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Article

Expression and Potential Function of Plasma Exosomal microRNAs (miR-483-5p, miR-671-5p, and miR-150-3p) in a Mouse Mode and in Mesial Temporal Lobe Epilepsy Patients

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Abstract:

Mesial temporal lobe epilepsy (mTLE) is one of the most common and refractory focal epilepsy syndromes. The molecular mechanisms of TLE are not completely understood. The aim of this study was to investigate the expression and potential function of plasma exosomal miRNAs (miR-483-5p, miR-671-5p, and miR-150-3p) in a mouse mode and in temporal lobe epilepsy patients. It was found that exosomal miRNAs were differentially expressed in three phases of the mouse mode, and exosomal miRNAs were down-regulated in mTLE patients compared with healthy controls. A bioinformatics analysis showed that target genes of exosomal miRNAs were significantly involved in the apoptotic process, cell adhesion, nervous system development, neurotrophin signaling pathway, PI3K-Akt signaling pathway, and metabolic pathways. The areas under the curve of miR-483-5p and miR-150-3p were 0.8714 (sensitivity = 75.00%, specificity = 91.65%) and 0.8213 (sensitivity = 67.50%, specificity = 90.00%), respectively. More importantly, the exosomal miRNAs were significantly associated with clinical parameters. Exosomal miRNAs may have the potential to become diagnostic and therapeutic biomarkers.

Key words: epilepsy; exosoma; microRNA

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Introduction:

Mesial temporal lobe epilepsy (mTLE) is one of the most common and refractory focal epilepsy syndromes，accounting for about 50% of all cases [1]. Hippocampal sclerosis is the most common and specific cause of mTLE-HS [2]. Recurrent seizures seriously affect quality of life of patients and even life.,Biochemical and molecular mechanisms of mTLE are not completely understood. Early diagnosis of epilepsy and timely treatment can achieve a good therapeutic effect. Therefore, it is urgent to find a reliable molecular target and study the pathogenesis of epilepsy.

Exosomes, about 30-100nm in size, are secreted by many kinds of cells such as tumor cells, macrophages, and neurons [3, 4]. Proteins, mRNA, tRNA, and microRNAs (miRNAs) are integral parts of the exosome content [5]. Exosomes may be a key mediator in cells to cells communication by transferring content [5], More importantly, exosomal miRNAs are involved in several diseases, including glioma, inflammation, and neurodegenerative changes [6-8]. For example, exosomal miR-21 levels can be a promising indicator for glioma diagnosis and prognosis, particularly by predicting tumor recurrence or metastasis [9]. Exosome-delivered miRNAs modulate the inflammatory response to endotoxins [10]. miRNAs found in nanovesicles have shown diagnostic potential in neurodegenerative diseases [8]. Together these data indicate that exosomal miRNAs play important roles in the pathophysiology of diseases by transferring cells to cells signals.

Our previous results showed that exosomal miRNAs profiles were different between mTLE patients and healthy controls and exosomal miRNAs (miR-483-5p, miR-671-5p, and miR-150-3p) were significantly down-regulated [1]. However, expression and function of exosomal miRNAs (miR-483-5p, miR-671-5p, and miR-150-3p) in the mouse model and in mTLE patients were not understood. In this study, we aim to analyze the expression and dynamic changes of exosomal miRNAs in the mouse model and in mTLE patients and further explore the function of exosomal miRNAs in mTLE.

