■ Original article

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Construction and analysis of a competing endogenous RNA network associated with circRNAs dysregulated in medial temporal lobe epilepsy

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ABSTRACT

Objective. The aetiology and pathogenesis of medial temporal lobe epilepsy (MTLE) remain unclear, and effective treatments are lacking. The involvement of a dysregulated competing endogenous RNA (ceRNA) network in MTLE is only partially understood. The purpose of this study was to investigate MTLE regulatory networks composed of messenger RNAs (mRNAs), circular RNAs (circRNAs), long non-coding RNAs (lncRNAs), and microRNAs (miRNAs) through a ceRNA network map.

Methods. RNA sequencing (RNA-seq) and small RNA-seq were used to detect mRNAs, circRNAs, miRNAs, and lncRNAs differentially expressed between post-operation hippocampal tissues of MTLE patients (n = 3) and paracancer tissues (n = 3). We performed bioinformatics analysis to identify differentially expressed RNAs and construct the corresponding ceRNA network. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of differentially expressed RNAs were conducted to explore the biological processes and pathways involved in MTLE.

Results. We identified 352 differentially expressed mRNAs, 179 circRNAs, and 42 miRNAs in MTLE. A ceRNA network composed of mRNAs, circRNAs, and miRNAs was constructed. GO and KEGG analysis of the network suggested a key role of synapses and mTOR, cAMP, ErbB, FoxO, and HIF-1 signalling pathways in MTLE.

Significance. We identify a new circRNA-miRNA-mRNA ceRNA network in MTLE. These results can help clarify the aetiology of MTLE and identify targeted molecular therapies.

Key words: medial temporal lobe epilepsy, sequencing, circular RNA, competing endogenous RNA network, biomarker

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Epilepsy is a recurrent neurodegenerative disease caused by hyperexcitability and hypersynchronization of the brain network [1, 2]. Around 50 million people worldwide suffer from epilepsy, and the number of new diagnostic cases annually ranges from 49 per 100,000 in high-income countries to 139 per 100,000 in low-and middle-income countries [3]. More than 20 new antiepileptic drugs have been developed in recent years, and the diagnosis and treatment of epilepsy have greatly improved. However, the disease is

drug-resistant in 30-40% of patients [4-9]. Medial temporal lobe epilepsy (MTLE) is the most common drug-resistant form of adult epilepsy. MTLE can occur repeatedly, reducing the patient's quality of life and potentially causing death, and it can place a huge financial burden on the family [10]. None of the antiepileptic drugs currently used can treat MTLE and, therefore, it is urgent to find new treatments for this form of epilepsy.

In recent years, new gene therapies and strategies targeting non-coding RNAs in epilepsy have aroused great interest [10]. Certain non-coding RNAs such as microRNAs (miRNAs) play a key role in the establishment of gene expression profiles in the brain (*e.g.* the hippocampus), and are important in both acquired and inherited epilepsy [11]. Compared with other epigenetic processes, miRNAs and other non-coding RNAs can be selectively targeted by using antisense oligonucleotides [12]. For example, injection of oligonucleotides targeting miRNAs can increase the surface expression of potassium channels and reduce excitability [10], which can attenuate induced or spontaneous seizures as well as reduce hippocampal damage in preclinical models [10, 13]. Neural networks and gene expression underlying pathophysiological networks in the brain structure (such as the hippocampus) are thought to be partially controlled by microRNAs [11]. This strategy may provide many other opportunities for the treatment of epilepsy, especially if other non-coding RNAs are considered.

For example, RNA-based therapy against long, non-coding RNAs (lncRNAs) can be used to down-regulate functional-gain mutant transcripts [14]. Down-regulating the long non-coding RNA (lncRNA), MALAT1 activates the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway to protect hippocampal neurons from autophagy and apoptosis in epileptic rats [15]. LncRNAH19 regulates the JAK/STAT pathway to activate hippocampal glial cells, making it a potential tool to treat epilepsy [16]. Oligonucleotides targeting the NAT lncRNA up-regulate *SCN1A*, which is a mutated gene associated with epilepsy, and this therapy is currently being explored in clinical trials against the severe myoclonic form of epilepsy known as Dravet syndrome [17].

Another type of non-coding RNA, so-called circular RNAs (circRNAs), may play regulatory roles in MTLE, but they have been rarely studied in the disease. To help clarify the interaction and roles of the various non-coding RNAs in disease, a competing endogenous RNA (ceRNA) network can be constructed involving lncRNAs, circRNAs, and miRNAs [18]. These networks are based on the principle that lncRNAs and circRNAs containing "miRNA response elements" bind to the corresponding miRNAs and regulate their activity. In this way, the lncRNAs and circRNAs act as "competitors" to enhance or weaken the function of miRNAs against their target genes. These RNAs may then be useful as diagnostic biomarkers and therapeutic targets for epilepsy therapy [19]. We are unaware of any reports of ceRNA networks in MTLE. circRNAs has a closed covalent ring structure and does not contain a 5 ′-end cap or a 3′ -end poly(A) tail structure, with diverse biological characteristics such as diversity, rich expression, stability, conserved, *etc.* [20].

In the present study, we used RNA sequencing (RNA-seq) and small RNA-seq to analyse the transcriptome in the hippocampus of patients with MTLE, and we compared the results between patients and controls without epilepsy. In addition, we constructed the ceRNA map linking the circRNAs, miRNAs, and their target genes. This study may provide new breakthroughs in the understanding of MTLE aetiology as well as the development of targeted molecular therapies.

Materials and methods

Patient selection and sample collection

The experimental group included hippocampal tissue from three patients with MTLE, which was collected after surgery at the Department of Neurosurgery of the First Affiliated Hospital of Kunming Medical University, in Kunming, China, from 2017 to 2018. MRI or magnetic resonance spectroscopy in all patients showed hippocampal sclerosis. The diagnosis of MTLE was determined by evaluating clinical seizure semiology, typical temporal auras, and interictal EEG recording of epileptiform discharges with a maximum over the temporal regions [21]. In the control group, paracancer tissues of three patients with temporal glioma were immediately stored in liquid nitrogen. Demographic characteristics were statistically tested using SPSS 24.0 (IBM, Armonk, NY, USA). The independent sample T test was used for normal distribution data, and the Mann-Whitney rank-sum test was used for non-normal distribution data.

