

Identification of serum miRNAs differentially expressed in human epilepsy at seizure onset and post-seizure

JIJUN SUN¹, WEIDONG CHENG¹, LIFENG LIU¹, SHUXIN TAO¹,
ZHANGYONG XIA¹, LIFENG QI¹ and MIN HUANG²

¹Department of Neurology, Liaocheng People's Hospital and Liaocheng Clinical School of Taishan Medical University, Liaocheng, Shandong 252000; ²Department of Neurology, Second Clinical College, Jinan University, Shenzhen, Guangdong 518020, P.R. China

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Abstract. MicroRNAs (miRNAs) function as potential novel biomarkers for disease detection due to their marked stability in the blood and the characteristics of their expression profile in several diseases. In the present study, microarray-based serum miRNA profiling was performed on serum obtained from three patients with epilepsy at diagnosis and from three healthy individuals as controls. This was followed by reverse transcription-quantitative polymerase chain reaction analysis in a separate cohort of 35 health volunteers and 90 patients with epilepsy. The correlations between miRNAs and clinical parameters were analyzed. The array results showed that 15 miRNAs were overexpressed and 10 miRNAs were underexpressed (>2-fold) in the patients with epilepsy. In addition, four miRNAs, including miR-30a, miR-378, miR-106b and miR-15a were found to be overexpressed in the serum of patients at seizure onset, compared with post-seizure. When the patients were at seizure onset, the expression of miR-30a was positively associated with seizure frequency. No significant differences were found between miR-30a and gender, age or number of years following diagnosis. The expression levels of miR-378, miR-106b and miR-15a were not associated with the clinical parameters in the patients with seizures. Calcium/calmodulin-dependent protein kinase type IV was a target of miR-30a, and its expression was increased following seizure and was negatively correlated with miR-30a in the patients with epilepsy. The present study provided the first evidence, to the best of our knowledge, that the expression levels of miR-378, miR-30a, miR-106b and miR-15a were enhanced in epileptic patients with seizures. miR-30a may be useful for prognostic prediction in epilepsy.

Introduction

Epilepsy is a common chronic neurological disease, which is characterized by recurring seizures. It has been reported that the morbidity rate of epilepsy is ~1% worldwide (1). Epilepsy affects >65,000,000 individuals in the world and >100,000 new cases are diagnosed every year (2). Electroencephalography (EEG) and magnetic resonance imaging (MRI) are the most widely used techniques for the diagnosis of epilepsy. EEG and MRI can detect and locate the epileptic seizures and zones (3). Usually, a typical EEG of epilepsy ranges between minutes and hours (4). However the exact etiology of epilepsy remains to be fully elucidated.

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) and are the most well-researched family of ncRNAs to date. Mature miRNA sequences are ~22 nucleotides in length (5). They are involved in depression of the expression of post-transcriptional target genes via binding to the 3'-untranslated region (3'UTR) (6). miRNAs are abundant in the nervous system where they serve as effectors of brain development and in maintenance of the neuronal phenotype (7).

The results of previous expression profiling have shown unique miRNA expression in acute brain injuries (8). Several studies have demonstrated that miRNAs affect the pathomechanisms in epileptogenic injuries (9). Previously, four studies have profiled the expression of miRNAs in experimental epilepsy, in which >100 different miRNAs were identified. These results confirmed that epilepsy is associated with widespread alterations in the expression of miRNAs (10-13). The expressed miRNAs were all detected in the brain tissues of patients or animal models. The present study aimed to examine the serum expression of miRNAs in patients with epilepsy, and to select differentially expressed miRNA between seizure onset and post-seizure to explore the function of selected miRNA in epilepsy. This was performed with the aim to identify the biomarker of epilepsy treatment.