Materials and methods

The kainic acid mouse mode

Male C57BL/6 mice (weighing 20-25 g) were purchased from Weitong Lihua (Beijing, China) SPF grade, micro-bucket l micro injector Micro liter syringes from Shanghai High-dove Workers Trade Co., Ltd., and kainic acid from Sigma (USA). The mice were anesthetized by intraperitoneal injection with 10% chloral hydrate (100 t/20 g). The mouse skull was fixed on a stereotaxic apparatus and the body was lying flat. The model was established with reference to the Kralic epilepsy model [11, 12]. The anterior fontanel was set as the origin of coordinates: anterior fontanel = 2.0 mm, medio-lateral (ML) = 1.8 mm, and the skull (dura) dorsoventral, DV) = 2.3 mm. Kainic acid (12 mg / kg) was injected into the right hippocampus. After the injection, the wound was sutured and the mice were placed in a cage for observation and feeding. Seizures were

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classified according to the Racine classification criteria. Grade I: chewing, blinking, and other facial muscle twitching; II: nodding movement mainly due to neck muscle twitching; III level: unilateral forelimb clonus, convulsions; IV: Bilateral forelimb clonus, convulsions with the body erected; V: bilateral hind limb rigidity, body back flexion, falls with systemic clonus. In the acute phase Ⅲ and above the onset of 1 h or more sustained behavioral showed that the mouse epilepsy model was successfully constructed. If the grade Ⅲ and above onset time was too long, diazepam was injected to alleviate the epileptic status. Mice were divided into the blank control group (n = 6) and kainic acid injection group (n = 24). The kainic acid injection group was divided into three groups according to the observed behavior: acute phase (24 hour, n = 8), latent phase (1 week, n=8), and chronic phase (1 week after injection, n = 8). After the mice were anesthetized with 10% chloral hydrate, and 0.8ml blood was collected from the eye vein. The blood samples were kept for 1 hour at room temperature. Then blood samples were centrifuged for ten minutes at 2500 RPM. The plasma was transferred into a new EP tube and stored at -80℃ until processing. All procedures were approved by the Institutional Animal Care and Use Committee of Tiantan Hospital.

MTLE patients and Controls

In this study, 40 patients diagnosed with mTLE-HS and 40 gender and age matched healthy volunteers were recruited in Tiantan Hospital (Beijing, China). Blood samples were collected. This study was approved by the Institutional Ethics Committee of Tiantan Hospital and written informed consent was obtained from the parents or patients before analysis

Isolation of exosomes and QPCR miRNA assay for individual miRNAs

Exosomes were isolated and a qPCR assay was done according to our previous reports [1]. The primers of miRNAs (miR-miR-483-5p, miR-671-5p, and miR-150-3p) were designed and synthesized by RIBO (Guangzhou, China). Real-time PCR for miRNAs was performed in triplicate for each sample. The relative amount of each miRNA was normalized against cel-miR-39-3p by the $2^{-\Delta\Delta Ct}$ method.

Bioinformatics analysis of differentially expressed exosomal miRNAs

The target genes of differentially exosomal miRNAs and bioinformatics analysis were done as by our previous report [1, 13]

Statistical analysis

All statistical analyses were done using the SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) or Graphpad Prism (version 5.0; Graphpad software). The difference between two groups was determined by a two-tailed Student's t test. Receiver Operator Characteristic (ROC) curves and area under the ROC curve (AUC) were used to evaluate the diagnostic value of exosome miRNAs for differentiating between mTLE-HS patients and healthy control groups. Clinical characteristics were compared using the $\chi 2$ test of independence for qualitative variables,

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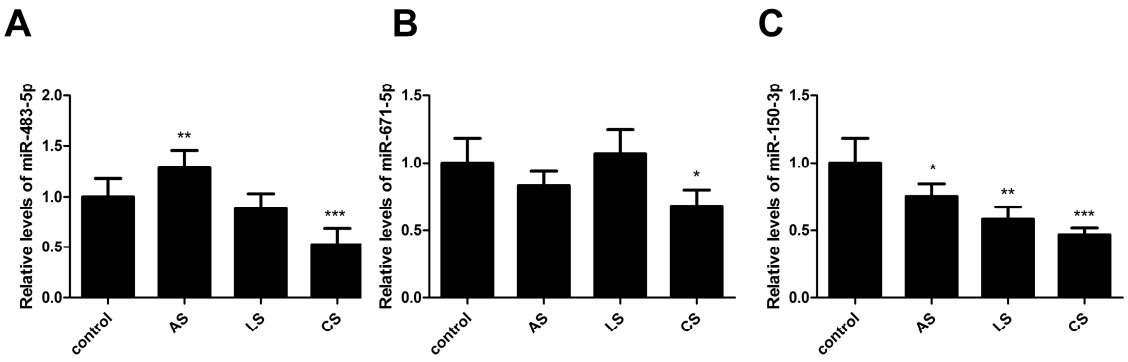
ANOVA, the t test of quantitative variables with normal distribution, and the non-parametric Kruskall-Wallis test or the Mann-Whitney U test of quantitative variables with skewed distribution, with P values of < 0.05 considered to be statistically significant.

