Identification of hsa-miR-1275 as a Novel Biomarker Targeting *MECP2* for Human Epilepsy of Unknown Etiology

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Epilepsy affects around 70 million people worldwide, with a 65% rate of unknown etiology. This rate is known as epilepsy of unknown etiology (EUE). Dysregulation of microRNAs (miRNAs) is recognized to contribute to mental disorders, including epilepsy. However, miRNA dysregulation is poorly understood in EUE. Here, we conducted miRNA expression profiling of EUE by microarray technology and identified 57 pathogenic changed miRNAs with significance. The data and bioinformatic analysis results indicated that among these miRNAs, hsa-microRNA (miR)-1275 was highly associated with neurological disorders. Subsequently, new samples of serum and cerebrospinal fluid were collected for validation of hsa-miR-1275 expression by TaqMan assays. Results show that hsa-miR-1275 in serums of EUE were increased significantly, but in cerebrospinal fluid, the miRNA was decreased. Moreover, the *MECP2* gene was selected as a hsa-miR-1275 target based on target prediction tools and gene ontology analysis. Validation of *in vitro* tests proved that MECP2 expression was specifically inhibited by hsa-miR-1275. Additionally, overexpression of hsa-miR-1275 can elevate expression of nuclear factor κB (NF-κB) and promote cell apoptosis. Taken together, hsa-miR-1275 might represent a novel biomarker targeting *MECP2* for human EUE.

INTRODUCTION

Epilepsy is one of the most frequent chronic neurological disorders characterized by recurring, unprovoked seizures associated with elevated mortality rate, decreased social participation, and quality of life. Epilepsy has a high prevalence, and around 70 million individuals worldwide are supposed to be suffering from epilepsy.[1] The International League Against Epilepsy (ILAE) seizure classification emphasizes consideration of etiology at each step of epilepsy diagnosis because of the potential therapeutic consequences. Etiology is broken into six nonhierarchical groups, including "genetic," "structural," "metabolic," "immune," "infectious," and "unknown" types.[2] Epilepsy of unknown etiology (EUE) is also known as epilepsy of unknown cause, with presumed symptomatic nature and no known identified genetic, structural, infectious, or metabolic etiology.[2,3] It was found that approximately 65% epilepsy patients have epilepsies of unknown etiology.[2]

The pathogenesis of different types of epilepsy involves many important molecular changes and biological pathways that contribute to the epileptogenic process, mainly including genetic variants,[4] DNA methylation,[5] and small noncoding RNAs.[6] MicroRNAs (miRNAs or miRs) are a family of small noncoding RNAs involved in the controlling process of the expression of various proteins in fundamental processes of epileptogenesis by reduction of mRNA stability and translation and could therefore be key regulatory molecular mechanisms with a therapeutic potential for epilepsy.[7,8] It has been recognized that mature miRNA forms base pairs with mRNA, often in the 3′ untranslated region (UTR), to induce mRNA degradation or translational repression.[8] miRNAs have been found to regulate translation of a wide range of processes in epilepsy.[9] Functional studies have identified contributions from miR-34a and miR-132 to seizure-induced neuronal death, whereas silencing miR-34 potently reduced status epilepticus, seizure damage, and the later occurrence of spontaneous seizures.[10,11] These studies show a fine correlation between miRNA regulation or dysregulation and inflammation, seizure-induced neuronal death, and other relevant biological pathways. However, the molecular etiologies for the majority of epilepsy patients remain elusive.[12] So far, few studies have investigated the possible role of miRNAs in the pathogenesis and epileptogenesis of EUE.

Table 1. Demographic and Clinical Data of Patients Included in the Study

	Numbering	Cohort	Diagnosis	Sample	Age	Gender
EUE Patients	S1	miRNA chip	EUE	serum	26	F
	S2	miRNA chip	EUE	serum	21	F
	S3	TaqMan assay	EUE	serum	24	M
	S4	TaqMan assay	EUE	serum	27	F
	S5	TaqMan assay	EUE	serum	22	M
	S6	TaqMan assay	EUE	serum	12	F
	S7	TaqMan assay	EUE	serum	27	M
	S8	TaqMan assay	EUE	serum	27	M
	S9	TaqMan assay	EUE	serum	21	F
	S10	TaqMan assay	EUE	serum	25	M
	S11	TaqMan assay	EUE	serum	32	M
	S12	TaqMan assay	EUE	serum	22	F
	S13	TaqMan assay	EUE	serum	27	M
	S14	TaqMan assay	EUE	CSF	37	M
	S15	TaqMan assay	EUE	CSF	46	F
	S16	TaqMan assay	EUE	CSF	24	F
	S17	TaqMan assay	EUE	CSF	21	F
	S18	TaqMan assay	EUE	CSF	28	F
	S19	TaqMan assay	EUE	CSF	21	F
Control	C1	microRNA chip	normal	serum	21	F
	C2	TaqMan assay	normal	serum	47	F
	C3	TaqMan assay	normal	serum	23	F
	C4	TaqMan assay	normal	serum	31	F
	C5	TaqMan assay	normal	serum	24	M
	C6	TaqMan assay	normal	serum	19	F
	C7	TaqMan assay	normal	serum	24	F
	C8	TaqMan assay	normal	serum	19	F
	C9	TaqMan assay	normal	serum	36	M
	C10	TaqMan assay	normal	serum	32	F
	C11	TaqMan assay	normal	serum	43	F
	C12	TaqMan assay	headache	CSF	48	F
	C13	TaqMan assay	gap cerebral infarction	CSF	40	F
	C14	TaqMan assay	viral meningitis	CSF	44	F

miRNA, microRNA; EUE, epilepsy of unknown etiology; CSF, cerebrospinal fluid; F, female; M, male.

Therefore, this study is aimed to broaden the knowledge about the role of miRNAs in EUE.

RESULTS
miRNA Profiling Analysis Reveals Differentially Expressed miRNAs in EUE

Here, we investigated miRNA expression patterns in blood serum of patients with EUE (S1 and S2, as listed in Table 1) as compared to healthy control (C1, as listed in Table 1), using a miRNA microarray that contains all 866 human miRNAs and miRNA star sequences deposited in the Sanger miRBase version 16.0. A total number of 57 significantly dys-regulated miRNAs in EUE were identified, which demonstrated that unique miRNA signatures in patients with EUE allow accurate differentiation from healthy controls. Principal-component analysis (PCA) of the changes of miRNA expression demonstrated that EUE miRNA expression profiles have similar expression patterns (shown in red) (Figure 1A). In contrast, mRNA-based PCA clearly separates EUE patients (S1 and S2) from the normal control sample C1 (shown in blue). Furthermore, based on the miRNA array results, we created a heatmap to better visualize the changes in miRNA expression between the normal control C1 (Figure 1B; first column) and the EUE patients S1 and S2 (Figure 1B; second and third columns). The clustering analysis