Materials and methods

Patients and serum samples. Following ethical approval and the provision of written informed consent, serum samples were collected from three seizure patients for miRNA microarray

Correspondence to: Dr Min Huang, Department of Neurology, Second Clinical College, Jinan University, 1017 North Dongmen Road, Shenzhen, Guangdong 518020, P.R. China
E-mail: huang_min_jnu@163.com

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analysis and from another 90 patients for confirmation of the miRNA expression. The patients were evaluated by an epileptologist and underwent a basal EEG and 3T MR brain imaging. Patients experiencing symptoms and EEG semiology were considered to be at seizure onset. Following treatment, when the symptoms and EEG semiology tended to be normal, patients were considered to be post-seizure. All samples were collected from consenting individuals from the Liaocheng People's Hospital, Taishan Medical College (Liaocheng, China) and Jinan University (Shenzhen, China). The sample collection was performed according to the protocols approved by the Ethics Committee of Jinan University. The blood samples were collected at seizure onset and post-seizure.

RNA isolation from human serum samples. Venous blood (5 ml) was collected and stored at room temperature for 1 h, followed by centrifugation at $1,600 \times g$ for 10 min at 4°C . The serum was gently collected and stored at -80°C or used in the following experiment. For all experiments, 500 μl of human serum was used and total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The concentration and the 260/280 nm absorbance ratio was detected using a Nanodrop 2000 (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA).

MicroRNA expression profiling. Total RNA (100 ng) was used to synthesize first strand cDNA at 42°C for 2 h using ArrayScriptTM reverse transcriptase, second strand cDNA was at 16°C for 1 h, 65°C for 10 min using reverse transcriptase and DNA polymerase, respectively. T7 Biotin IVT Mix was used to generate multiple copies of biotin-modified aRNA from the double stranded cDNA templates, incubated for 2–14 h at 40°C (MessageAmpTM Premier RNA Amplification kit; Thermo Fisher Scientific, Inc.). miRNAs were individually detected using specific oligonucleotides (Takara Bio, Inc.). A single miRNA-specific oligonucleotide was designed against each mature miRNA sequence, and miRNA-specific primers were extended using DNA polymerase. Universal primers were used to amplify the cDNA templates and the primer complimentary to the array was fluorescently labeled using miRCURY Hy3/Hy5 Power Labeling kit (Takara Bio Inc.). The labeled, single-stranded PCR products were hybridized to a Human v2.0 miRNA Expression BeadChip (Illumina, Inc., San Diego, CA) with 1,146 human miRNAs (97% coverage in the miRBase 12.0 database) (14,15).

Reverse transcription-quantitative PCR (RT-qPCR) analysis of the expression of miRNAs and mRNAs. RT for the individual qPCR analyses was performed using 500 ng of total RNA and a Reverse Transcription kit (Promega Corporation, Madison, WI, USA). RT-specific primers for human miRNAs miR-378, miR-30a, miR-106b and miR-15a (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for all miRNA RT procedures. Individual qPCR analyses were performed on the 7900HT Fast Realtime system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using miR-378-, miR-30a-, miR-106b- and miR-15a-specific Taqman miRNA assays (Applied Biosystems Thermo Fisher Scientific, Inc.). RNU6B was used for the normalization of miRNA expression levels. The mRNA levels of calcium/calmodulin-dependent protein

kinase type IV (CAMK4), were determined using TaqMan Real-Time PCR analysis, according to manufacturer's protocols (Applied Biosystems; Thermo Fisher Scientific, Inc.). To determine mRNA expression levels, total RNA (10 ng) were reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cDNA templates were amplified with TaqMan PreAmp Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR programs consisted in a hot start of 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C . The primers used were: CAMK4, sense 5'-AGC ACATTCAAACCACCACA-3', antisense 5'-GGACTCAGA GATCCGTCTGC-3'; miR-30a, sense 5'-TGTAACAT CCTCGAC-3', antisense 5'-ACATCCAGTGTAGCATA-3'; miR-378, sense 5'-ACACTCCAGCTGGGACTGGACTTG GAGTC-3', antisense 5'-TGGTGTCCGAGTCG-3'; miR-106a, sense 5'-CGGAATTCATCTCGAGACGCCAACTTG-3', antisense 5'-CGGGATCCCTTCATTCAAGGTCAATGAG-3'; miR-15a, sense 5'-GTCGTATCCAGTGCAGGGTCCGAG GTATTC-3', antisense 5'-GCACTGGATACGACCACA AA-3'; RNU6B, sense 5'-CTCGCTTCGGCAGCACA-3', antisense 5'-AACGCTTCACGAATTTGCGT-3'; and GAPDH, 5'-AAGGTCGGAGTCAACGGATT-3', antisense 5'-CTGGA AGATGGTGATGGGATT-3'. Each sample was analyzed in triplicate, in three independent experiments. The level of each mRNA was measured using the quantification cycle (Cq) and the level of the target was calculated as described above for the miRNAs. The gene expression levels were normalized against the expression of GAPDH. The relative fold change in expression of the target gene transcript was determined using the comparative quantification method ($2^{-\Delta\Delta\text{Cq}}$) (16). High or low expression of miRNAs and mRNAs were defined on the basis of the median expression, determined separately for each cohort.