Results:

Dynamic expression patterns of exosomal miR-miR-483-5p, miR-671-5p, and miR-150-3p in the three phases of TLE in mice.

To investigate dynamic expression changes of exosomal miRNAs in the three phases of mTLE in the mouse model, qPCRs were done. The results showed that exosomal miR-483-5p was significantly up-regulated in the acute phase and down-regulated in the chronic phase compared with controls. However, there was no significant difference between the latent phase and controls (Figure 1A). miR-671-5p was significantly down-regulated in the chronic phase while no significantly different expression was detected in the acute and latent phases (Figure 1B). At the same time, quantitative PCR (qPCR) results showed significant up-regulation of miR-150-3p expression in all plasma samples in the three phases of MTLE development in the immature mouse model（Figure 1C）. Plasma exosomal miR-483-5p, miR-671-5p, and miR-150-3p expressions were normalized to that of cel-miR-39-3p ($p<0.05$)



Expression of three exosomal miRNAs in TLE patients and the dynamic expression in the sera of pre- and 10 day post-operative TLE patients

Next, we analyzed the expression level of the exosomal miRNAs in 40 mTLE patients. It was found that expression levels of miR-miR-483-5p, miR-671-5p, and miR-150-3p were significantly decreased in TLE patients compared with healthy controls (Figure .2A). In addition, we analyzed the dynamic expression of exosomal miRNAs in the sera from a cohort of 20 pre- and 10 day post-operative TLE patients. The results showed that expression levels of miR-483-5p, miR-671-5p, and miR-150-3p were significantly increased post-operation compared with

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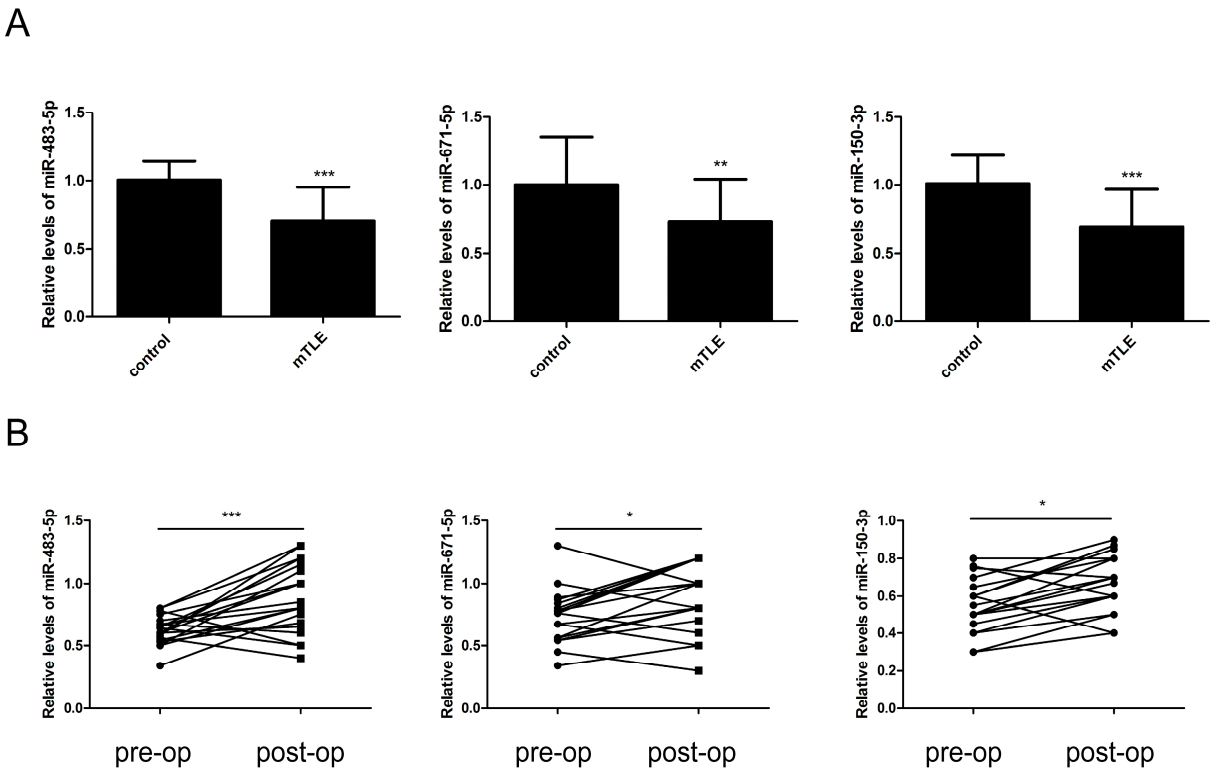
pre-operation (Figure .2B). The results indicated that exosomal miRNAs could be associated with the epileptic focus of mTLE patients.