A

C

B
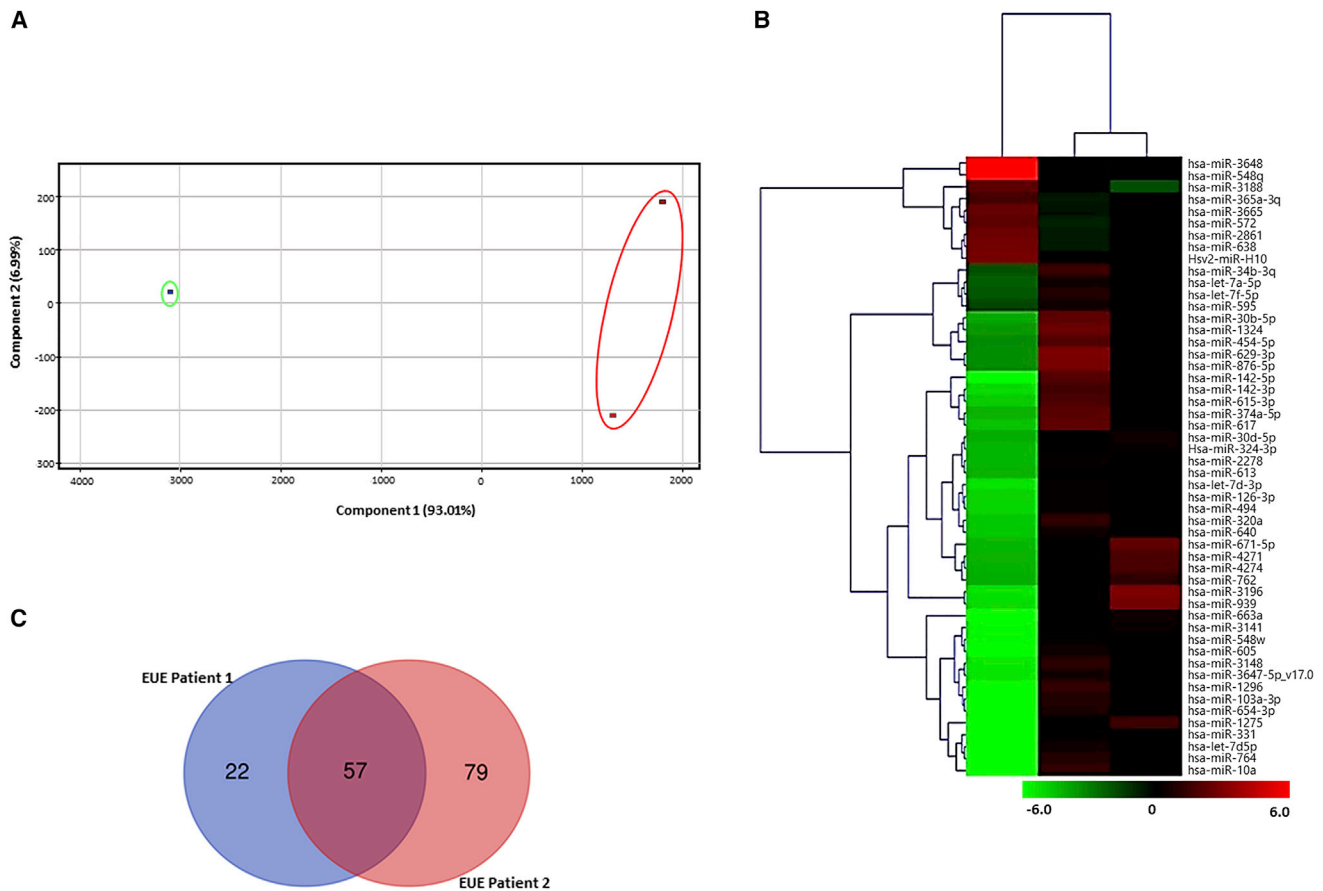
Figure 1. Differentially Regulated miRNA in Epilepsy of Unknown Cause (EUE) Samples

(A) Principal-component analysis (PCA) of all samples of the dataset. mRNA-based PCA clearly separates EUE patients (S1 and S2) (shown in red) from the normal control sample C1 (shown in blue). (B) Cluster dendrogram of control serum sample (C1) and EUE patients' serum samples (S1 and S2). (C) Venn diagram shows the overlap between the two EUE patients' serum samples.

reveals that the expression pattern of the EUE patients tends to be similar compared with normal control. The Venn diagram shows that there are 57 miRNAs with significant differences shared between the two EUE patients S1 and S2 (Figure 1C). Table 2 shows detailed information of the top 20 differentially regulated miRNA in EUE patients (fold change [FC] value > 30), all of which have not been recognized to be associated with EUE before. Altogether, changes to 57 different miRNAs were identified, providing compelling evidence that EUE is associated with widespread changes to miRNA expression. These data

suggest that miRNA expression signatures may represent a potentially useful biomarker for the diagnosis of EUE and that dysregulation of miRNA expression could play a role in the complex EUE pathogenesis.

hsa-miR-1275 Was Frequently Differentially Regulated in EUE

In an effort to illuminate the miRNA regulation of EUE, the cellular components of the top 20 miRNAs with significant differences were analyzed by ClueGO, and hsa-miR-1275 was found to participate in the regulation of neuronal and synaptic construction with significance (Figure 2A). miRNA functions are classified based on their targets. Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of miRNA target genes shows that hsa-miR-1275 was involved in the process categories of obsolete synapse part (Gene Ontology [GO]: 0044456, $p = 0.000929$), postsynaptic density (GO: 0014069, $p = 0.18404$), and postsynaptic membrane (GO: 0045211, $p = 0.113404$). Additionally, hsa-miR-1275 was subjected to coexpression meta-analysis (CoMeTa) to assigned functional annotations via the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Table 3) and GO (Table 4). Results show that hsa-miR-1275 has a