Construction of luc-UTR vectors. The full-length CAMK4 3'-UTRs were cloned into the *EcoRI* and *HindIII* sites of the pMIR-REPORT luciferase vector (Ambion; Thermo Fisher Scientific, Inc.) using a PCR-generated fragment. A Luc-mut vector, in which the first seven nucleotides complementary to the miR-30a seed-region were mutated, served as a mutant control. The binding sites of CAMK4 were UGUUUACA and these were replaced with the CACCCGUG in the Luc-mut vector.

Luciferase reporter assay. The Luc-wild-type (wt), Luc-mut and Luc-control vectors were co-transfected within miR-30a mimics into 293T cells (Cell bank of Chinese Academy of Sciences, Shanghai, China) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). The pMIR-REPORT β -galactosidase control vector (Thermo Fisher Scientific, Inc.) was transfected into cells to serve as a control. Luciferase activity was measured in the cell lysates 48 h following transfection using a dual-light luminescent reporter gene assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The results were normalized against the activity of β -galactosidase (17).

Western blot analysis. Cells were lysed in ice-cold lysis buffer (1% NP-40; 50 mM Tris-HCl, pH 8.0; 100 mM sodium fluoride; 30 mM sodium pyrophosphate; 2 mM sodium

Table I. Association between expression level of miRNAs and clinical parameters of epilepsy.

Parameter	miR-30a		miR-378		miR-106b		miR-15a	
	High	Low	High	Low	High	Low	High	Low
Gender								
Male (52)	36	16	28	24	27	25	37	15
Female (38)	25	13	26	12	21	17	20	18
Age								
≥39 years (41)	28	13	26	15	25	16	26	15
<39 years (49)	33	16	28	21	23	26	31	18
Years diagnosed								
≥16 (32)	20	11	17	15	18	14	19	13
<16 (58)	41	18	37	21	30	28	38	20
Seizure frequency								
≥5/month (37)	31	6 ^a	22	14	21	15	25	12
<5/month (53)	30	23	32	21	26	27	32	21

High and low expression were defined on the basis of the median expression. ^aP<0.01, compared with high expression. miR/miRNA, microRNA.