The potential function of exosomal miRNAs in TLE based on the bioinformatics prediction

MiRNAs inhibit mRNA expression by binging 3'UTR of the targeting gene. We predicted the potential targets of these exosomal miRNAs using bioinformatic tools. The number of predicted target genes for exosomal miRNAs (miR-483-5p, miR-671-5p, and miR-150-3p) was 6, 198, and 108, respectively. To assess the potential biological function of the differentially expressed miRNAs, GO annotations and KEGG pathway enrichment analysis were performed, based on three available databases (microRNA, miRBase, and TargetScan). The GO enrichment analysis revealed that target genes of the miRNAs (miR-150-3p, miR-671-5p, and miR-483-5p) were significantly related to the apoptotic process, cell adhesion, nervous system development. axon guidance, neurotrophin TRK receptor signaling pathway, ion transport, regulation of transcription, and were DNA-dependent (Figure 3A). At the same times, significant pathways were enriched for the target genes of miR-483-5p, miR-671-5p, and miR-150-3p, including the neurotrophin signaling pathway, PI3K-Akt signaling pathway, metabolic pathways, HIF-1 signaling pathway, and Dopaminergic synapse (Table 1). In addition, the miRNAs-mRNA network for the exosomal miRNAs was constructed, based on GO and KEEG (as shown in Figure 3B).

