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Original Article

Differential long non-coding RNA (lncRNA) profiles associated with hippocampal sclerosis in human mesial temporal lobe epilepsy

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Abstract: Objective: The aim of this study was to analyze the expression profiles of long non-coding RNA (lncRNAs) in human mesial temporal lobe epilepsy (MTLE) with hippocampal sclerosis (HS) and to detect the functions of lncRNAs in epileptogenesis in MTLE. Materials and methods: We used microarray analysis to analyze the differential expression of lncRNAs and mRNAs in three hippocampal sclerosis and three normal hippocampus samples. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to verify the microarrray results. A coding and non-coding gene co-expression network was constructed based on the correlation between the differential expression of lncRNAs and mRNAs. Gene ontology (GO) and pathway analyses were then performed to determine the potential roles of the differentially expressed mRNAs in the co-expression network. Lastly, to understand potential functions of lncRNAs in MTLE, cis-/trans-acting lncRNAs were predicted using bioinformatic analysis. Results: Compared with control hippocampus, 497 differentially expressed lncRNAs were identified in the hippocampal sclerosis samples, consisting of 294 up-regulated and 203 down-regulated lncRNAs (fold-change >2.0 or <-2.0, P<0.05). Similarly, 399 differentially expressed mRNAs were identified with 236 up-regulated and 163 down-regulated. There were 356 lncRNAs and 332 mRNAs in the non-coding and coding co-expression network, in which the highly enriched GO categories were related to the inflammatory response, and neuropeptide receptor activity. Nine pairs of lncRNAs and mRNAs (located within 10 kb of each other) were found to exert functional effects on epileptogenesis. Conclusion: Differential expression of lncRNAs of varying length and location were observed in human MTLE with hippocampal sclerosis. The dysregulated lncRNAs with co-dysregulated mRNAs in inflammatory response and neuropeptide receptor activity categories are predicted to play roles in epileptogenesis in MTLE. LncRNA *RP11-414J4* may contribute to epileptogenesis by targeting *CPLX3*.

Keywords: Long non-coding RNAS (lncRNAs), hippocampal sclerosis (HS), mesial temporal lobe epilepsy (MTLE), lncRNA *RP11-414J4*, *CPLX3*

Introduction

Mesial temporal lobe epilepsy (MTLE) is the most common epilepsy syndrome in adults, often developing into pharmacoresistant epilepsy and requiring surgical removal of the epileptic focus. The hallmark pathological change in MTLE is hippocampal sclerosis (HS), which is characterized by selective neuronal loss and gliosis in the Cornu Ammonis (CA)1, CA3, and CA4 (end folium) subfields [1]. HS has a significant role in epileptogenesis and the development of MTLE. Several processes are involved in HS progression, such as the inflammatory response, immune response, glial activation, synaptic transmission, ion transport, synaptic plasticity, and signal transduction [2]. However, the precise molecular mechanisms of epileptogenesis in MTLE remain unknown.

Long non-coding RNAs (lncRNAs) are generally defined as transcripts that exceed 200 nucleo-