Table 2. PCR Primer Sequences

Name	Forward Primers, 5′ to 3′	Reverse Primers, 5′ to 3′
Wild-type 3′ UTR of *MECP2*	CTCTACGCTCCC CCAGGCAAAGT	CTAGACTTTGCCTGGGGG AGTGCGTAGAGAGCT
Mutant 3′ UTR of *MECP2*	CTCTACGCGG CAAAGT	CTAGACTTTGCCG TGCGTAGAGAGCT
MECP2	TGTTCCGCCC AGTGGATTC	GCACGGGATGGTG GTAATAATCTC
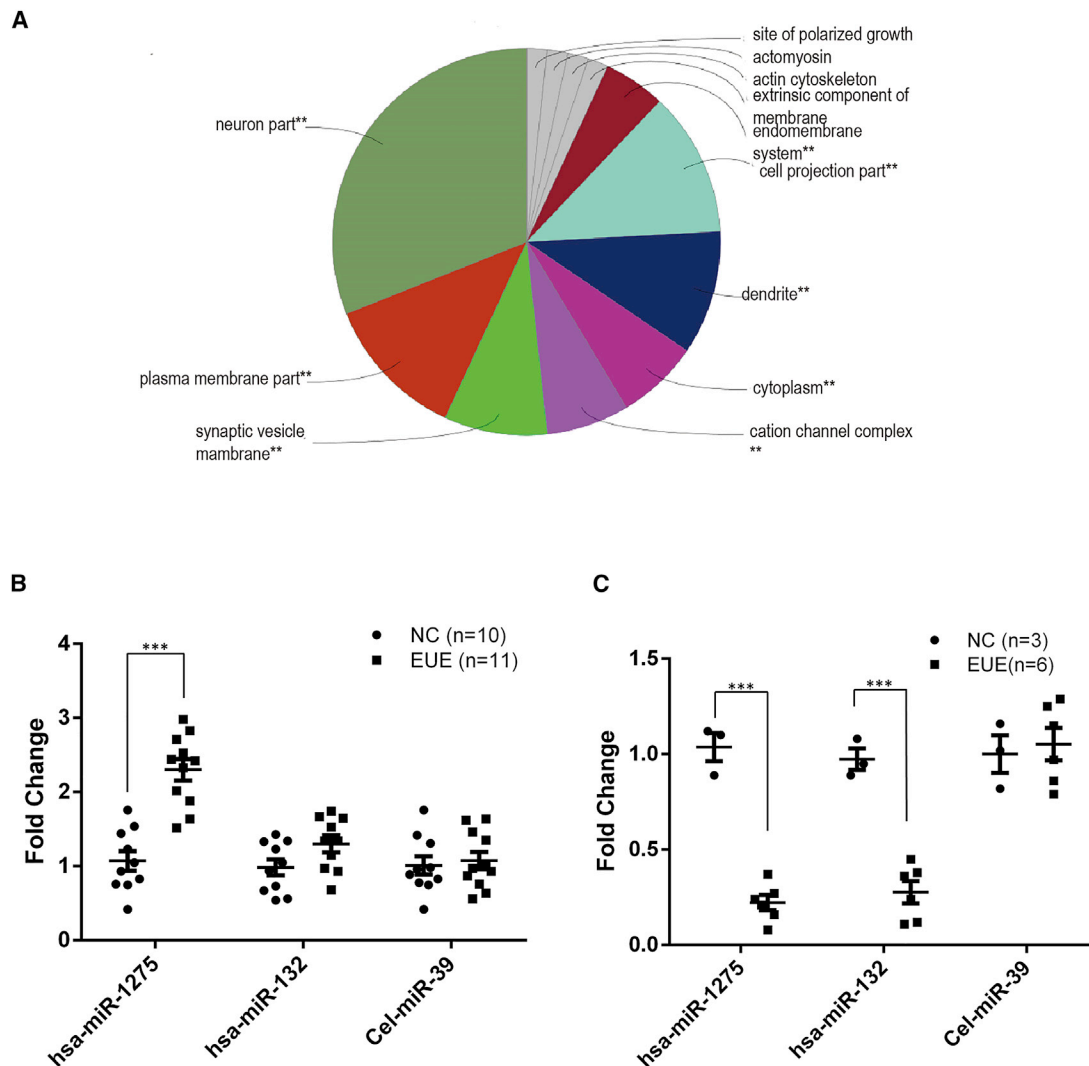
Figure 2. hsa-miR-1275 Was Frequently Differentially Regulated in EUE

(A) Analysis of cellular components of hsa-miR-1275 by ClueGO following a Benjamini-Hochberg correction. (B) Detection of hsa-miR-1275 in serum of EUE by TaqMan assays. (C) Detection of hsa-miR-1275 in cerebrospinal fluid by TaqMan assays. hsa-miR-132 was a positive control, and cel-miR-39 was used as the housekeeping gene for the detection of hsa-miR-1275 expression.

major function of neuronal regulation and participates significantly in mitogen-activated protein kinase (MAPK) signal pathway, calcium signaling pathway, cell adhesion, nerve impulse conduction, and neuronal differentiation, etc.

To validate the miRNA array findings and assess the biological role of hsa-miR-1275 in EUE, we compared hsa-miR-1275 expression by the TaqMan assay in serum with controls (n = 10) and EUE (n = 11) and in cerebrospinal fluid (CSF) with controls (n = 3) and EUE (n = 6). The hsa-miR-132 was a positive control since it has been reported to play an important role in epilepsy.[10] Caenorhabditis elegans miR-39 (cel-miR-39) was used as the housekeeping gene for hsa-miR-1275 expression detection.[13] As a result (Figures 2B and 2C),

hsa-miR-1275 increased significantly in the serum of EUE compared with normal control, which is consistent with the results of the miRNA microarray. However, hsa-miR-132 exhibited no significant difference in serum. Conversely, compared with CSF samples of patients suffering headache, gap cerebral infarction, and viral meningitis, the expression of hsa-miR-1275 in EUE patients' CSF samples decreased remarkably. hsa-miR-132 was also found to decrease significantly in CSF. These data suggested that the hsa-miR-1275 was frequently differentially regulated in EUE.

hsa-miR-1275 Targeting Inhibits MECP2 Expression *In Vitro*

The hsa-miR-1275-targeted genes were identified and analyzed by the common results of three miRNA target-predicting programs

Table 3. Differentially Expressed miRNA

Systematic Name	Regulation	FC ([cct] versus [css])	FC ([jf] versus [css])	Accession No.	chr Position
hsa-miR-548q	down	−200.89172	−213.36247	MIMAT0011163	chr10: 12767281–12767267[+]
hsa-miR-3648	down	−100.48062	−106.718155	MIMAT0018068	chr21: 9825868–9825879[−]
hsa-miR-3148	up	31.816053	49.91659	MIMAT0015021	chr8: 29814818–29814803[+]
hsa-miR-4274	up	33.671272	15.942899	MIMAT0016906	chr4: 7461824–7461835[−]
hsa-miR-548w	up	36.980785	40.145424	MIMAT0015060	chr16: 26036576–26036589[−]
hsa-miR-4271	up	37.7889	17.155443	MIMAT0016901	chr3: 49311595–49311609[−]
hsa-miR-605	up	40.99458	49.95323	MIMAT0003273	chr10: 53059355–53059370[−]
hsa-miR-103a-3q	up	41.106613	58.544506	MIMAT0000101	chr5: 167987970–167987963[+]
hsa-miR-1296	up	41.78882	67.24085	MIMAT0005794	chr10: 65132753–65132740[+]
hsa-miR-142-5p	up	42.252388	110.395546	MIMAT0000433	chr17: 56408628–56408611[+]
hsa-miR-654-3p	up	42.64155	56.577477	MIMAT0004814	chr14: 101506610–101506627[−]
hsa-miR-3141	up	43.907665	37.695004	MIMAT0015010	chr5: 153975599–153975587[+]
hsa-let-7d-5p	up	47.994743	59.989166	MIMAT0000065	chr9: 96941126–96941144[−]
hsa-miR-381	up	53.535255	56.77355	MIMAT0000736	chr14: 101512312–101512326[-]
hsa-miR-764	up	54.946342	77.98531	MIMAT0010367	chrX: 113873935–113873949[−]
hsa-miR-671-5p	up	57.011173	18.432907	MIMAT0003880	chr7: 150935548–150935557[−]
hsa-miR-1275	up	97.33257	52.137253	MIMAT0005929	chr6: 33967782–33967770[+]
hsa-miR-939	up	129.50027	28.656506	MIMAT0004982	chr8: 145619401–145619390[+]
hsa-miR-3196	up	161.56393	30.261171	MIMAT0015080	chr20: 61870146–61870157[−]
hsa-miR-663a	up	171.71622	139.51718	MIMAT0003326	chr20: 26188857–26188845[+]

chr, chromosome.

