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Expression profile of microRNAs in rat hippocampus following lithium–pilocarpine-induced status epilepticus

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ABSTRACT

Although microRNAs are expressed extensively in the central nervous system in physiological and pathological conditions, their expression in neurological disorder of epilepsy has not been well characterized. Here we investigated microRNA expression pattern in post status epilepticus rats (24 h after status). Rat MicroRNA array and differential analysis had detected 19 up-regulated microRNAs and 7 down-regulated microRNAs in rat hippocampus, and four randomly selected deregulated microRNAs (microRNA-34a, microRNA-22, microRNA-125a, microRNA-21) were confirmed by qRT-PCR, then their expression alterations in rat peripheral blood were analyzed. We found that these four deregulated microRNAs were also differentially expressed in rat peripheral blood, and trends for their blood expression alterations were just the same as their counterparts in rat hippocampus. Thus, our results have not only characterized the microRNA expression profile in post status epilepticus rat hippocampus but also demonstrated that some rat hippocampal microRNAs were probably associated with rat peripheral blood microRNAs. Moreover, targets of these deregulated microRNAs were analyzed using bioinformatics and the identified enriched MAPK pathway and long-term potentiation pathway might have been involved in molecular mechanisms concerning neuronal death, inflammation and epileptogenesis.

MicroRNAs (miRNAs) are single-stranded and non-coding RNAs recognized as endogenous regulators of post-transcriptional gene expression, as these small RNAs are capable of controlling gene expression by mediating either mRNA degradation or translation inhibition [5]. MiRNAs are involved in numerous physiological processes and increasing evidence suggests that miRNAs are dysregulated in several diseases. As the central nervous system (CNS) expresses the richest diversities of miRNAs in all human tissues [4], these small non-coding RNAs may provide opportunities for diagnosis and treatment of damaged nervous systems. So far, however, there are few available reports on roles of miRNAs in epilepsy—a major neurological disorder affecting fifty million people worldwide.

Epilepsy is a syndrome characterized by recurrent spontaneous seizures due to neuronal hyperactivity in the brain. Clinically, temporal lobe epilepsy is among one of the most frequent types of intractable epilepsy, and it is typically seen after an initial precipitating injury such as status epilepticus (SE) [10]. SE is a clinical emergency and it is defined as one continuous unremitting seizure lasting longer than 30 min, or recurrent seizures without regaining consciousness between seizures for greater than 30 min [2]. Although previous studies suggest the SE animal model as a reliable model for investigating temporal lobe epilepsy [12], the disease's molecular basis still needs more research effort. Considering the abundance and diversity of miRNAs in central nervous system (CNS), further investigations are needed to ascertain if there is an altered miRNA expression pattern associated with epilepsy.

MicroRNA microarray technology is a specific and efficient method to generate miRNA expression profiles. This approach has been applied to study the functional linkage between miRNAs and physiological/pathological processes [13]. We employed miRNA microarray technique on post status epilepticus rat hippocampus and showed an aberrant miRNA expression pattern. Then we also showed a probable correlation between the expression of hippocampal and peripheral blood miRNAs in post status epilepticus rats. Furthermore, we discussed some potential miRNA targets and their possible underlying molecular mechanisms.

Adult male Sprague–Dawley rats (6–8 weeks of age) weighing 230–270 g from Experimental Animal Center of Xiangya Medical College, Central South University were used. The animals were housed in a temperature (18–25 °C) and humidity (50–60%) controlled room which was kept on an altering 12 h light–dark cycle.

Abbreviations: miRNA, microRNA; SE, status epilepticus; MFS, mossy fiber sprouting; LTP, long-term potentiation.

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Food and water were available ad libitum. Experiments were performed in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals.

Lithium chloride (125 mg/kg, i.p., Sigma) was administered 18–20 h prior to pilocarpine injection. Pilocarpine was then intraperitoneally administered (20 mg/kg, Boehringer Mannheim, USA). The severity of convulsions was evaluated by Racine's classification, and only those animals that were classified higher than stage 3 were used in this study. SE was defined as continuous behavioral seizure activity lasting at least 30 min. Intraperitoneal pilocarpine administration (10 mg/kg) was repeated every 30 min 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. All SE rats were then intraperitoneally injected with chloral hydrate (10%, 3 mL/kg) to terminate seizure attack. The control rats received an injection of the same amount of normal saline as a replacement of pilocarpine. On a random basis, 15 rats received the lithium–pilocarpine treatment, whereas 5 rats received the normal saline treatment as control. After pilocarpine treatment, 13 rats (approximately 87%) had successfully developed SE, and 1 rat had died after SE onset (mortality rate 7%). For rat miRNA array, 6 randomly selected post-SE rats were included in the experiment group ($n = 6$) and 3 randomly selected normal rats were included in the control group ($n = 3$).

Animals were anesthetized by intraperitoneal injection of chloral hydrate (10%, 5 mL/kg) 24 h after SE onset. Hippocampus was quickly removed from the brain after decapitation and then reserved in RNAlater (−20 °C, 5 mL, Qiagen, Germany) for microarray.

Total RNA isolation for microarray was carried out using the Animal Tissue RNA Purification Kit (Norgen, Canada) according to the manufacturer's instructions. The purified RNA was quantified by determining the absorbance at 260 nm using a Du-640 Ultraviolet spectrophotometer (Beckman, USA).