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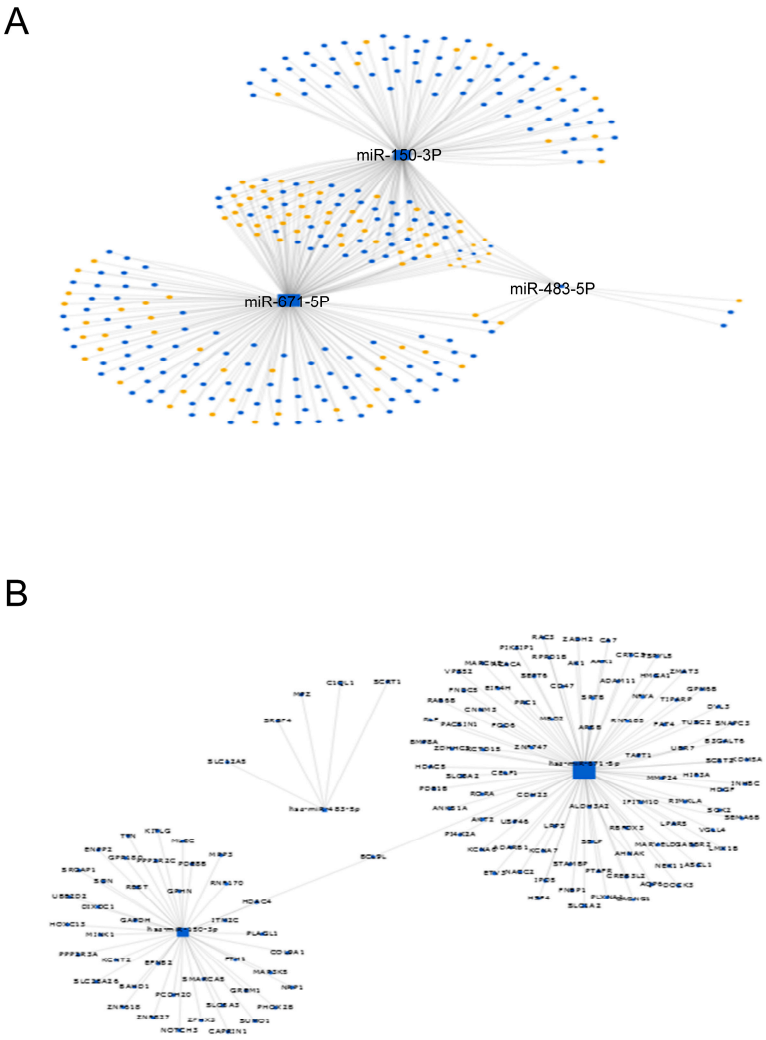

The diagnostic value of the exosomal miRNAs in TLE patients

To analyze the diagnostic value of the exosomal miRNAs, ROC curves were produced. The AUC curve areas for miR-483-5p, miR-671-5p, and miR-150-3p were found to be 0.8714 (95% CI, 0.7788–0.9651), 0.7281 (95%, 0.5928-0.8635), and 0.8213 (95% CI, 0.7185–0.9254), respectively (Figure 4 and Table 2). The results suggested that miR-483-5p and miR-150-3p have good diagnostic value in distinguishing mTLE patients from healthy controls. More importantly, when the cutoff value for miR-483-5p was 0.7281, the sensitivity and specificity were 75% and 91.67%. At the cutoff value of 0.8213 for miR-150-3p, the sensitivity was 67.5% and the specificity was 90%. These results suggested that exosomal miRNAs may be used as reliable diagnostic markers for TLE.

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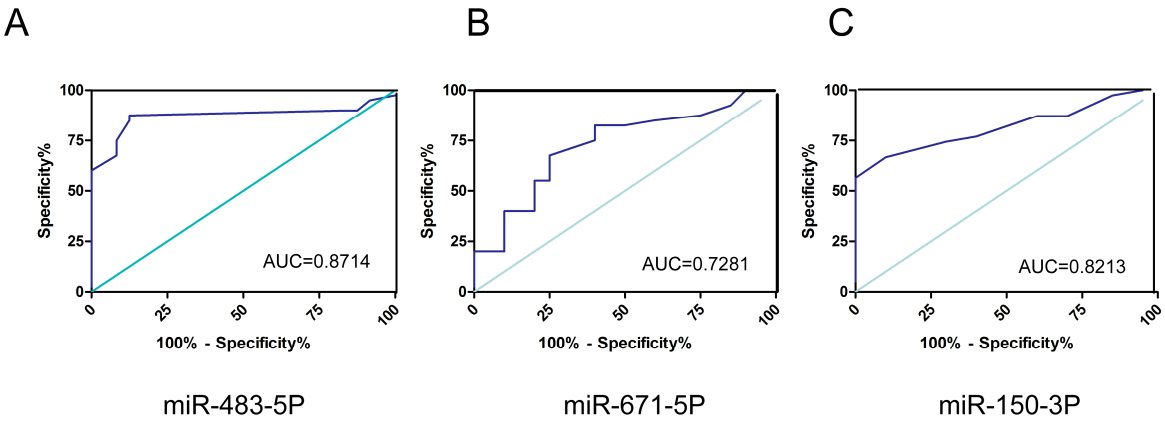