(TargetScan, miRWalk, and miRanda). Pathway analysis was performed using ClueGO to understand the biological importance of the hubs. Then, the obtained genes and pathways were used for extended network construction, analysis, identification of hub, and pathway enrichment analysis. Figure 3A shows the chart of the clustering and visualization of functionally connected genes. The enrichment was analyzed by the right-sided hypergeometric test with a Benjamini-Hochberg correction, and the terms were linked with a κ score of 0.3. The key gene *MECP2*, one of the target genes of hsa-miR-1275, was identified based on the analysis approach, which has extensive interactions with hsa-miR-1275 in neuronal development and neuromodulation-related signal pathways, such as nervous system development, negative regulation of the neuron apoptotic process, long-term synaptic potentiation, sensory perception of pain, negative regulation of smooth muscle cell differentiation, behavioral fear response, histone methyl-

ation, cell proliferation, and apoptosis process regulation. Therefore, *MECP2* was selected as a candidate target of hsa-miR-1275 for further testing.

To explore the interaction region of *MECP2* and hsa-miR-1275, three algorithms (microrna.org, DIANA Lab, and TargetScan) were applied. It was predicted that the 3′ UTRs of *MECP2* might be the potential binding site for hsa-miR-1275 identification. After a multispecies alignment of 3′ UTRs of *MECP2* (Figure 3B), it was found that the regulated sequence is highly conserved in humans and apes. Figure 3C shows the complementary relationship of the seed sequence of hsa-miR-1275 and 3′ UTR of *MECP2* (425–431).

To evaluate the biological function of hsa-miR-1275 with *MECP2*, we first examined the expression of MECP2 after transfection with the

Table 4. hsa-miR-1275 KEGG Pathway

Signaling Pathway	Number of Genes	Percentage	p Value (%)	Fold Enrichment	Bonferroni	Benjamini	FDR
MAPK signaling pathway	22	4.24	0.0002	2.44	0.03	0.03	0.24
Ca$^+$ signaling pathway	17	3.28	0.0002	2.86	0.03	0.02	0.29
Cell adhesion molecule	14	2.70	0.0004	3.14	0.06	0.02	0.51
Receptor-ligand interactions of neuronal activity	20	3.85	0.0009	2.31	0.11	0.03	1.01
Axon orientation	12	2.31	0.0038	2.75	0.40	0.08	4.41
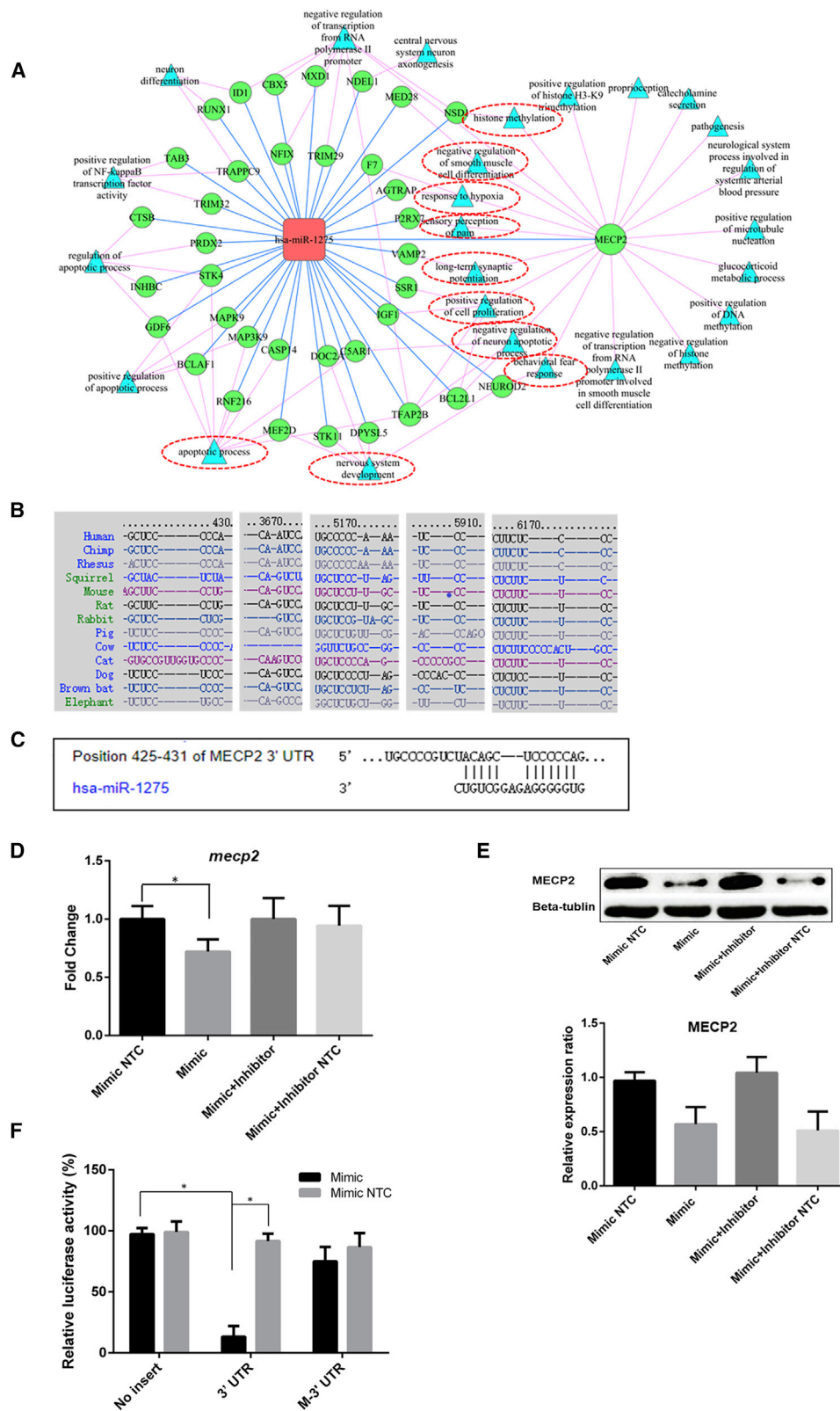
(legend on next page)

hsa-miR-1275 mimic (50 nM) after 24 h and then cotransfection with inhibitor (200 nM) after 48 h in the U251 cells. Results in Figure 3D show that the transcription of *MECP2* was significantly inhibited by the hsa-miR-1275 mimic compared to the negative control (mimic NTC), and the inhibition function was rescued by the inhibitor after 48 h. The inhibition of MECP2 expression by the hsa-miR-1275 mimic was further proven by western blot (Figure 3E). After transfection with the inhibitor of hsa-miR-1275, the inhibition of MECP2 expression was reversed.

