutes; the reaction was terminated at 85°C for 5 seconds, and then held at 4°C.

qPCR was performed using the SYBRR® Premix Ex Taq™ II (TAKARA, Dalian, China) kit. qPCR was performed in triplicates. The 10μl PCR reaction contained the following: 5 μl SYBRR® Premix Ex Taq™ II, 0.4 μl Uni-miR qPCR Primer, 2 μl miR Specific Primer (GeneCopoeia, Rockville, MD, USA), 1 μl cDNA, and 1.6 μl DEPC-treated water. The qPCR reactions were incubated at 50°C for 2 minutes (UDG incubation) and 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds, followed by melting curve analysis from 65.0 to 95.0°C (increment 0.5°C every 5 seconds). The relative expression level for each miRNA was calculated using the comparative CT method. The expression of the U6 small nucleolar RNA gene was used as internal control.

Statistical analysis

All of the data are expressed as means ± standard deviation. A Student's t test was performed to determine significant differences between two groups. One-way analysis of variance was utilized to determine significant differences among multiple groups. The value of $p<0.05$ was considered to be statistically significant.

Results

Dynamics of relative expression patterns of miR-9, miR-138, miR-181a, miR-221 and miR-222 in the three stages of MTLE in immature rats

qPCR results showed significant upregulation of miR-9 expression in hippocampal tissues in the acute and chronic stages of MTLE development, with an acute stage mean of 0.857±0.140 compared to the control group mean of 0.380±0.104, and a chronic stage mean was 0.883±0.202 compared to the control group mean of 0.343±0.101. In the latent stage, the expressions were nearly equal with a mean of 0.367±0.057 for the epileptic group and a mean of 0.323±0.087 for the control group (Figure 1a).

MiR-138 expression showed significant downregulation in the acute and chronic stages and slight but not significant downregulation in the latent stage. The acute stage mean was 0.183±0.076 compared to the control group mean of 0.383±0.076, while in the chronic stage, the mean was 0.167±0.029 compared to the control group mean of 0.433±0.115. In the latent stage, mean was 0.323±0.108 compared to the control group mean of 0.423±0.068 (Figure 1b).

MiR-181a expressions showed significant downregulation in the acute stage, upregulation in the chronic stage and almost equal expression in the latent stage. The acute stage mean was 0.090±0.036 compared to the control group mean of 0.397±0.020, while in the chronic stage, the mean was 0.833±0.104 compared to the control group mean of 0.357±0.129. In the latent stage, mean was 0.350±0.087 compared to the control group mean of 0.323±0.025 (Figure 1c).

MiR-221 expression showed significant downregulation in all three stages of MTLE. The acute stage mean was 0.190±0.085 compared to the control group mean of 0.433±0.153, while in the chronic stage, the mean was 0.207±0.090 compared to the control group mean of 0.540±0.066. In the latent stage, mean was 0.117±0.029 compared to the control group mean of 0.433±0.058 (Figure 1d).

MiR-222 expression showed also significant downregulation in all three stages of MTLE. The acute stage mean was 0.150±0.050 compared to the control group mean of 0.473±0.046, while in the chronic stage, the mean was 0.183±0.126 compared to the control group mean of 0.513±0.081. In the latent stage, mean was 0.190±0.036 compared to the control group mean of 0.430±0.066 (Figure 1e).

In rat tissues, miR-9, miR-138, miR-181a, miR-221 and miR-222 expressions were normalized to that of the rat U6B small nuclear RNA gene (rnu6b) ($p<0.05$).

Relative expression of miR-9, miR-138, miR-181a, miR-221, and miR-222 in children with MTLE

qPCR results showed significant upregulation of miR-9 in the hippocampal tissues of children with MTLE compared to the tissues from the control group, with a miR-9 mean of 0.733±0.076 compared to the control group mean of 0.373±0.117 (Figure 2a). MiR- 138 showed significant downregulation with the mean of 0.175±0.075 compared to control