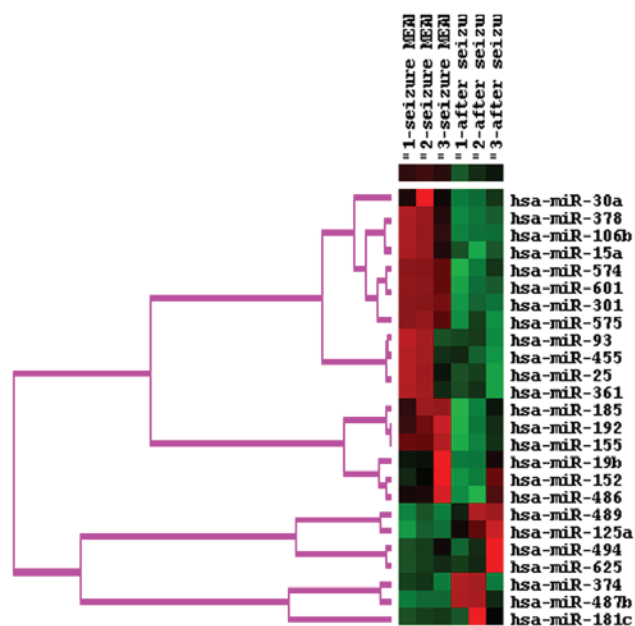


Figure 1. miRNAs differentially expressed in patients with epilepsy at seizure onset and post-seizure. Heat map representation of the average expression levels of the 25 miRNAs differentially expressed at seizure onset and post-seizure in serum from three patients. The colors of the genes represented on the heat map correspond to the expression values normalized against the mean expression of miRNA across all samples. Green indicates downregulated expression and red indicates upregulated expression in the serum. miRNA/miR, microRNA.

molybdate; 5 mM EDTA and 2 mM sodium orthovanadate). Lysates were centrifuged at 10,000 x g for 15 min at 4°C. The concentration was determined by Pierce bicinchoninic acid Protein Assay kit (Thermo Fisher Scientific, Inc.). Protein lysates (50 µg) were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA).

Membranes were blocked in 5% nonfat milk in TBS-Tween (TBST) for 1 h at room temperature. The primary rabbit anti-human CaMK4 antibody (cat. no. 4032; Cell Signaling Technology, Inc., Danvers, MA, USA) was diluted 1:1,000 in 5% milk with TBST and incubated with the membrane at 4°C overnight. Then blots were washed three times with TBST. Secondary goat anti-rabbit IgG (cat. no. ab6721; Abcam, Cambridge, UK) was diluted at 1:3,000 in milk with TBST and incubated with the membrane at 37°C for 1 h. The hybridization signals were detected by chemiluminescence (Invitrogen; Thermo Fisher Scientific, Inc.) and captured using a ChemiDOC (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are presented as the mean ± standard error of the mean. Analysis was performed using SPSS software (version 17; SPSS, Inc., Chicago, IL, USA). Comparisons between two groups were made using Student's *t*-test, whereas multigroup comparisons were made using analysis of variance followed by appropriate post-hoc testing. Pearson's correlation analysis was used to test the correlation between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical parameters. Serum from three patients at seizure onset and post-seizure were used for microarray analysis. The patients included two men and one woman. Serum from an additional 90 patients at seizure onset and post-seizure were used for RT-qPCR analysis. The patients had a mean age of 39 years (range, 21-60), a mean number of years following epilepsy diagnosis of 16 years (range, 5-31) and a mean seizure frequency of 5 (range, 1-20) times per month.

miRNA microarray. The expression levels of 15 miRNAs were increased and 10 miRNAs were decreased at seizure onset,