Inclusion criteria:

- a diagnosis of medial temporal lobe epilepsy;
- MRI showing unilateral or bilateral hippocampal sclerosis;
- and patients requiring surgical treatment based on evaluation by a neurologist. Patients meeting these three criteria were included in the epilepsy group in this study.

Exclusion criteria included:

- pregnant and lactating women;
- and patients with obvious cardiopulmonary, liver and kidney failure, or blood system diseases and other obvious contraindications.

All patients provided signed informed consent for their tissues and anonymized medical data to be analysed and published for research purposes. This study was approved by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University.

Transcriptome analysis

Total RNA in tissue samples was extracted for high-throughput RNA-seq. The entire transcriptional library was built using the TruSeq Stranded Total RNA Library Prep Kit (Illumina, USA). The rRNA probe was incubated with total RNA, and the captured rRNA probe was modified by biotin. Magnetic beads coated with streptavidin were combined with the probe and rRNA complex to remove rRNA. SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, USA) was used to synthesize the first-strand cDNA, then a second-strand synthesis kit was used to obtain double-stranded cDNA with the addition of a single adenosine at both ends. The library was purified using the Agencourt SPRI select reagent kit (Beckman Coulter, USA), and fragments were screened by size at the same time. PCR amplification was performed with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) to determine the size distribution of the library fragments, and the library was sequenced using an Illumina high-throughput sequencing platform and 2×150-bp double-terminal sequencing strategy.

A small RNA library was constructed using a phosphate group at the 5′ end and a hydroxyl group at the 3′ end of the mature miRNA. Connectors of known sequence were added at both ends using truncated T4 RNA ligase 2 (NEB, UK) and T4 RNA ligase (NEB, UK). SuperScript IV reverse transcriptase (Thermo Fisher Scientific, USA) was used to synthesize the first strand of cDNA using reverse transcriptase primers that were complementary to the sequence on the joint. Next, high-resolution polyacrylamide gel electrophoresis (PAGE) was performed to isolate the miRNA library with insert sizes of 22-24 nucleotides. The transcriptome sequencing workflow is shown in *supplementary figure 1*.

Assessment of raw data and alignment with reference sequences

FastQC software and R (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) were used to evaluate the quality of the RNA-seq data. We used fastp software for quality filtering of miRNA sequencing data, generating clean reads for follow-up analysis. The filtered reads were compared with reference sequences using STAR software, and the results of the STAR alignment were analysed using Picard. The distribution of each sample sequence in coding regions, untranslated regions, introns, and gene spacer regions was calculated to construct a database. A saturation curve was used to describe the accuracy of gene expression detection according to the extent of sequencing. Sequence uniformity was analysed based on redundant sequences, and sequencing homogeneity and bias were evaluated using gene coverage analysis.

CircRNA description and analysis of differential RNA expression

CIRCexplorer2 software was used to predict the start and end positions of circRNAs and to annotate the source genes. The distribution of numbers and lengths of circRNAs on each chromosome was determined. After the gene expression was quantified, the Poisson correlation coefficient was calculated between patients and controls based on circRNA expression, and a correlation heat map was drawn.

Differentially expressed circRNAs, miRNAs, and mRNAs between cases and controls were analysed using Deseq2 software. Differential expression was defined as $|\log2 \text{ (fold change)}| > 1$ and $p<0.05$.

- ***Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed circRNAs***

GO analysis examines three ontologies: molecular functions of genes, cellular components, and biological processes. The hypergeometric distribution test was used to identify meaningful items that were enriched compared with all gene backgrounds, and the string graph in R software was used to display the four most enriched items in each ontology. During KEGG analysis, pathways were considered significantly enriched in differentially expressed genes if the pathways were associated with $p<0.05$.

- ***Construction of a ceRNA network (circRNA-miRNA-mRNA)***

Associations between circRNA, miRNA, miRNA and mRNA were based on the following criteria:

- a significant difference between the two types of gene groups;
- and a targeting relationship between the two types of genes. The correlations associated with $p < 0.05$ were selected based on the cor.test function in R. A ceRNA network was constructed using Cytoscape 3.8.0 in order to satisfy the following conditions:

– significant differences among circRNAs, miRNAs and mRNAs;
– a targeted relationship between miRNA and circRNA, and a negative correlation with $p < 0.05$ and cor < =-0.8;
– a targeted relationship between miRNA and mRNA, and a negative correlation with $p < 0.05$, cor < =-0.8;

– a targeting relationship with an mRNA and a circRNA for a given miRNA;
– and significant correlation between the circRNA and mRNA, linked to the same miRNA in the network ($p < 0.05$).

• ***Luciferase reporter gene assay***
PGL3-CMV-LUC-H_ATP6V1C2 3′UTR WT and PGL3-CMV-LUC-H_ATP6V1C2 3′UTR mutant (MT) reporter plasmids were constructed. HEK-293 cells were seeded into 24-well plates and after 24 h incubation, confluence reached 60-70%. HEK-293 cells were transiently cotransfected with hsa-miR-1908-5p mimic or hsa-miR-1908-5p inhibitor together with 0.1 µg ATP6V1C2 3′UTR WT or ATP6V1C2 3′UTR MT reporter plasmids using Lipofectamine 3000 (L3000008, Invitrogen, USA). Samples were collected 48 hours after transfection. Moleculardevices (SpectraMax L, USA) was used to detect firefly and renilla luciferase activities.