To further confirm that hsa-miR-1275 directly targets *MECP2* by interaction with the 3′ UTR, the binding region from the 3′ UTR of *MECP2* was inserted downstream to the luciferase reporter gene in the pmirGLO vector. A mutant construct for the hsa-miR-1275 binding site was also prepared in which the predicted binding sequence had been deleted. Results show that luciferase activity was markedly decreased when cells were transfected with hsa-miR-1275 mimics. On the other hand, luciferase activity showed no obvious change when mutant constructs with deletion of seed binding sites of the 3′ UTRs were transfected. All of these findings indicate direct targeting and transcriptional inhibition of the target genes by hsa-miR-1275.

Overexpression of hsa-miR-1275 Promotes Cell Apoptosis

We next investigated the effects of hsa-miR-1275 on phenotypes of U251 cells. hsa-miR-1275 mimic (50 nM) was transiently transfected into U251 cells. As a control, cells were transfected with mimic NTC without specifically targeting any human gene products. After 48 h, cells transfected with the hsa-miR-1275 mimic were found to grow more slowly than the NTC. Several apoptotic features, such as chromatin condensation, nuclear membrane dissolution, rupture of the nucleus, and nuclear disintegration, were observed (Figure 4A). We further detected cell apoptosis by flow cytometry (Figure 4B). After transfection with the hsa-miR-1275 mimic, the cell number in late stage of apoptosis increased by 8.2%. These results indicated that the inhibition of cell growth by hsa-miR-1275 was associated with increased apoptosis.

It has revealed from the bioinformatics analysis above that hsa-miR-1275 participates significantly in the MAPK signal pathway. Nuclear factor κB (NF-κB), as one of the most important downstream factors of MAPK, was detected by transfection of pNFκB-TA-luc into U251 cells. Results show that NF-κB was positively regulated by hsa-miR-1275, which might be an inducible factor for cell apoptosis (Figure 4C).

DISCUSSION

EUE, which is presumed to be symptomatic in nature and of an unidentified cause, is a significant disease burden with limited treatment.[3] Improved understanding of the mechanisms coordinating gene expressions or regulations in patients and maintenance of the epileptic state consequential upon EUE may help to identify novel therapeutic targets and biomarkers. Large-scale alterations are thought to be involved in EUE pathogenesis, including gene expressions of controlling neurotransmitter signaling, ion channels, synaptic structure, neuronal death, gliosis, and inflammation.[2,3] It has been described that a number of serum neurological autoantibodies in association with autoimmune encephalopathy or epilepsy were detectable in EUE.[14] A report has further demonstrated common anatomic abnormalities in patients with EUE, including a volume reduction in the thalamus, cerebellum, hippocampus, caudate, and cerebral white matter.[15] The reduction of pre-existing cerebellar volume in patients with EUE may be associated with the underlying epileptic pathogenesis that has led to seizures.[15] Despite these significant observations, the molecular etiologies for EUE remain elusive.

Emerging evidence has shown that miRNAs appear to be the key in regulation of neuronal development and apoptosis, synaptic functions, neuronal microstructure, and inflammation.[16] Therefore, miRNA-mediated gene regulations provide new insights into the etiology of human neurological diseases.[7] One of the first miRNAs recognized to be differentially expressed in neuron regulation is miR-132, a cyclic AMP (cAMP)-response-element binding protein (CREB)-regulated miRNA involved in dendritic growth and morphology in hippocampus.[17,18] A previous study has elaborated that the status epilepticus induced by pilocarpine resulted in increasing levels of miR-132.[10] Additionally, miR-132 antagomirs significantly reduced the damage of the hippocampus.[19] However, the target genes of miR-132 responsible for the facilitation of neuronal death remain to be determined. Among the functional studies involving miRNAs, miR-34a is upregulated during seizure-induced neuronal death or apoptosis, and targeting miR-34a is associated with an inhibition of an increase in activated caspase-3.[11] Besides, it has been reviewed that hsa-miR134, -miR-146a, -miR-124, -miR-199a, -miR-128, -miR-15a-5p, and -miR-194-5p, etc., could be potential noninvasive biomarkers for the epilepsy diagnosis in future.[20] Taken together, miRNAs represent a potentially important mechanism to interrupt epileptogenesis pathways. A following issue is whether common miRNA profiles are present in EUE.