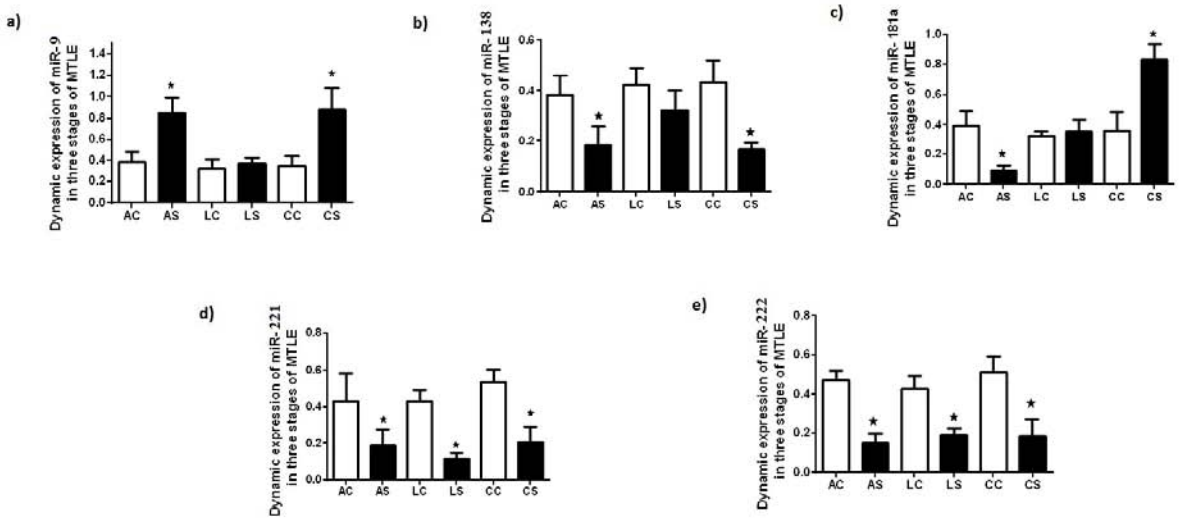

Figure 1. Relative dynamic expressions of miR-9, miR-138, miR-181a, miR-221 and miR-222 in the three stages of MTLE development in an immature rat model. a) miR-9 was significantly upregulated in the acute and chronic stages, while in the latent stage, it is nearly equal to the control. b) miR-138 was significantly downregulated in the acute and chronic stages and slightly downregulated, but not significantly, in the latent stage. c) miR-181a was significantly downregulated in the acute stage, upregulated in the chronic stage and unaltered in the latent stage. d) miR-221 was significantly downregulated in all three stages of MTLE. e) miR-222 expression showed also significant downregulation in all three stages of MTLE. n=8/group. The asterisk denotes $p<0.05$.

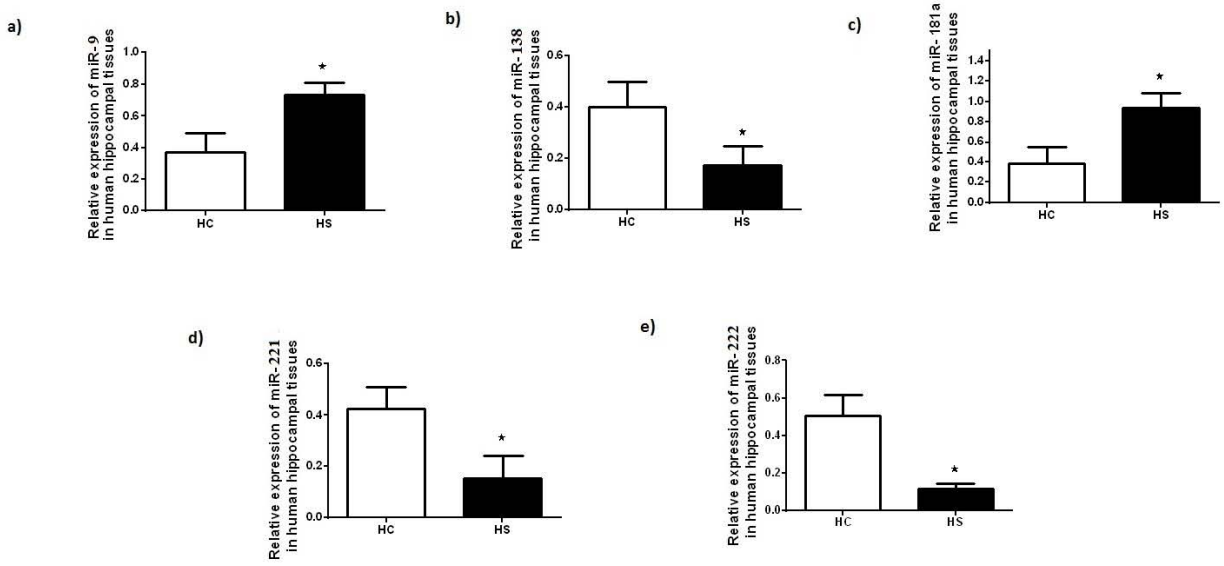

Figure 2. Relative expressions of a) miR-9, b) miR-138, c) miR- 181a, d) miR-221 and e) miR-222 in the hippocampal tissues obtained from MTLE children and normal controls. miR-9 and miR-181a showed significant upregulation in children with MTLE relative to normal controls, while miR-138,miR-221 and miR-222 were significantly downregulated in children with MTLE relative to normal controls. n=8/group. The asterisk denotes $p<0.05$.

group mean 0.400±0.100 (Figure 2b). MiR- 181a showed significant upregulation with the mean of 0.940±0.144 compared to control group mean 0.383±0.161 (Figure 2c). MiR-221 showed significant downregulation with the mean of 0.150±0.132 compared to control group mean 0.423±0.087 (Figure 2d). Finally, miR-222 showed also significant downregulation in the hippocampal tissues of children with MTLE compared to the tissues from the control group, with the mean of 0.117±0.029 compared to control group mean 0.570±0.110 (Figure 2e).

In human tissues, miR-9, miR-138, miR-181a, miR-221 and miR-222 expressions were normalized to that of the human U6B small nuclear RNA gene (rnu6b) ($p<0.05$).