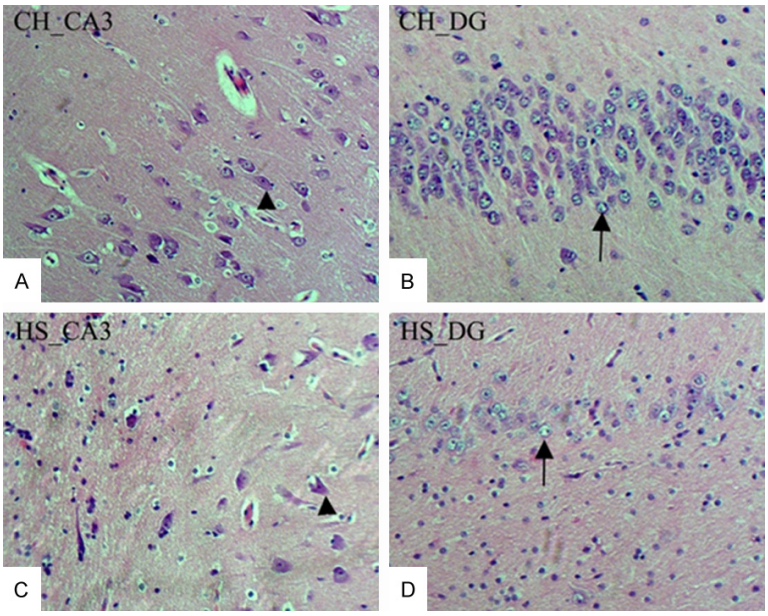

Figure 1. Pathological differences between CH and HS in hippocampal subfields (CA3 and DG). Compared with CH, the numbers of CA3 pyramidal neurons (arrowheads in A and C) and DG granule cells (arrows in B and D) were decreased in HS. Scale bar in (A-D): 80 μm.

tides in length with no protein-coding capacity [3, 4]. They have significant regulatory functions in neural stem/progenitor cell proliferation and differentiation, neurite elaboration and synaptogenesis, and neuronal plasticity [5]. lncRNA involvement has been detected in various neurological diseases, including epilepsy [6, 7]. However, the expression and function of lncRNAs in human MTLE with HS are still unclear. In the present study, we used the Agilent Human lncRNA+mRNA Array V4.0 (CapitalBio Corp, Beijing, China) to detect the expression of lncRNAs and mRNAs in hippocampi from MTLE patients and controls. The coding and non-coding gene co-expression network, gene ontology and pathway analyses, and cis-/trans-acting lncRNA prediction were used to reveal the potential functions of lncRNAs in epileptogenesis in human MTLE with HS.

Materials and methods

Patients and controls

This study was approved by the Medical Ethical Committee of the Chinese PLA General Hospital and appropriate informed consent was obtained from all patients or their families. Resected hippocampal tissues from three patients with refractory MTLE were divided into two samples: one was snap-frozen at -80°C in liquid nitrogen and the other was formalin-fixed and paraffin-embedded. Control hippocampal (CH) specimens from subjects without a history of seizures or other neurological diseases were obtained from three individuals who had died from cardiac arrhythmia, injuries caused by a car accident, and acute pancreatitis. Autopsies were performed within 12 h after death. All six samples were stained with hematoxylin and eosin for pathological analysis (**Figure 1**). The clinical information of the patients and controls is summarized in **Table 1**. The pathology of hippocampal samples was evaluated according to the Watson grading system for HS [8].

Microarray analysis of lncRNAs and mRNAs

Total RNAs were extracted from the six specimens using Trizol (Invitrogen, USA) and purified using the mirVana RNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturers' instructions. The isolated RNAs were used to synthesize double-stranded complementary DNA (cDNA), which was then labeled and hybridized to the Agilent Human lncRNA+mRNA Array V4.0 (CapitalBio Corp, Beijing, China) according to the manufacturer's instructions. The Agilent Human lncRNA+mRNA Array V4.0 was designed with four identical arrays per slide (4 × 180 K format), with each array containing probes for 41,000 human lncRNAs and 34,000 human mRNAs. Next, the hybridized images were scanned and the lncRNA+mRNA array data were subjected to data summarization, normalization, and quality control. Differentially ex pressed genes were defined as those that underwent changes in threshold values ≥2-fold or ≤-2-fold, with Benjamini-Hochberg-corrected *P*-values <0.05. The expression of nine lncRNAs and one mRNA were measured by RT-PCR using SYBR Premix Ex Taq (Takara Bio, Japan) on a Thermal Cycler Dice TP800 (Takara Bio, Japan). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. All experiments were performed in triplicate.

Table 1. Characteristics of MTLE patients and controls

Identifier	Gender	Age (years)	Duration of epilepsy (years)	Antiepileptic drugs	Side of resected hippocampus	Pathology (Watson,1996)
H1	M	32	31	CBZ, PB	Right	HS grade 4
H2	F	23	15	CBZ, VPA	Left	HS grade 4
H3	F	26	18	CBZ, LEZ	Right	HS grade 4
Identifier	Gender	Age (years)	Postmortem interval (hours)	Cause of death	Side of resected hippocampus	
C1	F	36	12	Cardiac arrhythmia	Left	
C2	M	25	9	Car accident	Right	
C3	F	27	11	Acute pancreatitis	Left	

H1-H3 represent patients of MTLE with HS; C1-C3 represent autopsy controls without HS; CBZ, carbamazepine; PB, phenobarbitone; VPA, valproate; LEZ, levetiracetam; HS, hippocampal sclerosis.