Figure 3. Effect of Exogenous hsa-miR-1275 on MECP2 Expression *In Vitro*

(A) Gene and pathway interaction networks. The green nodes are the hub genes; the blue triangles are signal pathways. The red circles were for the corresponding edges to highlight the crosstalk between hsa-miR-1275 and *MECP2*. All pathways represented here are highly significant following a Benjamini-Hochberg correction. Pathway enrichment analysis and visualization were performed in Cytoscape version 3.0.2. (B) Binding sites of 3′ untranslated region (3′ UTR) with *MECP2* and sequence alignment between multiple species. (C) The complementary relationship of the seed sequence of hsa-miR-1275 and 3′ UTR of *MECP2* (425–431). (D) qRT-PCR detection of the transcription of *MECP2* after transfection with hsa-miR-1275 mimic, mimic negative control (mimic NTC), and cotransfection with the inhibitor of hsa-miR-1275 and inhibitor NTC. (E) Representative western blot results of MECP2 and semiquantifications of the western blot bands by ImageJ software. (F) Luciferase reporter assays to validate the targeting 3′ UTR of *MECP2* by hsa-miR-1275. The 3′ UTR of *MECP2* (and mutant) with the hsa-miR-1275 binding region was cloned and inserted into the pmirGLO vector. The U251 cells were cotransfected with hsa-miR-1275 mimics or mimic NTCs. Cells were analyzed for luciferase activity after 48 h. For each transfection, luciferase activity was averaged from three replicates. Data were expressed as mean ± SD. *p < 0.05.
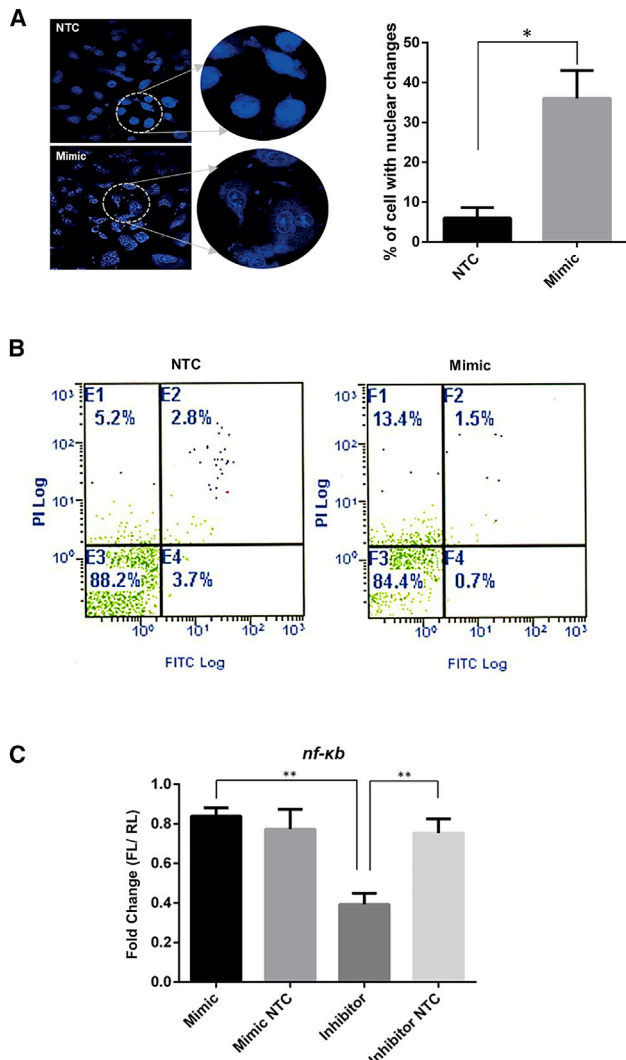
Figure 4. Overexpression of hsa-miR-1275 Promotes Cell Apoptosis
(A) DAPI attaining nucleus of U251 transfected with (top) hsa-miR-1275 mimic NTC (100 nM) and (bottom) mimic (100 nM). Cells: original magnification, 100×. (B) Detection of cell apoptosis by flow cytometry (Annexin V FITC-propidium iodide [PI]). (C) The transcription level of NF-κB affected by hsa-miR-1275. U251 cells were cotransfected with pNFκB-TA-luc and hsa-miR-1275 mimic (50 nM), inhibitor (100 nM), or NTCs. For each transfection, luciferase activity was averaged from three replicates. Data were expressed as mean ± SD. *p < 0.05, **p < 0.01.

At the beginning of this study, we manipulated miRNA profiling to uncover the emerging potential of serum-detected miRNAs as biomarkers and to improve the understanding of the pathogenesis of EUE. miRNA profiling data revealed that a series of unique miRNA responses have been generated in EUE. Almost all of the miRNAs with significant differences identified in the EUE serum have, so far, not been associated with seizures. Some of them were involved in neurological disease regulation. For example, hsa-miR-4271 was downregulated to promote neuronal differentiation in Parkinson's disease,[21] miR-572 was identified to improve early postoperative cognitive dysfunction by downre-
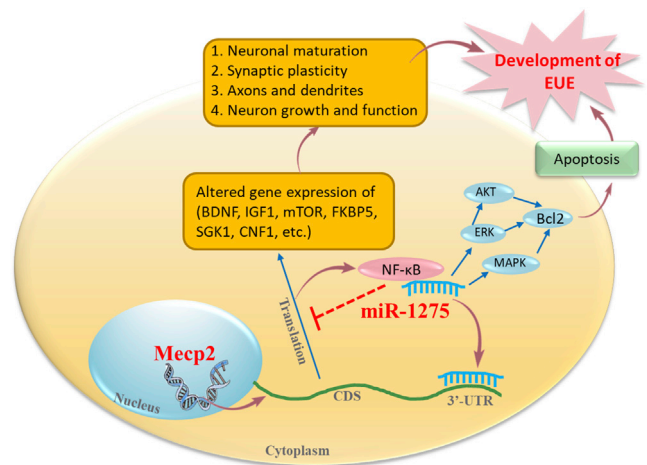
Figure 5. Involvement of the hsa-miR-1275-*MECP2* Interaction in Human EUE
Network of the predicted hsa-miR-1275-*MECP2* signal in human EUE. *MECP2* was supposed to be a potential target for hsa-miR-1275 in EUE regulation. MECP2 plays a critical role in neuronal maturation, synaptic plasticity, axons and dendrites, and neuron growth by alteration of brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF1), serum glucocorticoid-inducible kinase 1 (SGK), FK506-binding protein 5 (FKBP5), cytotoxic necrotizing factor 1 (CNF1), and mechanistic target of rapamycin (mTOR), etc. By binding to the 3′ UTRs of MECP2, hsa-miR-1275 might inhibit *MECP2* expression. Simultaneously, hsa-miR-1275 positively regulates NF-κB expression and promotes cell apoptosis.

gulating neural cell adhesion molecule 1,[22] and miR-494 exacerbated neurodegeneration by reduction of an oxidative sensor.[23] Although the roles of miRNAs in epilepsy pathophysiology remain controversial, our results provide additional evidence for potential involvement of miRNAs in this process. In all, these findings suggest an alternative mechanism of EUE under miRNA regulation.

Among dysregulated miRNAs with significant differences, hsa-miR-1275, with a 17-base pair (bp) intergenic miRNA, is encoded by chromosome 6.[24] A previous study demonstrated that hsa-miR-1275 can function as a tumor suppressor and control hepatocellular carcinoma (HCC) tumor growth by targeting several oncogenic members of the insulin-like growth factor (IGF) axis.[25] Transcription of hsa-miR-1275 could be regulated by the response of tumor necrosis factor (TNF)-α and interleukin (IL)-6 through the NF-κB pathway in obesity.[24] hsa-miR-1275 can inhibit the differentiation of human visceral preadipocytes by suppressing ELK1.[26] The expression of hsa-miR-1275 was downregulated in the serum of pregnant women with fetuses having neural tube defects.[27] In neurological diseases, hsa-miR-1275 has been identified as highly expressed in stroke patients' peripheral blood.[28] Furthermore, hsa-miR-1275 has been identified to be differentially expressed in mesial temporal lobe epilepsy (mTLE) with hippocampal sclerosis (HS).[29,30] However, the knowledge about the regulation of miR-1275 in epilepsy is limited. In this study, hsa-miR-1275 was chosen based on DAVID analysis for target genes, which reveals that hsa-miR-1275 is involved in the regulation of obsolete synapse part, postsynaptic density, and postsynaptic membrane. With the use

of biological validation of hsa-miR-1275 in serum and CSF of EUE patients, we have discovered that hsa-miR-1275 was elevated in serum of EUE. The discovery has revealed new possibilities for EUE diagnosis.