Results

Data quality evaluation and alignment with reference sequences

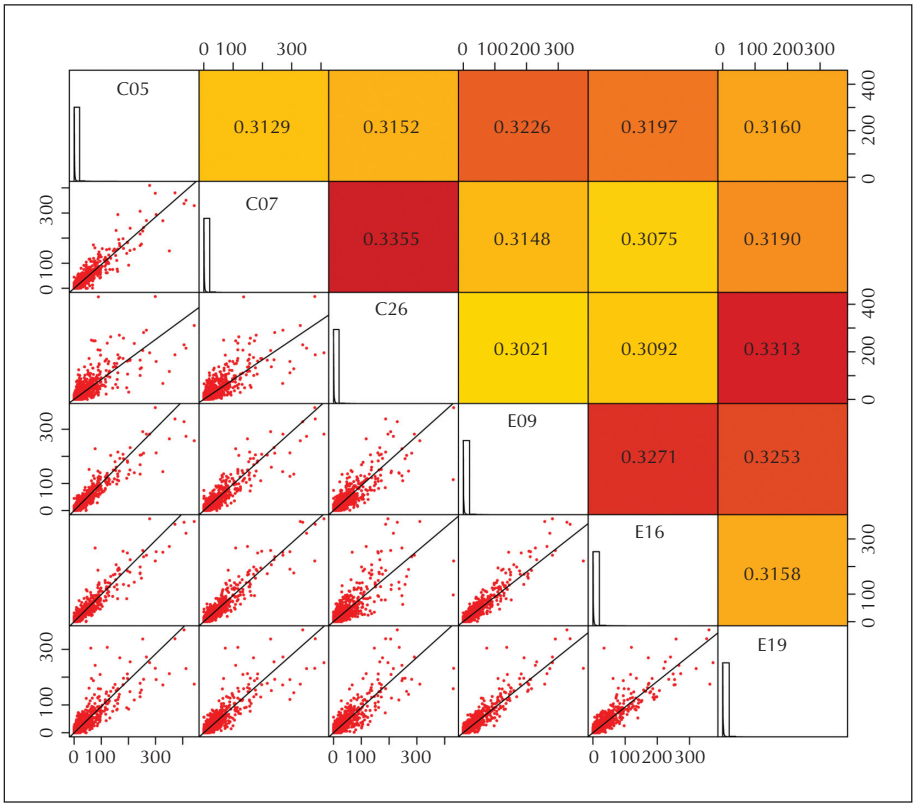
We compared the demographic and clinical characteristics between MTLE patients and control group patients (*table 1*). There was no significant difference in age between the two groups ($p>0.05$). Second-generation sequencing was performed on six samples. The originally obtained sequence contained some low-quality reads. The raw reads were filtered to obtain clean reads. The accuracy of base recognition was high, and the quality of reads was good in this study (*supplementary tables 1, 2*). Alignment with RNA reference sequences showed that the percentage of sequences located in exons (including coding and untranslated regions) was higher than that at other genomic positions (*supplementary figure 2*), confirming that our sequencing results and subsequent analysis were reliable.
The saturation curve showed that high detection accuracy of gene expression (*supplementary figure 3*). Redundant sequence analysis showed that the sequence was uniform during library building and amplification (*supplementary figure 4*), and gene coverage analysis showed that the sequencing results were unbiased (*supplementary figure 5*). The above results show that the data obtained by RNA-seq were accurate and reliable.

Analysis of differential expression of circRNAs, miRNAs, and mRNAs

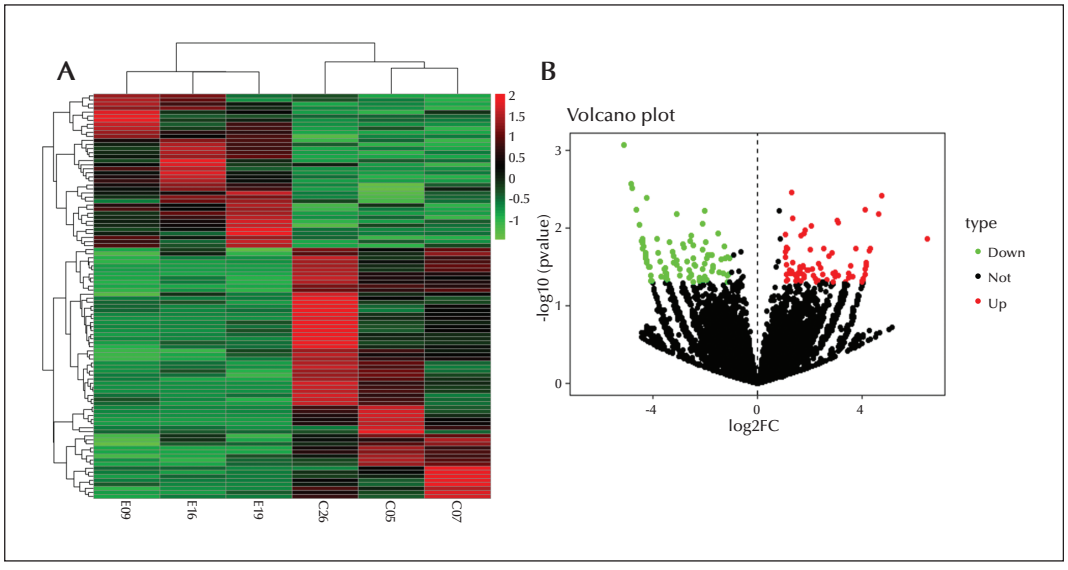
In this study, CIRCexplorer2 software was used to predict the start and end position, chromosome distribution, length, circRNA type, and source genes of circRNA. There were 60,253 circRNAs in total. The most frequent circRNA length was 2,000-4,000 nucleotides (*supplementary figure 6*). CircRNAs were derived from 22 autosomes and two sex chromosomes, and were distributed on chromosomes with a decreasing trend (*supplementary figure 7*).
The Pearson correlation heat map of gene expression showed good grouping and classification between the experimental and the control groups (*figure 1*). A total of 179 differentially expressed circRNAs were identified, of which 84 were up-regulated and 95 down-regulated in MTLE (*figure 2*). The top five up-regulated and down-regulated circRNAs were selected (*table 2*). Among the 42 differentially expressed miRNAs, 27 were up-regulated and 15 down-regulated (*figure 3*).