Correlation analysis between miRNAs and clinical-pathological features

Next, we investigated the correlation between exosomal miRNAs and clinical-pathological features in mTLE patients. As shown in table 3, a significant association was found between the exosomal miRNAs and clinical parameters, including miRNA-483-5p and the course of epilepsy and seizure time; miRNA-671-5p with seizure frequency and seizure time; and miRNA-150-3p with the course of epilepsy and seizure frequency. These results indicated that exosomal miRNAs are involved in the epileptogenesis of mTLE.

Discussion:

Epilepsy is one of the common nerve disorder syndromes. About 65 million people worldwide suffer from epilepsy [1]. However, the pathogenesis of epilepsy remains unclear. A better understanding of molecular and functional mechanisms underlying the mechanism of mTLE development is helpful for diagnosis and therapy [14]. In this study, we focused on expression and dynamic expression of plasma exosomal miR-150-3p, miR-671-5P, and miR-483-5p in the three phases of mTLE development in the mouse model of SE and mTLE patients and explored the function of miRNAs in the pathologensis of mTLE.

Previous studies on the effects of miRNAs in epilepsy mainly focused on the tissues and circulation, however, exomal miRNAs were less studied. For example, miRNA-129-2-3p is up-regulated in cortical brain tissue and plasma samples from patients with refractory TLE [15]. Interestingly, in FCD with refractory epilepsy, hsa-miR-4521 is up-regulated in the serum compared with healthy controls [16] . Altered plasma exosomal miRNA expression is involved in the pathology of several disease, including cancer, inflammation , neurodegenerative diseases, and epilepsy. Endogenous miR-155 and miR-146a, two critical miRNAs that regulate inflammation, are released from dendritic cells and are subsequently taken up by recipient dendritic cells.[10] Specially, exosomal proteins and miRNAs, hold great promise as novel biomarkers for clinical diagnosis in neurodegenerative diseases [8]. Our previous paper also demonstrated that exosomal miRNA profiles of mTLE patients are significiantly different from healthy controls

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Dysregulation of miRNA expression as well as its involvement in the pathophysiology of epilepsy has been previously described. Brain-specific miR-124 and miR-134 are significantly up-regulated in the seizure-related phases, suggesting that both can be potential targets for anticonvulsant drugs in epileptic developing brains [14]. The dysregulation of TNF-α and miR-155 in the three phases of mTLE development indicated that miRNAs play roles in the development of the epileptogenic process in immature brains [17]. Interestingly, the exosomal miRNAs were different in the phases of TLE, including the mouse model and TLE patients. The results indicated that the exosomal miRNAs could play important roles in development of TLE.

The mechanism of miRNAs in the pathogenesis of TLE are involved in the inflammatory reaction, apoptosis, and regeneration of nerve synapses [18-20]. In this study, the exosomal miRNAs were closely related to the apoptotic process, cell adhesion, nervous system development. axon guidance, the neurotrophin TRK receptor signaling pathway, ion transport, neurotrophin signaling pathway, PI3K-Akt signaling pathway, metabolic pathways, HIF-1 signaling pathway, and Dopaminergic synapse. Exosomal miRNAs can exist in the peripheral circulation, which could serve as diagnostic and progression biological markers, As we previously reported, miR-8071 had the best diagnostic value for mTLE-HS with 83.33% sensitivity and 96.67%, specificity [1]. In this study, miR-483-5p and miR-150-3p had better diagnostic value for distinguishing between mTLE patients and control healthy. Especially, the exosomal miRNAs were associated with clinical parameters, which could reflect the severity of TLE. All these results indicated that the exosomal miRNAs were involved in pathogenesis of TLE.