In the results of hsa-miR-1275 validation, it was moreover observed that hsa-miR-1275 was downregulated in CSF with significance. The controversial distribution pattern of hsa-miR-1275 in blood serum and CSF might attribute to the dysfunction of the blood-brain barrier (BBB). As known, blood is a systemic circulation fluid, and its composition may reflect pathological changes in different tissues or organs besides nervous system. On the other hand, CSF is protected by the BBB, which might have a higher predictive accuracy for neurodegenerative diseases since blood molecules can rarely cross the BBB.[31] It is evident that BBB disruption can induce epileptogenesis and promote the generation of seizures.[32–34] BBB leakage is observed after perinatal asphyxia, which is associated with the presence of seizures.[35] However, the complex interactions between BBB leakage and epileptogenesis are not fully clarified. One of the explanations is that BBB leakage and brain inflammation can lead to a lower threshold for seizures and create a vicious cycle that ultimately leads to epilepsy.[34] On the other hand, BBB dysfunction can be caused by seizure activity.[36] Interestingly, it is puzzling whether the BBB dysfunction during status epilepticus is just a consequence of seizures or that it can also contribute to the seizure's occurrence.[36] One of the most obvious consequences of BBB leakage is that the serum albumin diffuses into the brain tissue, which is associated with an astroglia response.[37] This evidence suggests a potential mechanism that BBB dysfunction is associated with molecular transmission across BBB during epilepsy. So far, no correlation has been elucidated between the expression of miRNAs in blood and CSF and their relationship with BBB dysfunction.[31,38] During specific phases of epilepsy development, it has been found that miRNAs are altered in patients with epilepsy.[39] miRNA expression in CSF is also observed to be altered in the development of Alzheimer's disease.[40] Based on these facts, we hypothesize that the controversial distribution pattern of hsa-miR-1275 in blood serum and CSF in this study might be associated with hsa-miR-1275 transmission cross the BBB and diffuse into blood because of BBB leakage. The dysregulation of hsa-miR-1275 expression is thus not only reflective of cell-based changes in EUE pathogenesis but may also provide novel insights to assess disease progression mechanism and therapeutic efficacy.

The functions of main target genes of hsa-miR-1275 were classified into neuron system regulation, cell apoptosis, and proliferation processes regulation (Figure 3). Among these target genes, MECP2 shows a wide range of neuron system effects intersecting with hsa-miR-1275. Interestingly, almost all of the overlapping signals of hsa-miR-1275 and the MECP2 are related with seizure disorders. Therefore, MECP2, as one of the preferred target genes, was chosen for the subsequent detection of the hsa-miR-1275 regulation mechanism.

It is common that proteins involved in regulation of basic biologic processes, such as cell development and differentiation, often contain limited miRNA target binding sites in their 3′ UTRs.[16,41] Even partial complementary binding of the miRNA to the 3′ UTR of the target mRNA can result in post-transcriptional inhibition. In experimental studies, levels of specific miRNAs can be increased by miRNA "mimics" and decreased by antagomirs, which bind to miRNAs and thereby inhibit their function.[16] In this study, five predicted consequential parings of the target region of 3′ UTR of MECP2 were analyzed, and the hsa-miR-1275 mimic was designed for further exploration. All of these findings suggested that hsa-miR-1275 might targetedly inhibit the expression of MECP2. However, the confirmation of the biological functions of hsa-miR-1275 and its relationship with the MECP2 needs further corroboration.

It has been recognized that MECP2 plays fundamental roles in synaptic and neuronal plasticity and in maintenance of central nervous system development.[42] MECP2 deficiency in the brain has been shown to decrease the expression of the brain-derived neurotrophic factor (BDNF), a neurotrophic factor essential for neuronal survival, differentiation, and synaptic plasticity,[43] and IGF1, which is essential for neural progenitors proliferation, neuronal survival, and synapse formation;[44] increase the serum glucocorticoid-inducible kinase 1 (SGK) and FK506-binding protein 5 (FKBP5), which are stress-responsive genes glucocorticoid in the brain;[45] and activate cytotoxic necrotizing factor 1 (CNF1) to enhance neurotransmission and synaptic plasticity.[46] MECP2 plays a critical role in the maintenance of neuronal structure by modulation of mechanistic target of rapamycin (mTOR) protein kinase in neuronal activity and synaptic inputs.[47] All of these factors are involved in neuronal maturation, synaptic plasticity, axons and dendrites, and neuron growth and function. Thus, we hypothesized that the interaction of hsa-miR-1275-MECP2 might play a crucial role in the regulatory network of human EUE (Figure 5).

MECP2 is reported to be associated with a severe epileptic seizure phenotype in Rett syndrome (RTT) and in MECP2 duplication syndrome (MDS).[48,49] RTT is an X-linked neurodevelopmental disorder that primarily occurs in females.[50] Mutations of the gene encoding MECP2, which have been identified in 90%–95% of RTT cases, are considered to be the foremost cause of typical RTT.[50] MDS has been identified in males characterized by moderate to severe intellectual disability, spasticity, and inconstant epileptic seizures.[49] MECP2 has been proved to be critically responsible for the progressive neurological disorder[51] and encode an essential epigenetic regulator in postnatal brain development.[52] Among a wide range of neurologic manifestations of RTT, epilepsy is reported to occur frequently and represents a significant, commonly accompanied clinical feature in RTT.[53] However, epileptic seizures in RTT and MDS are poorly elaborated because of their inconsistency and scarcity of cases.[54] Moreover, the correlation between EUE and RTT or MDS has not been previously recognized. The only intersection report is that the expression of MECP2 is homeostatically regulated by miR-132,[55] which is also a critical regulator in EUE. In this study, MECP2 was identified as the major target of hsa-miR-1275 by bioinformatics analysis and biological valuation. As hsa-miR-1275 was significantly regulated in EUE, it may lead to an inference that MECP2 is a critical regulator in neuron dysregulation of EUE and that there might be a potential relevance between EUE and RTT or MDS regulation. In summary, our findings are promising and provide several potential insights of

fundamental processes involved in EUE, as well as processes leading to epileptogenesis for further functional investigation to understand the complex molecular mechanism underlying EUE disorders.