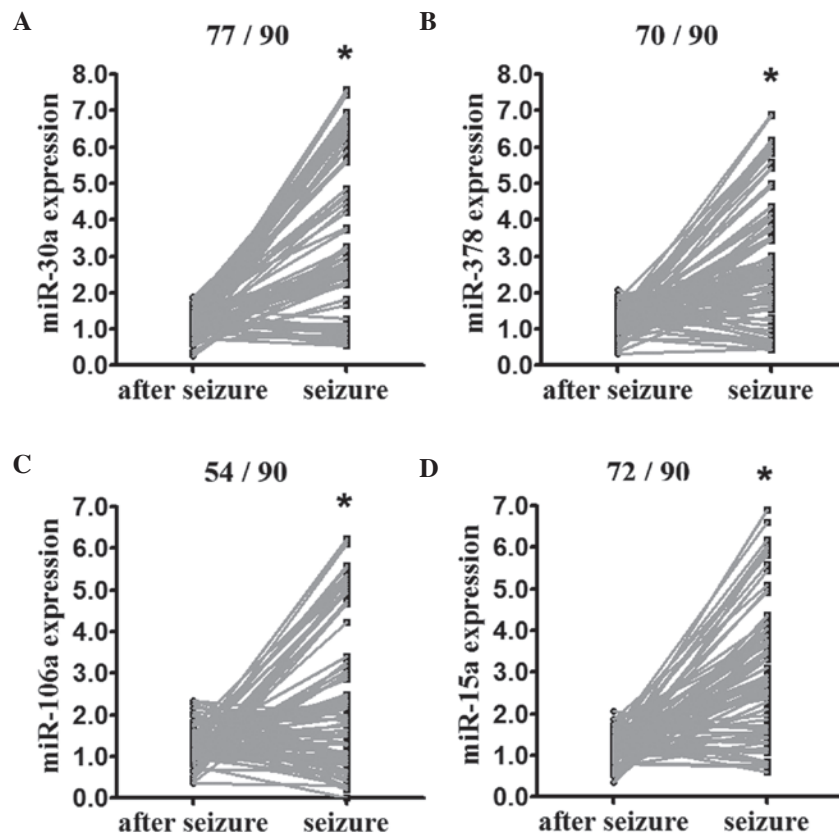


Figure 2. Relative expression of (A) miR-30a, (B) miR-378, (C) miR-106b and (D) miR-15a, evaluated using reverse transcription-quantitative polymerase chain reaction analysis, in the additional cohort of patients with epilepsy (90 patients per group). Values (above graphs) indicate the number of samples that the miR was decreased in after seizure out of the total number of samples examined. The expression levels of miR-30a, miR-378, miR-106b and miR-15a were detected at seizure onset seizures and post-seizure. * $P < 0.01$. miR, microRNA.

compared with post-seizure in the patients with epilepsy, as determined using miRNA microarray analysis (Fig. 1). The expression of miR-30a, miR-378, miR-106b and miR-15a was confirmed in the extended cohort of the three original patients and the additional 90 patients (indicated in Table I), using RT-qPCR analysis. The expression levels of miR-30a, miR-378, miR-106b and miR-15a, the top four overexpressed miRNAs identified, were enhanced at seizure onset, compared with post-seizure (Fig. 2).

Correlation between upregulated miRNAs and clinical parameters in patients experiencing seizure. The correlation between upregulated miRNAs and clinical parameters was statistically analyzed. The expression levels of miR-378, miR-106b and miR-15a were not associated with the age, gender, years following diagnosis or seizure frequency. The expression of miR-30a was positively associated with seizure frequency (Table I).

CAMK4 is a target of miR-30a. The online software program, Targetscan 6.0 (www.targetscan.org/vert_71/), was used to assist in identifying miR-30a targets. The miR-30a mimics decreased the luciferase activity of the CAMK4-3'-UTR-wt reporter (Fig. 3A). The results from the RT-qPCR and western blot analyses showed that the enhanced expression of miR-30a by the miR-30a mimics in the 293T cells led to a decrease in endogenous mRNA and protein levels of CaMK4 (Fig. 3B). The mRNA expression of CAMK4 was significantly reduced

post-seizure, compared with at seizure onset (64/90; Fig. 3C). The expression of miR-30a and CAMK4 were demonstrated to be negatively correlated (Fig. 3D).

Discussion

The pathogenesis of different types of epilepsy involves several important biological pathways, a number of which have been shown to be regulated by miRNAs. In the present study, the expression of miRNAs was compared at seizure onset with expression post-seizure in patients with epilepsy. The miRNA microarray revealed that the expression levels of 15 miRNAs were increased and 10 miRNAs were decreased at seizure onset, compared with at post-seizure in patients with epilepsy. The present study then confirmed that the expression levels of miR-30a, miR-378, miR-106b and miR-15a were higher at seizure onset, compared with levels post-seizure in the serum of patients with epilepsy.

miR-30a is involved in tumorigenesis, inflammation and myoblast differentiation and miR-30a is known to function as a tumor suppressor in breast cancer, small cell lung cancer and colorectal carcinoma (18). miR-30a has been found to be increased in cerebral arteries following subarachnoid hemorrhage (SAH), compared with the levels in rats subjected to sham surgery, and may be involved in the vascular wall changes observed following SAH (19). Wen *et al* (20) demonstrated that miR-30a-5p is significantly overexpressed in hepatitis B virus (HBV)-positive patients