In conclusion, the differentially expressed exosomal miRNAs (miR-150-3p, miR-671-5P, and miR-483-5p) might play important roles in the pathogenesis of TLE by regulating their target genes. Furthermore, exosomal miRNAs might help to distinguish between diseased patients and healthy controls and be related to the severity of diseases. The study of these miRNAs may provide a clearer understanding of the pathogenesis of mTLE-HS and indicate that exosomal miRNAs may be potential therapeutic targets and diagnostic biomarkers for mTLE-HS. In our next study, expression levels of important target genes will be investigated by integrated miRNA and mRNA analysis to find more potential value for TLE.

Abbreviations

mTLE: mesial temporal lobe epilepsy (mTLE), AUC: area under the receiver operating characteristic curve, CI: confidence interval, GO: The Gene Ontology (GO), KEGG: Kyoto Encyclopedia of Genes and Genomes, PCa: prostate cancer. qRT-PCR: quantitative reverse transcriptase polymerase chain reaction,

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CONFLICTS OF INTEREST

All the authors declare that there are no conflicts of interest.

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Figure legends:

F1
Dynamic expression patterns of exosomal miRNAs from the plasma in the three phases of TLE in mice.
A: miR-483-5p was significantly up-regulated in the acute phase and down-regulated in the chronic phase, while in the latent phase no significantly different expression was detected.
B: miR-671-5p was significantly down-regulated in the chronic phase, while no significantly different expression was detected in the acute and latent phases.
C: miR-150-3p was significantly down-regulated in the three phases of TLE
* P<0.05,**P<0.01,P<0.001

F2: Expression of exosomal miRNAs in the plasma of patients with TLE and. dynamic expression pre-op and post-op
A: qPCR results showed that significant down-regulation of exosomal miRNAs occurred in the TLE patients compared to healthy controls.
B: The results showed that expression levels of exosomal miRNAs were significantly increased post-operation compared with pre-operation.
* P<0.05,**P<0.01,P<0.001

F3: Prediction of functions for exosomal miRNAs

A: GO categories and distribution for the predicted miRNA targets of related differential miRNAs .The squares represent miRNA. The circles represent functional elements (the yellow marker represents up-regulated functional elements and the blue marker represents

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down-regulated functional elements). The relationship between miRNA and functional elements is represented by a green line.
B: MiRNA-gene network.
The gray circles represent gene (mRNA), the squares represent miRNA (the red marker represents up-regulated miRNA and blue marker represents down-regulated miRNA). The relationship between the miRNA and gene is represented by a green line.

F4:
ROC curves for exosomal miRNAs that were significantly different in TLE patients as compared to healthy controls.
ROC curves for exosomal miR-483-5p, miR-671-5p, and miR-150-3p were used to discriminate patients and healthy controls and the area under the curve (AUC) was used to evaluate the level of discrimination.

Table1: Enriched pathways for target genes of the differentially expressed exosomal miRNAs (miR-483-5p, miR-671-5p, and miR-150-3p).

miR-483		
GABAergic synapse	Cell adhesion molecules (CAMs)	Spliceosome
miR-671-5P		
Axon guidance	Insulin signaling pathway	HTLV-I infection
Melanogenesis	Circadian entrainment	MAPK signaling pathway
Cytokine-cytokine receptor interaction)	Neurotrophin signaling pathway	Neurotrophin signaling pathway
Lysine degradation	Salivary secretion	Amyotrophic lateral sclerosis (ALS)
PI3K-Akt signaling pathway	Melanogenesis	Protein processing in endoplasmic reticulum
Dopaminergic synapse	Long-term potentiation	Pathways in cancer
mRNA surveillance pathway	Alcoholism	Pathways in cancer
Tight junction	Gastric acid secretion	Dilated cardiomyopathy
Chagas disease (American trypanosomiasis)	Estrogen signaling pathway	Hypertrophic cardiomyopathy (HCM)
Hepatitis C	Vascular smooth muscle contraction	Ubiquitin mediated proteolysis
HIF-1 signaling pathway	Glioma	Protein processing in endoplasmic reticulum 95Shigellosis
Alzheimer's disease	Oocyte meiosis	Huntington's disease