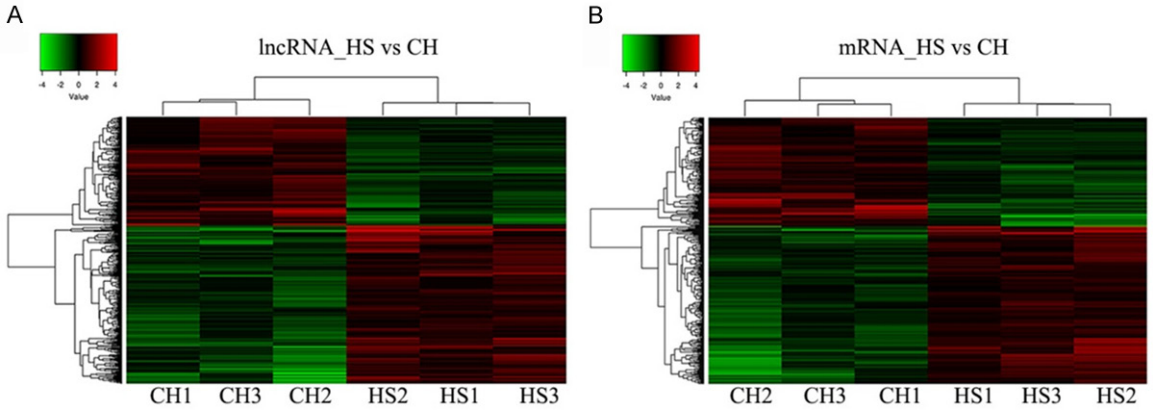

Figure 2. Differential lncRNA expression profiles between HS and CH. A. Dendrogram of the hierarchical clustering analysis of differentially expressed lncRNAs between the two groups. Samples were arranged into groups based on their lncRNA expression levels and dendrograms were subsequently constructed. B. Differential mRNA expression profiles between HS and CH. Dendrogram of the hierarchical clustering analysis of differentially expressed mRNAs between the two groups. Samples were arranged into groups based on their mRNA expression levels and dendrograms were subsequently constructed.

Relative gene expression was represented as fold-change using the $2^{-\Delta\Delta Ct}$ method.

Construction of the coding and non-coding gene co-expression network, gene ontology and pathway analysis, and cis-/trans-acting lncRNA prediction

A coding and non-coding gene co-expression network was constructed based on the correlation between the differential expression of lncRNAs and mRNAs. For each pair of genes, a Pearson correlation coefficient was calculated and significant pair coefficients were selected (correlation >0.99 or <-0.99 and P<0.05) to construct the network using the open-source bioinformatics software program, Cytoscape (Institute of Systems Biology, Seattle, USA). Based on the differential expression of mRNAs in the lncRNA-mRNA co-expression network, Gene Ontology (GO) and pathway analysis were performed using the KEGG Orthology-Based Annotation System (KOBAS). Fisher's exact test was used to identify the significance of each categorization. P<0.05 was considered significant in GO and pathway terms.

Cis-acting lncRNAs were predicted, based on tight correlation (minimum Pearson's correlation coefficient of 0.99) to a group of expressed protein-coding genes. lncRNAs generally reside within 10 kb of a protein-coding gene [9]. Trans-acting lncRNAs were predicted using blat tools (Standalone BLAT v. 35 × 1 fast sequence search command line tool, http://hgdownload.cse.ucsc.edu/admin/exe/) to compare the full