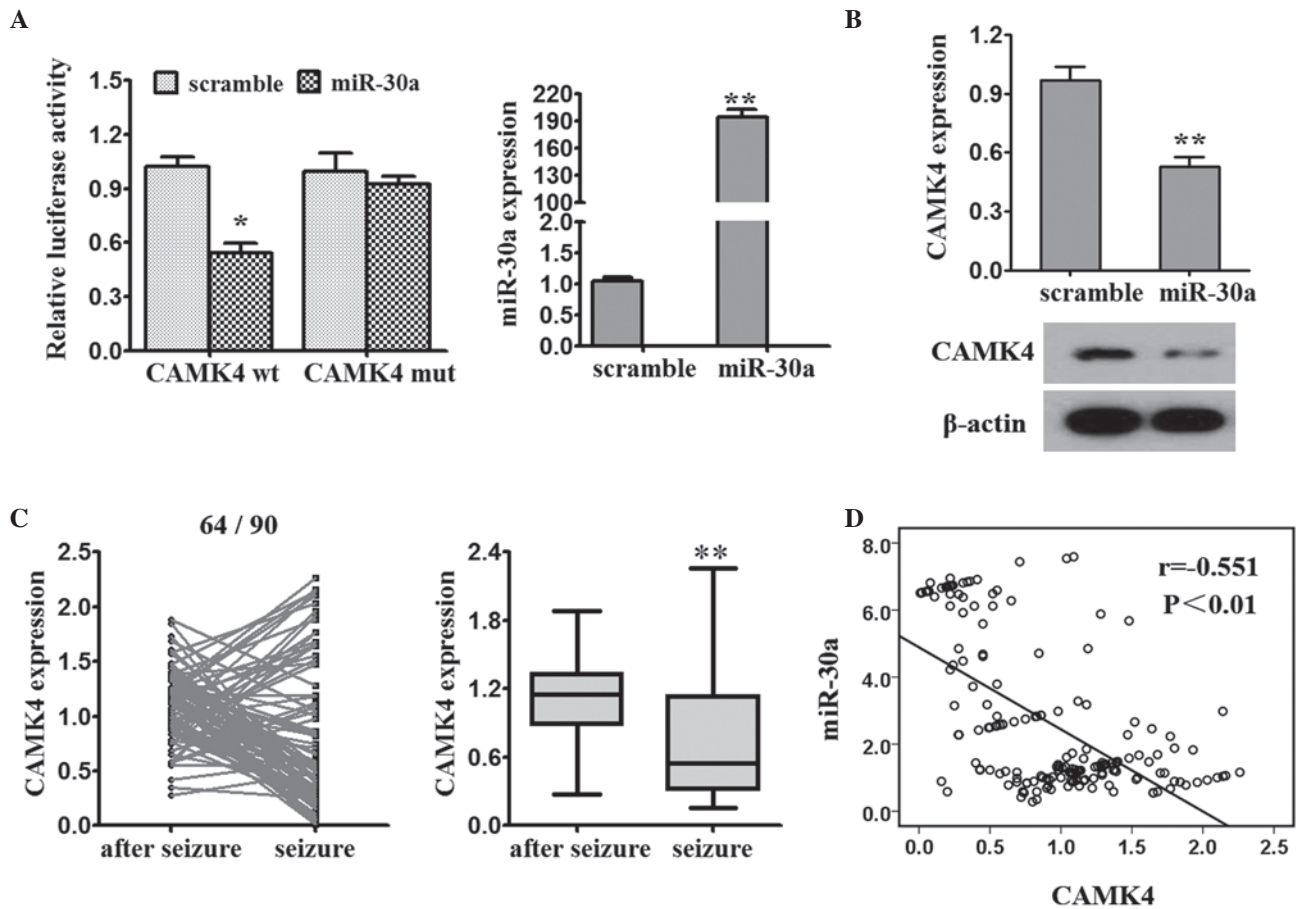


Figure 3. CAMK4 is a target of miR-30a in epilepsy. (A) miR-30a was found to bind to the CAMK4 3' untranslated region, as determined using a luciferase reporter assay. (B) Expression of CAMK4 was decreased by miRNA-30a, determined using reverse transcription-quantitative polymerase chain reaction and western blot analyses. * $P < 0.05$, ** $P < 0.01$ vs. scramble. (C) Expression of CAMK4 in patients with epilepsy. CAMK4 expression was decreased in 64/90 samples after seizure compared with at seizure onset. (D) Correlation between miR-30a and CAMK4 in epilepsy. * $P < 0.01$ vs. after seizure. miR, microRNA; CAMK4, calcium/calmodulin-dependent protein kinase type IV.

with hepatocellular cancer, compared with HBV-positive controls without cancer. This study revealed that plasma miR-30a offers potential as an early biomarker for detecting hepatocellular carcinoma. Overexpression of miR-30a-5p promotes the differentiation of myoblasts, whereas its inhibition restricts the differentiation of myoblasts *in vitro* (21). Circulating levels of miR-30a have been found to be markedly downregulated in patients with ischemic stroke until 24 weeks (22). As all four miRNAs detected in the present study were decreased following seizures, the present study analyzed the association between the expression of miRNAs in patients with epilepsy with seizures and the clinical parameters. The results of the present study revealed that the expression of miR-30a was higher in the sera of patients at seizure onset, with that post-seizure, and was associated with seizures frequency. Several studies have indicated that miR-30a functions in multiple biological processes via targeting a number of genes. For example, miR-30a can target insulin receptor substrate 2 in colorectal tumorigenesis (23). The overexpression of miR-30a upregulates the expression levels of B cell lymphoma 2-related protein A1 immediate early response 3 and cyclin D2 by inhibiting forkhead transcription factor ligand 2 (24). The downregulation of miRNA-30a alleviates cerebral ischemic injury through enhancing beclin 1-mediated autophagy (25). In the present