▼ **Table 1.** Demographic and clinical characteristics of MTLE patients and control patients.

Patient number	Gender	Age (years)	Family history	Drug-resistant epilepsy	Diagnosis	Position
E09	Male	28	No	Yes	General tonic-clonic seizures	Hippocampus
E16	Female	34	No	Yes	General tonic-clonic seizures	Hippocampus
E19	Female	30	No	Yes	General tonic-clonic seizures	Hippocampus
C05	Male	35	No	-	Protoplasmic astrocytoma (WHO II)	Temporal lobe
C07	Male	31	No	-	Fibrillary astrocytoma (WHO II)	Temporal lobe
C26	Female	26	No	-	Subependymoma (WHO II)	Temporal lobe



■ **Figure 1.** Heat map showing correlation of circular RNAs in patients with MLTE (E09, E16, E19) and controls (C05, C07, C26).



■ **Figure 2.** Heat map (A) and volcano map (B) showing the expression of circular RNAs in MTLE patients relative to expression in controls. Green indicates down-regulated genes (Down), red indicates up-regulated genes (Up), and black indicates unchanged genes (Not).

▼ **Table 2.** The top five up-regulated and down-regulated circRNAs in patients with MTLE.

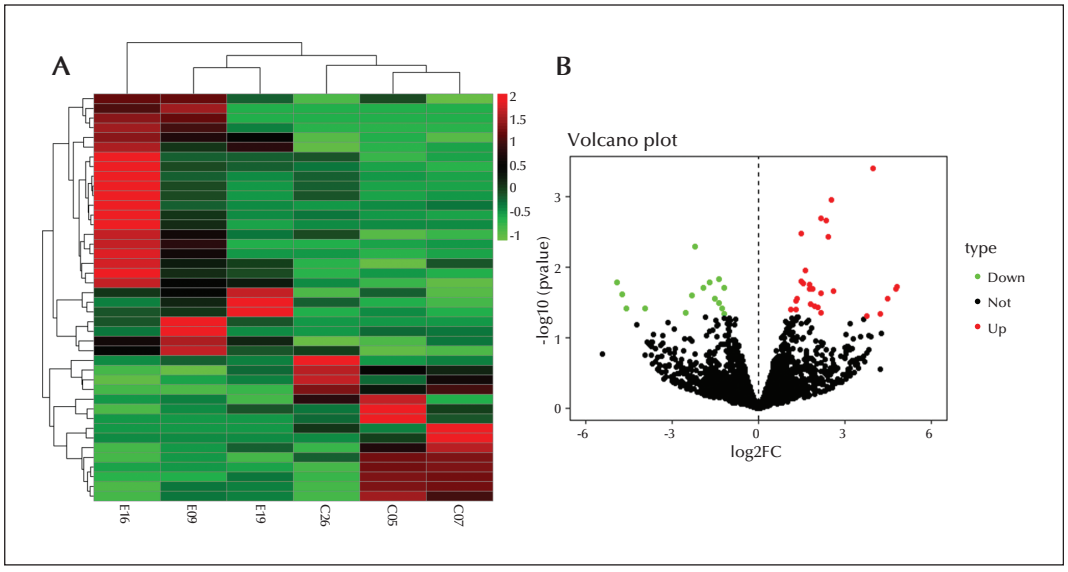
circRNA_ID	Differential expression	Log2 fold change	P value	circBase_ID	Host gene
chr2:98158542-98165954:-	Up-regulated	6.4997248	0.014		ANKRD36B
chr15:66048478-6048810:-	Up-regulated	4.7335993	0.004	hsa_circ_0004393	DENND4A
chr4:151604703-51753128:-	Up-regulated	4.6349112	0.007	hsa_circ_0071173	LRBA
chrX:112048175-12075598:-	Up-regulated	4.3349288	0.019		AMOT
chr2:20526022-20527139:-	Up-regulated	4.2847229	0.020	hsa_circ_0004011	PUM2
chr2:48573340-48573890:+	Down-regulated	-5.1012796	0.001	hsa_circ_0001002	FOXN2
chr1:72241855-72400994:-	Down-regulated	-4.8116661	0.003		NEGR1
chr5:139818046-39828890:+	Down-regulated	-4.7620441	0.003	hsa_circ_0007402	EIF4EBP3
chr5:37115062-37120442:-	Down-regulated	-4.6308035	0.006	hsa_circ_0072202	C5orf42
chr3:196778448-96786869:-	Down-regulated	-4.4864045	0.009		DLG1

There were 352 differentially expressed mRNAs, including 224 up-regulated and 128 down-regulated mRNAs (*figure 4*).

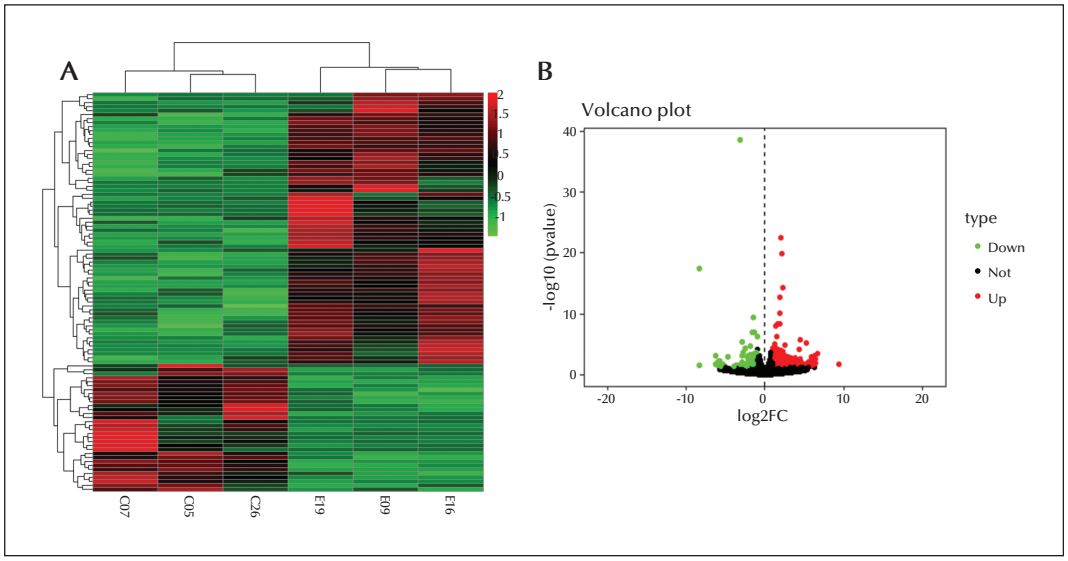
GO and KEGG enrichment analysis of differentially expressed circRNAs