Discussion

MTLE is the most prevalent form of refractory symptomatic epilepsy [21]. Despite the high prevalence and extensive research, mechanisms of the cause and progression of epilepsy are still unknown [22]. It has been well established that early in life the brain is more susceptible to seizures. About 50% of cases the onset of epilepsy occurs in childhood and in elderly, with half of those being under one year of age [23]. Molecular changes triggered by an epileptogenic insult can continue to progress after the epilepsy diagnosis, even though they might qualitatively and quantitatively differ at various phases of the epileptic process [24,25].

Interestingly, it is now clear that miRNAs regulate many aspects of the pathogenic mechanisms underlying MTLE development. Selective reduction in Dicer levels in tissue from TLE patients with severe hippocampal sclerosis (HS), suggested that Dicer loss may contribute to the pathogenesis of TLE-HS [26]. MiR-184 has been identified as a seizure-regulated miRNA and its inhibition increases neuronal death after seizures [27]. MiR-34a is upregulated and contributed in seizure-induced neuronal death [28]. Kan *et al.* (2012) profiled the miRNAs changes in human TLE [17]. Our previous experiments supported the role of inflammation-related miRNAs (miR-146a, -155, -21, and -132) and brain-specific miRNAs (miR-124 and -134) in the pathogenesis of MTLE in immature rat models and children [20,29,30]. On the therapeutic level, miR-134a silencing produces neuroprotective and prolonged seizure-suppressive effects [31], while targeting miR-132 reduces seizure-induced neuronal death and protects the hippocampus [32].

In this experiment, we assessed for the first time the dynamics of miR-9, miR-138, miR-181a, miR-221, and miR-222 expression during the three stages of MTLE development in PN25 immature rats, which nearly equal those of a human infant, and then tested them for differential expression in children with MTLE relative to controls.

Our results revealed an upregulation of brain-specific miR-9 in the seizure-related acute and chronic stages, but not during the seizure-free latent stage; this pattern of expression was the same for brain-specific miRNAs miR-124 and miR-134 in the same animal model [30]. MiR-9 was previously found to be upregulated in response to epileptic preconditioning [27] and in the chronic stage of MTLE [33]. Induction and involvement of miR-9 in the fine tuning of NF-κB-dependent inflammatory response [8] may explain this pattern of expression, based on the same pattern of expression for NF-κB in response to convulsant stimulation [34]. MiR-9 upregulation in the seizure-related stages and in children with MTLE makes it a potential target for anticonvulsant drugs in the developing brain.

MiR-138 showed downregulation in the three stages of MTLE in an immature rat

model of MTLE. In association with our results, miR-138 has previously been shown to be downregulated in the acute stage of MTLE development [28,35]. The different expression pattern of brain-enriched miR-138 from the other brain-enriched miRNAs tested, miR-124, miR-134, and miR-9, which are upregulated in the seizure-related stages [30], needs further experiments to explore its specific function in this disease. Consistent with the chronic stage results in rat, we found downregulation of miR-138 in children with MTLE, which has also been described in adult MTLE patients [17].

Inflammation-related miR-181a showed a different pattern of expression in the three stages of MTLE development: it was downregulated during the acute stage, not significantly altered during the latent stage, and upregulated during the chronic stage. Risbud and Porter [35] also found downregulation of miR-181a in the acute stage of MTLE. Other studies showed dramatic changes in miR-181a expression during the various stages of T-cell differentiation [13,36]. Our observations suggest that these changes are related to the disease process and not the developmental stage of the animal model. We found also significant upregulation for miR-181a in the children with MTLE.

In our study, the hippocampal-enriched miR-221 and miR-222 were significantly downregulated in the three stages on MTLE; the same expressions were also observed in the acute stage of MTLE in Risbud and Porter study [35]. Both were downregulated in children with MLTE, which is consistent with Kan *et al*. (2012) demonstration of downregulation of miR-221 and miR-222 in humans with MTLE and HS [17]. Being hippocampal-enriched and being strikingly downregulated in the three stages of MTLE development and in tissues obtained from surgically treated children raises the possibility that miR-221 and miR-222 may serve unique functions during the process of MTLE development in the developing brains.

In conclusion, our study shows that miR-9, miR-138, miR-181a, miR-221 and miR-222 showed different expression patterns in the immature rat model and children with MTLE. Modulation of these miRNAs expressions may be new targets for antiepileptic and anticonvulsant therapies in the developing brains.

Acknowledgments

This study was approved by the Institutional Ethics Committee of Central South University, and written informed consent was obtained from the parents of all patients before analysis. All procedures on experimental animals were approved by the Institutional Animal Care and Use Committee of Central South University. This work was kindly supported by the National Natural Science Foundation of China (30901631) and the Scientific and Technological Department of Hunan Province (2011FJ3163). All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design MUA, AO, NA, and HK. Acquisition of data: MUA and AO. Analysis and interpretation of data: MUA, AO and JP. Writing the draft of the manuscript: AO and MUA. Critical revision of the manuscript for important intellectual content: JP, AO and MUA. Statistical analysis: MUA and HK. Administrative, technical, and material support: JP and FY. Study supervision: FY. None of the authors have any conflict of interest to disclose.

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