study, the hypothesis that CAMK4 was a target of miR-30a was confirmed. The inhibition of CAMK4 is detrimental in cerebral ischemia (26). The present study analyzed the correlation between the expression levels of miR-30a and CAMK4. The expression of CAMK4 was negatively associated with that of miR-30a in patients with epilepsy. However, the function of CAMK4 in epilepsy was not investigated, and CAMK4 may be involved in the miR-30a-mediated pathway in epilepsy.

miR-378 promotes the migration of liver cancer cells by downregulating the expression of Fus (27). miR-378 is also considered to be a diagnostic biomarker in cancer, including renal cell carcinoma (28) and colorectal cancer (29). miR-378 may be a potential biomarker for characterizing non-small cell lung cancer brain metastasis (30). The overexpression of miR-378 attenuates high glucose-suppressed osteogenic differentiation through targeting caspase 3 and activating the phosphoinositide 3-kinase/Akt signaling pathway (31). Circulating levels of miR-378 predicts left ventricular hypertrophy in patients with aortic stenosis (32). miRNA-378 controls classical brown fat expansion to counteract obesity (33). miR-106b promotes hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice (34). miR-106b is decreased in patients with chronic myeloid leukemia in the chronic phase (35).

A previous study found that the relative levels of miR-106b prior to and following *Helicobacter pylori* eradication were significantly higher in the high-risk group, compared with the control (36). miR-15a enhances the radiosensitivity of breast cancer cells by targeting G2 checkpoints (37). Maudet *et al* (38) identified the miR-15a miRNA family as cellular restriction factors for *Salmonella* infection using functional high-throughput screening. The miR-15 family is a regulator of cardiac hypertrophy and fibrosis, acting by inhibiting of the transforming growth factor β -pathway (39). However, the functions of miR-378, miR-106b and miR-15a in the nervous system have not been reported. In the present study, it was found that miR-378, miR-106b and miR-15a were increased in the sera of patients experiencing seizures. The mechanisms underlying the regulation of the expression of these miRNAs require further investigation. The present study suggested that miR-30a may be useful for predicting prognosis following seizure.

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