Based on the GO enrichment analysis of the source genes of differentially expressed circRNAs, we found that the most common biological processes were associated mainly with synaptic-related motility. Regarding cellular components, the 10 most significant items based on the GO enrichment analysis were distributed mainly in presynaptic and synaptic structures, while their molecular functions were Rab GTPase binding and serving as structural components of synapses (*figure 5*).

The results of the KEGG enrichment pathway showed that the source genes of differentially expressed circRNAs may be involved in signalling pathways mediated by mammalian target of rapamycin (mTOR), cAMP, hypoxia inducible factor-1 (HIF-1), and forkhead



■ **Figure 3.** Heat map (A) and volcano map (B) showing the expression of microRNAs in MTLE patients relative to expression in controls. Green indicates down-regulated genes (Down), red indicates up-regulated genes (Up), and black indicates unchanged genes (Not).



■ **Figure 4.** Heat map (A) and volcano map (B) showing the expression of mRNAs in MTLE patients relative to expression in controls. Green indicates down-regulated genes (Down), red indicates up-regulated genes (Up), and black indicates unchanged genes (Not).

transcription factor of the O class (FoxO), among others (*figure 6*). These pathways have been linked to the occurrence and development of epilepsy [22-24].

Construction of a ceRNA network (circRNA-miRNA-mRNA)

In total, 456 targeting relationships were predicted according to the differences between circRNAs and miRNAs, of which 125 relationships were up-regulated by circRNAs and miRNAs, 82 were up-regulated by miRNAs and circRNAs, 153 were up-regulated by miRNAs but down-regulated by circRNAs, and 96 were down-regulated by both circRNAs and miRNAs. Correlation analysis based on gene expression values identified 69 meaningful targeting relationships. These interactions were visualized using Cytoscape (*figure 7*). Using the same analysis method, we explored the targeting relationship between differentially expressed miRNAs and mRNAs, leading to the prediction of 20 targeting relationships, of which seven were up-regulated by mRNAs and miRNAs, one was up-regulated by mRNAs but down-regulated by miRNAs, nine were down-regulated by mRNAs but up-regulated by miRNAs, and three were down-regulated by both mRNAs and miRNAs. The interaction diagram of miRNAs and mRNAs is shown in *figure 8*.

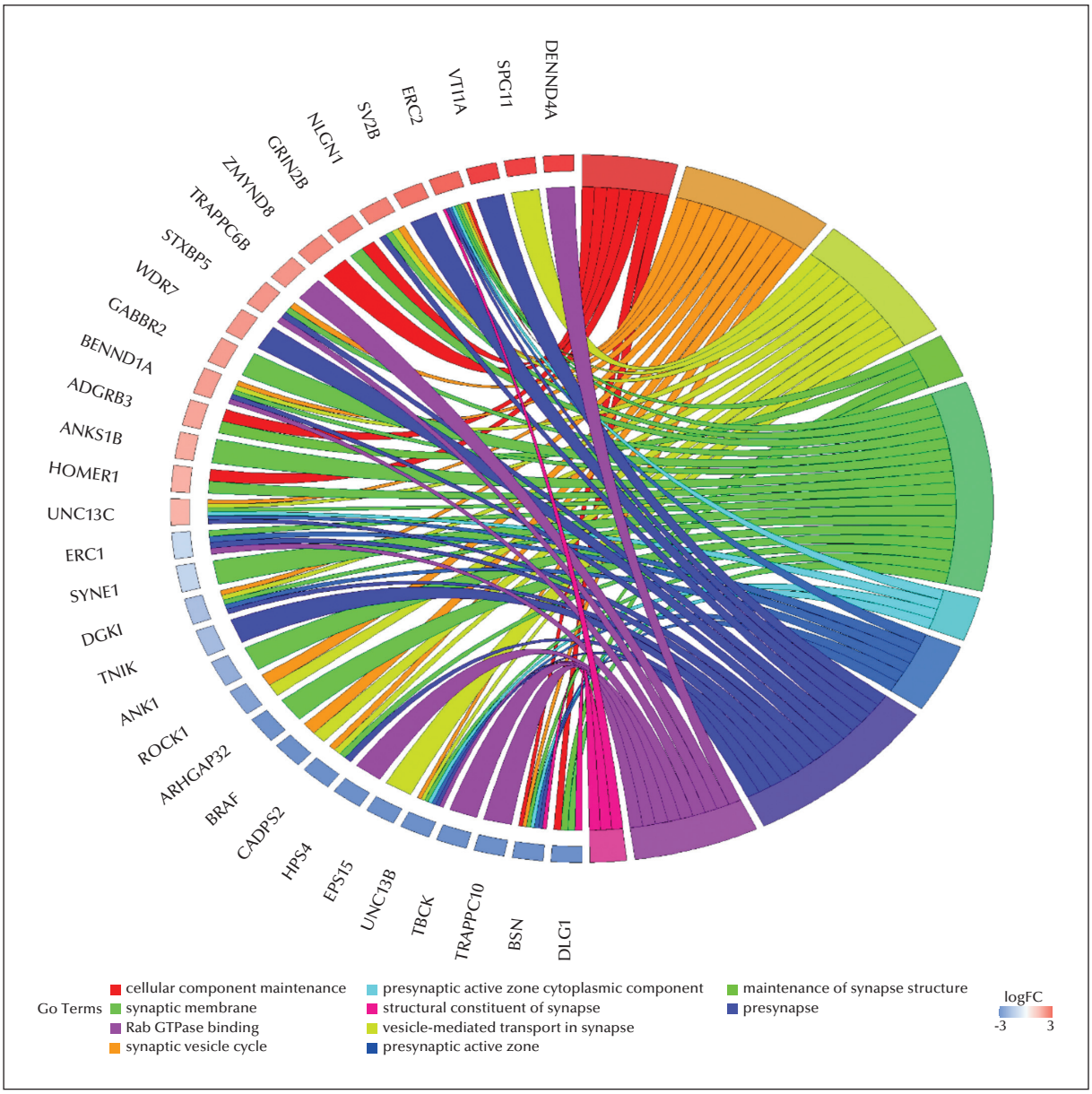
Interestingly, when constructing the ceRNA network based on the differentially expressed circRNAs, miRNAs and mRNAs, we identified the following networks: hsa-circ-0006737-hsa-miR-1908-5p-ATP6V1C2 and hsa_circ_0003575 or hsa_circ_0009027 or has_circ_0003342 or hsa_circ_0048002-hsa-miR-6131-VGLL2 (*figure 9*). Therefore, these RNAs may play an important role in the pathogenesis of epilepsy.