Rat MicroRNA array (Signosis, USA) that includes 113 known rat miRNAs was used to identify the deregulated miRNAs in post status epilepticus rat hippocampus. The experimental procedure was performed as described in detail on the website of Signosis BioSignal Capture at http://www.signosisinc.com. In brief, 5 μg total RNA from each samples were annealed with oligo mix and miRNA/oligo hybrids were selected. Then the ligation of miRNA-directed oligos formed a singe molecule, and it was transcribed by T7 RNA polymerase before ready for hybridization. Following hybridization of miRNAs with the miRNA array, the array was detected with Alpha-Innotech chemiluminescence imaging system. RUN48 was used as internal control in the image. Microarray images were analyzed using MICROTEK ScanMaker 8700. Background was subtracted, the intensity data were extracted and normalization was performed based on mean array intensity for inter-array comparison. To determine the significant differentially expressed miRNAs, the Cochran and Cox approximation (t test without homogeneity of variance) was used since it was an improvement over the standard form of the t test as it permitted sharing information among genes without assuming that all genes share equal variances. The significantly deregulated miRNAs are defined as miRNAs that have p-values less than 0.05 and with either a greater than 2.0-fold increase or a less than 0.5-fold decrease in expression compared with controls. Normalized data were hierarchically clustered by gene and plotted as a heatmap.

To validate the rat miRNA array results in this study, four deregulated miRNAs (miR-34a, miR-22, miR-125a and miR-21) were selected for qRT-PCR confirmation, with 5 post-SE rats and 5 normal rats included in either experiment or control ($n = 5$ in each group). The hippocampal samples for qRT-PCR were reserved in liquid nitrogen, peripheral blood from post-SE rats and normal rats were obtained prior to decapitation, followed by immediate RNA isolation. Total RNA was isolated using Trizol reagent (Invitrogen, USA).

Each reaction mixture of RT containing RNA samples included 1 μL purified total RNA, 1 μL MiR-RT primers, 10 μL DEPC-treat water (12 μL, total volume A) and 4 μL 5× reaction buffer, 2 μL 10 mM dNTPs, 1 μL RNase inhibitor (20 U/μL) (Takara), 1 μL MMLV reverse transcriptase (200 U/μL) (Invitrogen). The 12 μL total volume A was blended and centrifuged at 4 °C, incubated for 5 min at 65 °C and ice-bathed for 25 min. The 20 μL reactions were then incubated for 30 min at 16 °C, 30 min at 42 °C 10 min at 85 °C and then held at 4 °C.

qPCR reaction was performed using the Hairpin-it™ miRNAs qPCR Quantitation Kit (catalog no. QPM-010), Sybr Green I (Invitrogen, Lot no. 30033W), Rox (Invitrogen, Lot no. 303260) and ABI Mx3000P QPCR System (Stratagene). qPCR was performed in triplicates. The 20 μL PCR reaction contained: 10 μL 2× Realtime PCR Buffer, 0.4 μL MiR specific Primer set (5 μM), 0.4 μL Rox, 2 μL MiRNA RT Product, 0.2 μL Tag DNA polymerase (5U/μL) and 7 μL ddH_2O. The 1× Realtime PCR Buffer contained 3 mM Mg^{2+}, 0.2 mM dNTP and the SYBR Green. The PCR reactions were incubated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 12 s, 62 °C for 60 s.

The relative expression level for each miRNA was calculated by the comparative CT method. The expression of the U6 small nucleolar RNA gene was used as internal control. To account for possible differences in expression levels of miRNAs between groups, statistical analysis was performed by two-tailed Student's t-test, and p-values less than 0.05 were considered significant.

Deregulated miRNAs were analyzed using bioinformatics algorithms. Potential targets of deregulated miRNAs were predicted by three microRNA target prediction databases including Sanger, TargetScan and microrna.org. Then targets of deregulated miRNAs were integrated. Functional classification was performed by Gene Ontology so as to determine the biological significance of these targets, and the intersections of up-regulated and down-regulated miRNA targets are associated with relevant GO terms. An accompanying p-value calculated using Fisher's Exact Test indicates which functions are over represented in the targets. A pathway analysis on the targets was performed using the KEGG pathway database to identify the enriched pathways. To identify the significance of pathway, the p-value was calculated for each pathway using a hyper-geometric distribution.

Rat MicroRNA array and differential analysis resulted in 26 deregulated miRNAs (Table 1). This result had demonstrated that miRNAs were differentially expressed between normal and post status epilepticus rats. Among the deregulated miRNAs, 19 miRNAs (miR-213, -132, -30c, -26a, -375, -99a, -24, -124a, -22, -34a, -125a, -101-1, -29b, -125b, -199a, -196b, -150, -151, -145) were significantly up-regulated (fold change >2.0, $p < 0.05$), and 7 miRNAs (miR-29a, -181c, -215, -181b, -25, -10b, -21) were significantly down-regulated (fold change <0.5, $p < 0.05$). To better demonstrate the differential expression of these miRNAs, a hierarchical clustering/heatmap of the 26 deregulated rat miRNAs was shown in Fig. 1.

To verify the accuracy of microarray results above, miR-34a, miR-22, miR-125a and miR-21 were selected for further confirmation using qRT-PCR. The results were consistent with that of the microarray analysis. Hippocampal expression amounts of miR-34a, miR-22 and miR-125a had increased after SE (Fig. 2A–C, left), whereas hippocampal expression amount of miR-21 had decreased (Fig. 2D, left). Furthermore, to demonstrate the possible correlation between hippocampal and peripheral blood miRNAs in the post-SE rats, peripheral blood miRNAs from the post-SE rats were also analyzed using qRT-PCR. As can be seen in our data, peripheral blood expression amounts of miR-34a, miR-22, miR-125a had increased after SE (Fig. 2A–C, right), whereas peripheral blood expression amount of miR-21 had decreased (Fig. 2D, right) after SE, sug-