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Metabolic pathways	Phototransduction	Morphine addiction
Mineral absorption	Alzheimer's disease	Purine metabolism
Axon guidance	GnRH signaling pathway	Amphetamine addiction
Protein digestion and absorption	Pertussis	Dopaminergic synapse
Calcium signaling pathway	Tuberculosis	Cardiac muscle contraction
Long-term potentiation	Phosphatidylinositol signaling system	Axon guidance
miR-150-3P		
Long-term potentiation	Dopaminergic synapse	Protein digestion and absorption
Amphetamine addiction	Insulin signaling pathway	Calcium signaling pathway
Circadian entrainment	Long-term potentiation	Pertussis
Neurotrophin signaling pathway	Alcoholism	Tuberculosis
Salivary secretion	Gastric acid secretion	Phosphatidylinositol signaling system
Melanogenesis	Estrogen signaling pathway	PI3K-Akt signaling pathway
Chagas disease (American trypanosomiasis)	Vascular smooth muscle contraction	Dopaminergic synapse
Hepatitis C	Glioma	mRNA surveillance pathway
Axon guidance	Oocyte meiosis	Huntington's disease
HTLV-I infection	Phototransduction	Morphine addiction
Axon guidance	Alzheimer's disease	Purine metabolism
MAPK signaling pathway	GnRH signaling pathway	PI3K-Akt signaling pathway
Neurotrophin signaling pathway	Pathways in cancer	Dopaminergic synapse
Amyotrophic lateral sclerosis (ALS)	Melanogenesis	mRNA surveillance pathway
Protein processing in endoplasmic reticulum	Cytokine-cytokine receptor interaction	Tight junction
Lysine degradation	PI3K-Akt signaling pathway	HIF-1 signaling pathway
Alzheimer's disease	Metabolic pathway	Mineral absorption
Axon guidance		

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Table2 Area under the receiver operating characteristic curve (AUC), 95% confidence interval (CI), and P values of the differentially expressed microRNAs.

Exomal- miRNAs	AUC	95%CI	P value
483-5p	0.8714	0.7788-0.9651	<0.001
671-5p	0.7281	0.5928-0.8635	0.004
150-3p	0.8213	0.7185-0.9240	<0.001

Table3 Demographic and clinical data of patients with mTLE.

Characteristic	miR-483-5P		P	miR-671-5P		P	miR-150-3P		P
	High	Low		High	Low		High	Low	
Male: Female	11:9	13:7	NS	10:10	14：6	NS	12:8	12:8	NS
Age (range)	23.2y (11-38)	24.50 (13-45)	NS	21.20 （12-29）	25.60 （15-45）	NS	22.32 (11-40)	24.52 (13-45)	NS
Family history of epilepsy	3	1	NS	0	4	NS	2	2	NS
Course of epilepsy (range)	11.20 (1-30y)	16.35 (3-35y)	*	10.05 (2-28y)	15.32 (1-35y)	*	9.5 (1-35y)	14.20. (3-27y	*
Onset of seizure	10	8	NS	12	13	NS	11	13	NS
Seizure frequency (range) （months）	28,3 5-300	30.5 1-300	NS	25.2 2-300	60.54 10-300	***	34.32 10-280	72.32 5-300	**
Seizure time (range)	0.8m 10s-2m	1.5m 3s-3.2m	*	1.2m 3s-1.5m	1,5m 15s-3.2m	*	1.15m 3s-1,5 m	1.35m 10s-3. 2m	NS
Laterality of epileptogenic zone									
Right	7	7	NS	8	6	NS	5	7	NS
Left	9	10		7	12		12	7	
Bilateral	4	3		5	2		2	5	

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AED therapy at the last clinic visit									
Two AEDs	7	6	NS	4	5	NS	6	7	NS
More than two AEDs	13	14		16	15		14	13	

y; year, m: minutes, s: seconds, * P<0.05, **p<0.01, ***p<0.001, NS: not significant