miR-29b-3p targets the 3′-UTR of TRAF3

To identify the putative mechanisms of miR-1908-5p-mediated regulation of *ATP6V1C2*, bioinformatics predictions were performed using miRanda and RNAhybrid. Subsequently, dual-luciferase reporter assays were performed to determine the relative luciferase activity for miR-1908-5p mimic or miR-1908-5p inhibitor with ATP6V1C2 3′UTR WT or ATP6V1C2 3′UTR MT reporter plasmids. As shown in *figure 10*, a significant decrease in relative luciferase activity was noted for miR-1908-5p in the presence of ATP6V1C2 3′UTR WT, but not in the presence of ATP6V1C2 3′UTR MT.

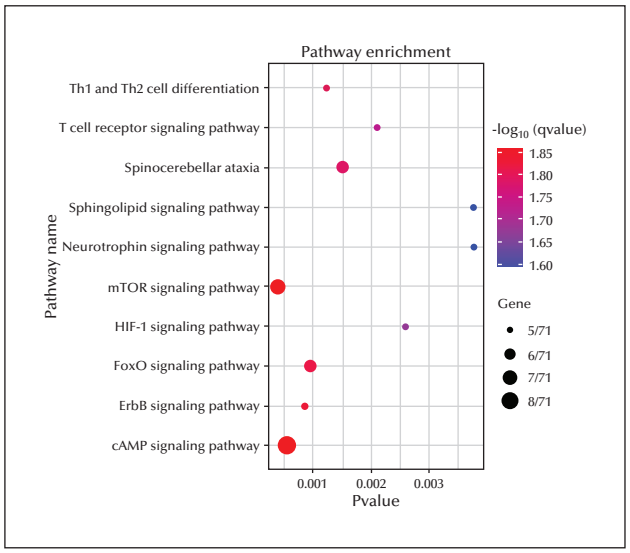
Discussion

MTLE is one of the most common drug-resistant epilepsies in humans. The study of high-throughput MTLE-specific gene expression may reveal molecular changes in the pathogenesis of the disease and new therapeutic targets. In our study, we found 179 circRNAs, 42 miRNAs (27 up-regulated and 15 down-regulated), and 352 mRNAs (224 up-regulated and 128 down-regulated) that were differentially expressed in the hippocampus of MTLE patients compared with controls. Based on the differentially expressed



■ **Figure 5.** Gene Ontology enrichment analysis of the source genes of differentially expressed circRNAs (string diagram).

circRNAs and miRNAs, 456 targeting relationships were predicted, among which 125 circRNAs and miRNAs were both up-regulated, 82 circRNAs were up-regulated, 153 circRNAs were down-regulated, and 96 circRNAs and miRNAs were both down-regulated. CircRNAs can prevent miRNAs from inhibiting translation through competitive binding, therefore up-regulating target genes [25]. Although the prediction and identification of circRNAs has been quite extensive for some diseases, little is known about MTLE-related circRNAs. CircRNAs have the characteristics of structural stability, a high level of expression, and a high level of sequence conservation, which suggest their potential as disease biomarkers. GO analysis of the parent genes of identified circRNAs revealed many items related to synapses. In the transcriptome map of the MTLE hippocampus, 24 enriched pathways were found, including signalling

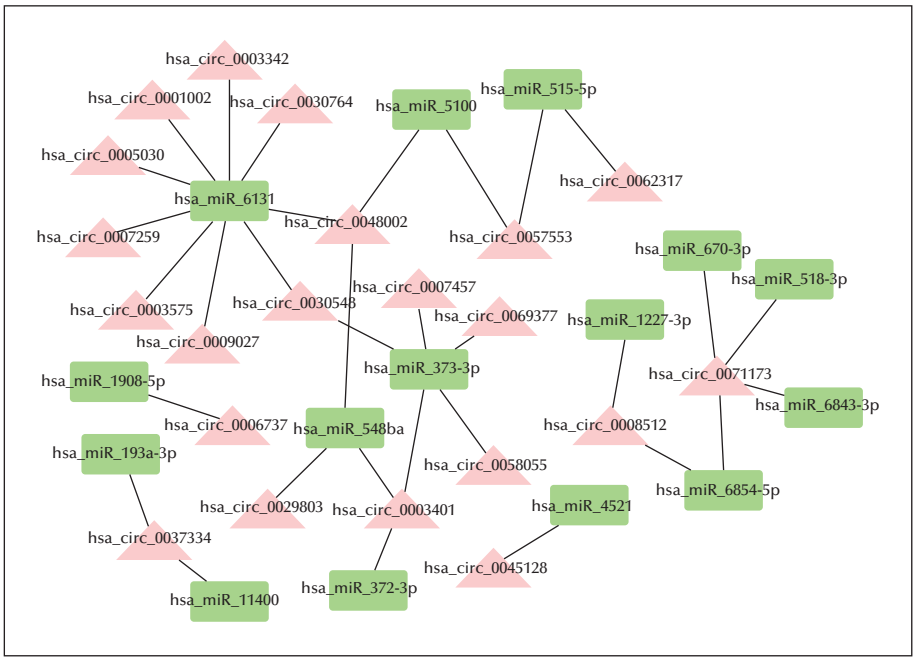


■ **Figure 6.** Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the source genes of differentially expressed circular RNAs.