MATERIALS AND METHODS

Study Participants and Chip Experiment

The study was approved by the Committee of First Affiliated Hospital of Xiamen University (Fujian, China). All patients were referred to the Department of Neurology of First Affiliated Hospital of Xiamen University for their medical intractability and fulfilled the diagnostic criteria for EUE. The diagnosis was according to ILAE criteria. Brain structural abnormalities were not detected in any of the patients by magnetic resonance imaging (MRI) scans, and none of the patients underwent previous intracranial surgery. All patients are well informed of the use of their tissue for scientific purposes, particularly in this study, by signing informed consent. Tissue samples of the blood and CSF were taken during their diagnosis and treatment. The time period from seizure episode to sample collection was less than 3 days. Samples were not collected simultaneously. The analysis of a miRNA chip experiment was conducted from two women patients' serum samples with the first diagnosis of EUE compared with one normal control. For the lack of sample numbers in the chip experiment, EUE blood serum (n = 11) and healthy control blood serum (n = 10) were collected for validation using the TaqMan assay. Validation was also performed in CSF samples from six EUE patients, comparing three patients suffering headache, gap cerebral infarction, and viral meningitis as control. The clinical characteristics of the sample are described in Table 1.

Each blood specimen was processed individually for microarray hybridization. Briefly, total RNA was extracted from whole-blood specimens using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), subjected to DNase treatment, and purified using the RNeasy Plus Kit (QIAGEN, Valencia, CA, USA). RNA purity was assessed by the A260/A280 ratio, and quality and integrity were assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

Microarray Data Analysis

As described above, miRNA data come from human blood serum obtained from independent epilepsy donors and healthy control. The chip experiment was conducted according to Agilent Human miRNA Microarray Kit Release 16.0 (G4870A; Agilent Technologies) and Microarray Scanner System (Agilent Technologies). PCA and hierarchical clustering were performed as described.[56,57] PCA is an unbiased analysis performed in GeneSpring to look for similar expression patterns and underlying cluster structures. Hierarchical cluster analysis of differentially expressed miRNA was conducted using the smooth correlation distance measure algorithm (GeneSpring) to identify samples with similar patterns of gene expression. The Cytoscape-ClueGO plugin is used to analyze the gene list in the context of cell-component level.[58] We compared treatment and control groups using the t test (p values) and the Benjamini-Hochberg false discovery rate (FDR) correction (adjusted p values).

Quantitation of hsa-miR-1275 by TaqMan Assay

The hsa-miR-132 was selected as a positive control because it has been reported to play an important role in epilepsy.[10] cel-miR-39 was used as the housekeeping gene for the detection of hsa-miR-1275 expression.[13] The primers for hsa-miR-1275, hsa-miR-132, and cel-miR-39 were included in TaqMan miRNA assays (4427975; Applied Biosystems, Life Technologies, NY, USA). Sequence of miRNA and references used in the paper were hsa-miR-132 (catalog [Cat.] number 4427975-000468) and cel-miR-39 (Cat. number 4427975-000377). The reverse primers were also used for reverse transcription with the TaqMan MicroRNA Reverse Transcription Kit (4366596; Applied Biosystems, Life Technologies, Grand Island, NY, USA) in a total volume of 10 μL. Real-time qPCR for miRNA was performed with the ABI StepOne qPCR system.

Bioinformatics

Bioinformatics algorithms microrna.org (https://integbio.jp/dbcatalog/en/record/nbdc01585), DIANA Lab (http://diana.imis.athena-innovation.gr/DianaTools/index.php), and TargetScan (http://www.targetscan.org/) were used to predict miRNAs, which can simultaneously target the 3′ UTRs of MECP2. These algorithms were also used to predict whether hsa-miR-1275 may bind to the 3′ UTR of MECP2.

Cell Culture, Transfection, and 4′,6-Diamidino-2-Phenylindole (DAPI) Staining

The U251 cell line (glioblastoma-derived human cell line; Cat. number TCHu 58) was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM/F12, supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The hsa-miR-1275 duplex mimic, oligonucleotide duplex mimic NTC, and inhibitor were designed and provided by RiboBio (Guangzhou, China). 30%–50% confluent cells were transfected with miRNAs by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

After being transfected with hsa-miR-1275 mimic and NTC for 48 h, cells were stained with DAPI (Sigma-Aldrich, USA), and those with fragmented or condensed nuclei were defined as apoptotic cells. At least five visual fields were observed under a fluorescence microscope for each sample.

Western Blot Analysis

Proteins extracted from cells were immunoblotted with different antibodies following a published protocol.[59] The primary antibodies used were MECP2 (1:1,000 dilution) (Santa Cruz Biotechnology, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5,000 dilution) (Cell Signaling Technology, Danvers, MA, USA).

Vector Cloning and Luciferase Assay

The 3′ UTR of MECP2 with the hsa-miR-1275 binding region was cloned and inserted into the pmirGLO Dual-Luciferase MicroRNA Target Expression Vector (Promega, USA). The mutant construct

Table 5. hsa-miR-1275 Gene Ontology

Gene GO	Number of Genes	Percentage	p Value (%)	Fold Enrichment
GO: 0019226 ~ nerve impulse conduction	43	8.29	0	3.90
GO: 0007268 ~ synaptic transmission	35	6.74	0	3.73
GO: 0007267 ~ intercellular signal	52	10.02	0	2.75
GO: 0016337 ~ intercellular adhesion	28	5.39	0	3.22
GO: 0021700 ~ maturation	16	3.08	0	5.03
GO: 0030182 ~ neuronal differentiation	35	6.74	0	2.54

with deletion of the binding region of hsa-miR-1275 was an NTC. Primers used for PCR are shown in Table 5. 24 h after transfection with 2 μg of these constructs, the U251 cells were then cotransfected with hsa-miR-1275 mimics, inhibitor, and mimic NTCs. Cells were analyzed for luciferase activity after 48 h by the Luciferase Reporter Assay Kit (Promega, USA), and each construct was compared to that of the pmirGLO vector no-insert control. For each transfection, luciferase activity was averaged from three replicates.

Flow Cytometric Analysis

At 72 h post-transfection, cell aliquots were incubated with primary antibody (MECP2; Santa Cruz Biotechnology, USA) for 20 min, followed by washing with PBS. Cells were incubated with secondary antibody (goat anti-mouse immunoglobulin G [IgG]-fluorescein isothiocyanate [FITC]; Santa Cruz Biotechnology) for 20 min in the dark, then washed and resuspended in PBS, and analyzed immediately using a Coulter Epics XL flow cytometer (Beckman Coulter, FL, USA). The mean fluorescence signal intensity (MFI) was analyzed using WinMDI software (https://www.scripps.edu) (La Jolla, CA, USA) and calculated as the percentage of that of mock cells.

Statistical Analysis

Statistical analyses were performed by two-tailed unpaired t tests and one-way analysis of variance (ANOVA) using inStat version 5.0 for Windows (GraphPad, San Diego, CA, USA). Data in bars represent mean ± SD in histograms of three independent experiments. Differences were considered statistically significant at $p < 0.05$.

AUTHOR CONTRIBUTIONS

Y.Z., C.L., and C.-M.T. conceived experiments and analyzed the data. C.L., Q.L., L.C., and H.W. performed all experiments. Y.Z., H.W., L.C., Q.L., F.M., E.B.T., H.-C.L., and C.-M.T. wrote and reviewed the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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