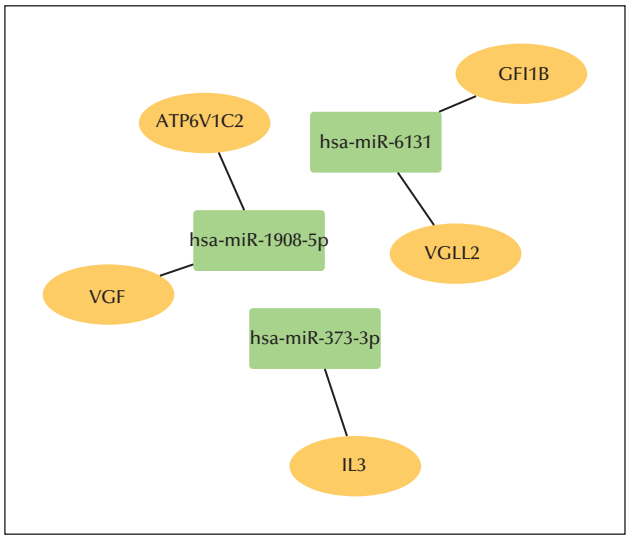
pathways mediated by mTOR, cAMP, ErbB, FoxO, and hypoxia inducible factor-1 (HIF-1), as well as other pathways closely related to epilepsy. These results suggest that differentially expressed circRNAs are highly likely to be involved in the occurrence and development of MTLE. We next discuss the mechanisms by which these pathways may play a role in epilepsy, and provide a theoretical basis for further research on the mechanism of MTLE.

In human symptomatic epilepsy, NRG1-ErbB4 signal inhibits the phosphorylation of GluN2B at position 1472 by Src kinase and reduces the phosphorylation of GluN2B and Src [26]. Loss of NRG1 or ErbB4 function or inhibition of NRG1 signalling affects the development of nerve cells and the formation of synapses in the brain, resulting in changes in nerve transmission and cortical function, giving rise to epileptic symptoms and cognitive impairment [26].

In a previous study, the inhibition of the PI3K/AKT/mTOR pathway reduced the expression of related proteins, as well as synaptic remodelling and abnormal neuronal discharges induced by magnesium-free medium or IL-1β [22]. In different animal models, the mTOR signal pathway is activated during status epilepticus [27, 28]. The PI3K/Akt/mTOR signalling pathway may play a critical role in regulating synapse activity and electrophysiology of hippocampal neurons. Inhibitors of mTOR can reduce the pathological changes associated with epilepsy, especially mossy fibre sprouting [29], reduce the frequency of seizures, and improve cognitive impairment in epileptic models with brain injury [30]. In fact, the PI3K/Akt/mTOR pathway is activated in the rat MTLE model [22]. Future studies could focus on whether cirRNA/mime play a role in MTLE by regulating these pathways.

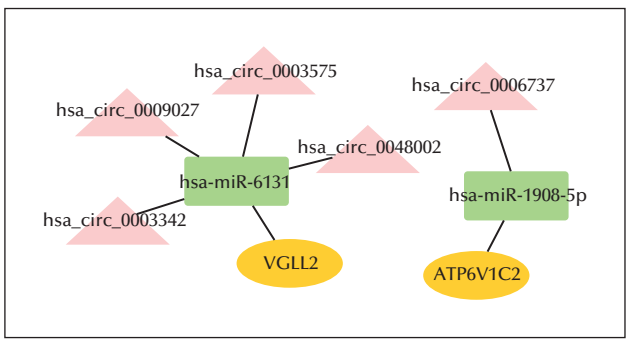


■ **Figure 7.** Combinations of differentially expressed circular RNAs (circRNAs) with microRNAs (miRNAs).



■ **Figure 8.** Combinations of differentially expressed miRNAs with mRNAs.

FoxO is involved in apoptotic injury, and its activity is regulated by the PI3K-AKT pathway. The interaction between FOXO3a and Akt has been linked to activation of FOXO3a and apoptosis in the hippocampus after status epilepticus [2]. HIF-1α is significantly increased in patients with epilepsy and animal models of epilepsy during the acute and chronic phases [31]. HIF-1α activates the Notch signal pathway through its physical interaction with the Notch intracellular domain (NICD), enhancing the neurogenesis of acute epilepsy [32]. During hypoxia, HIF-1α can up-regulate multidrug resistance 1 and thereby contribute to refractory epilepsy [23]. In this study, we also found that differentially expressed genes in the hippocampus of MTLE patients were enriched in the HIF-1α and FoxO3a pathway.



■ **Figure 9.** A competing endogenous RNA (ceRNA) network comprising circRNAs, miRNAs, and mRNAs.

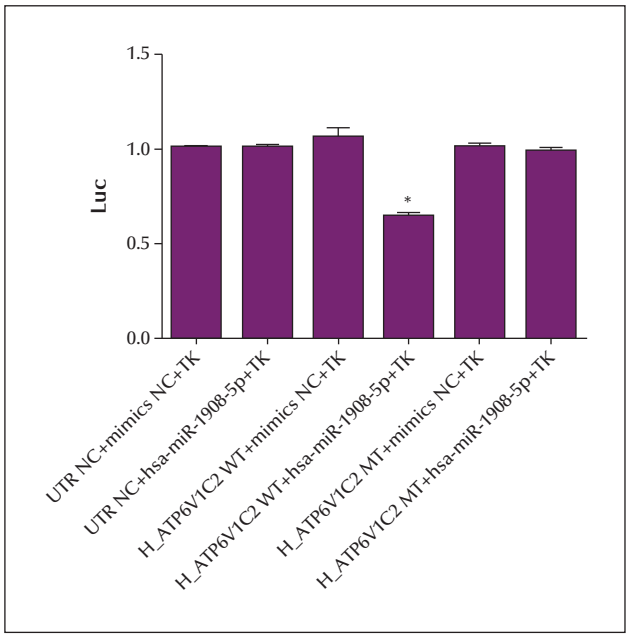
The level of cAMP in the brain increases during seizures, and this increase seems to lower the threshold for epilepsy induction [33]. In animal models of epilepsy, pilocarpine perturbs a well-characterized cAMP-dependent signalling pathway involved in presynaptic forms of long-term potentiation [34]. At present, only two anti-epileptic drugs, carbamazepine and valproate, as well as some benzodiazepines have been found to directly affect the cAMP signalling system [24]. Continuously elevated cAMP levels increase long-term synaptic enhancement through CREB, promoting excitatory nerve transmission, which can be reversed by carbamazepine and valproate [35]. In addition, the calcium signal phenotype of "enhanced" astrocytes common in epileptic tissues may also affect cAMP signalling dynamics [36]. In order to identify new drug targets for the activation of these signalling pathways, we need to characterize the upstream regulatory pathways involved. Therefore, further study of our newly discovered circRNA/miRNA/mRNA may provide a new therapeutic target for MTLE.

In our study, we further analysed the relationship between circRNAs and miRNAs or mRNAs. First, we analysed the relationship of circRNAs with miRNAs and mRNAs independently, and then we constructed the ceRNA network of circRNA-miRNA-mRNA through the intersection of miRNAs. Finally, two ceRNA regulatory networks emerged:

– has-circ-0006737/has-miR-1908-5p/ATP6V1C2;
– and has-circ-0003575 or has-circ-0009027 or has-circ-0003342 or has-circ-0048002 / has-miR-6131 / VGLL2).

These networks may play a key role in the pathogenesis of epilepsy. The hsa-circ-0003575, which localizes primarily in the cytoplasm rather than the nucleus, may function as an miRNA 'sponge' [37]. Previous studies have reported that the transcriptional cofactor VGLL2 can regulate IGF-1/PI3K signalling and PGC-1α in skeletal muscle [38]. It is well known that PI3K and PGC-1α are plays an important role in epilepsy [39]. VGLL2 may play a role in epilepsy by regulating PI3K/PGC1α, but further studies are needed to confirm this.

In another ceRNA network, miR1908-5p was predicted to regulate the expression of genes involved in glutamatergic synaptic function [40]. Targeting glutamate uptake has emerged as a promising strategy for the treatment of epilepsy. Targeted overexpression of glutamate transporter-1 reduces seizures and attenuates pathological changes in a mouse model of epilepsy [41]. When extracellular glutamate concentrations rise to abnormal levels, glutamate receptor over-activation and subsequent calcium influx into postsynaptic neurons trigger the cell death pathway. Regulation of excitatory amino acid transporter



■ **Figure 10.** Specificity of miR-1908-5p for *ATP6V1C2*. Relative luciferase activity in HEK-293 cells transfected with miR-1908-5p and miR-1908-5p inhibitor along with ATP6V1C2 3'UTR WT or ATP6V1C2 3'UTR MT. NC: negative control; TK : pGMLR-TK, that is the sea kidney luciferase reporter gene plasmid. PGMLR-TK luciferase reporter gene is based on HSV-thymidine kinase promoter (TK), which is suitable for constitutive expression in sea kidney luciferase reporter gene vector.

upregulation through transcription and translation has been shown to successfully reduce spontaneous recurrent epileptic seizures and provide neuroprotective effects *in vivo* [42].

However, the has-circ-0006737/has-miR-1908-5p/ATP6V1C2 ceRNA network has not been reported so far in MTLE. Our study confirms, for the first time, that *ATP6V1C2* is the target gene of miR-1908-5p based on a luciferase reporter gene assay. Our future research should be validated with more brain tissues and animal models of epilepsy for temporal lobe epilepsy. This study provides a new insight into the pathogenesis of temporal lobe epilepsy.

In our study, the method of total RNA extraction (removing rRNA) combined with miRNA was used to construct an RNA-seq library in order to allow multidimensional analysis of circRNAs. This method may not detect low abundance circRNAs, and linear RNAs may interfere with the identification of circRNAs. On the other hand, our method allows other RNAs to be obtained and combined with circRNAs for comprehensive analysis. Nevertheless, our study presents several limitations. First, the sample was small, and more second-generation sequencing studies are needed. Second, all patients in our study received anti-epileptic drugs and, therefore, we cannot exclude the potential influence of such medication on our results. Despite these limitations, our results may provide a strong theoretical basis for identifying potential therapeutic targets in MTLE. Further research is needed to verify the results of our high-throughput sequencing analysis in tissue samples and animals.

Conclusions

We used RNA-seq to analyse the differential expression of mRNAs, miRNAs, and circRNAs in MTLE patients compared with controls, and we performed a comprehensive identification and analysis of circRNAs. We investigated the GO/KEGG pathways enriched for differentially expressed circRNA parent genes in order to further explore the pathogenesis of epilepsy. Finally, we established a ceRNA network of circRNAs, miRNAs and mRNAs to reveal potential interactions in the disease. We hope that this work will provide valuable resources for the clinical diagnosis and treatment of epilepsy in the future. ■

Supplementary material.
Supplementary data and summary slides accompanying the manuscript are available at www.epilepticdisorders.com.

Disclosures.
The authors have no conflict of interest to declare.

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TEST YOURSELF

(1) What are the clinical features of MTLE?

(2) What are the possible mechanisms by which CircRNAs influence medial temporal lobe epilepsy?

Note: Reading the manuscript provides an answer to all questions. Correct answers may be accessed on the website, www.epilepticdisorders.com.