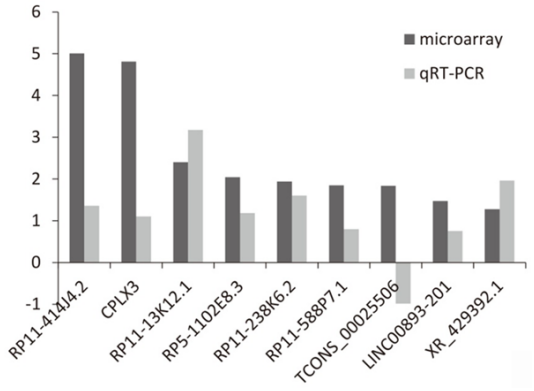

Figure 3. Comparison between microarray and qRT-PCR results for lncRNAs and mRNAs. Except for *TCONS_00025506*, the microarray data of all lncRNAs and one mRNA correlated with the qPCR results.

sequence of the lncRNA with the 3'-UTR of its co-expressed mRNA, using default parameter settings.

Results

Differential expression profiles of lncRNAs and mRNAs: HS vs. CH

In HS versus CH samples, 497 significantly dysregulated lncRNAs were identified; 294 were upregulated, and 203 were downregulated (fold-change >2.0 or <-2.0, P<0.05), of which 11 were up-regulated >10-fold and 13 were down-regulated >5-fold. Compared with CH, *RP11-414J4* (ENST00000488000) (32-fold) and *linc-CBLB-9* (TCONS_00006599) (8-fold) were the most extensively up- and down-regulated lncRNAs in HS patients (**Figure 2A**). Between HS and CH groups, 399 differentially expressed mRNAs were identified (fold-change >2.0 or <-2.0, P<0.05); 236 were up-regulated and 163 down-regulated. Among them, 15 mRNAs were up-regulated or down-regulated by more than 7-fold. Compared with CH, Complexin III (*CPLX3*) (30-fold) and doublecortin domain-containing 5 (*DCDC5*) (18-fold) were the most highly up- and down-regulated mRNAs in HS patients (**Figure 2B**).

Nine RNAs were chosen for validation: one up-regulated mRNA (*CPLX3*) and eight up-regulated lncRNAs (*RP11-414J4*, *RP11-13K12*, *RP5-1102E8*, *RP11-238K6*, *RP11-588P7*, *TCONS_00025506*, *LINC00893-201* and *XR_429392*). Except for *TCONS_00025506*, the patterns for all lncRNAs and the one mRNA were consistent between the qRT-PCR and microarray results (**Figure 3**).

Co-expression lncRNA-mRNA network

The coding and non-coding gene co-expression network showed that one lncRNA was associated with one to several dozen mRNAs, and vice versa. There were 332 mRNA nodes and 356 lncRNA nodes in the co-expression network, which interacted in positive or negative correlations.

GO and pathway analyses and cis-/trans-acting lncRNA prediction

GO analysis revealed the 30 most significantly enriched terms, which were related to inflammation, and neuropeptide and cell receptor (**Figure 4A**). In the pathway analysis, the most significantly enriched terms corresponded to metabolic disorders of oxidation enzymes, neuroactive ligand-receptor interaction, the Wnt signaling network, lipoprotein metabolism, and the calcium signaling pathway (**Figure 4B**). Cis-/trans-acting lncRNA prediction showed nine lncRNAs had neighboring mRNAs (within 10 kb), including *lnc-HSCB-5* and *LOC102724900*, *lncRNA RP11-414J4* and *CPLX3*, *lncRNA RP11-404I7* and *A_21_P0003507* (**Table 2**).

Discussion

Over the past decade, extensive research on lncRNAs has increased our understanding of their diverse structural and regulatory functions in various biological processes. In epilepsy, however, there are limited studies on lncRNAs. Recently, it has been demonstrated that expression of lncRNAs is altered in animal models of epilepsy. In 2015, Lee reported a genome-wide lncRNA profiling study in both the kainic acid and pilocarpine mouse models of temporal lobe epilepsy, where 279 and 384 lncRNAs were significantly dysregulated, respectively, compared with control mice (3.0-fold, P<0.05) [10]. They found two up-regulated lncRNAs, carboxypeptidase N polypeptide 2 (*CPN2*) and chromobox homolog 7 (*CBX7*), which might mediate the progression or induction of epilepsy. In 2017, Han et al. used high-throughput microarray analysis to explore the influence of lncRNA H19 on gene expression in an epileptic rat model. There were 1841 differ-

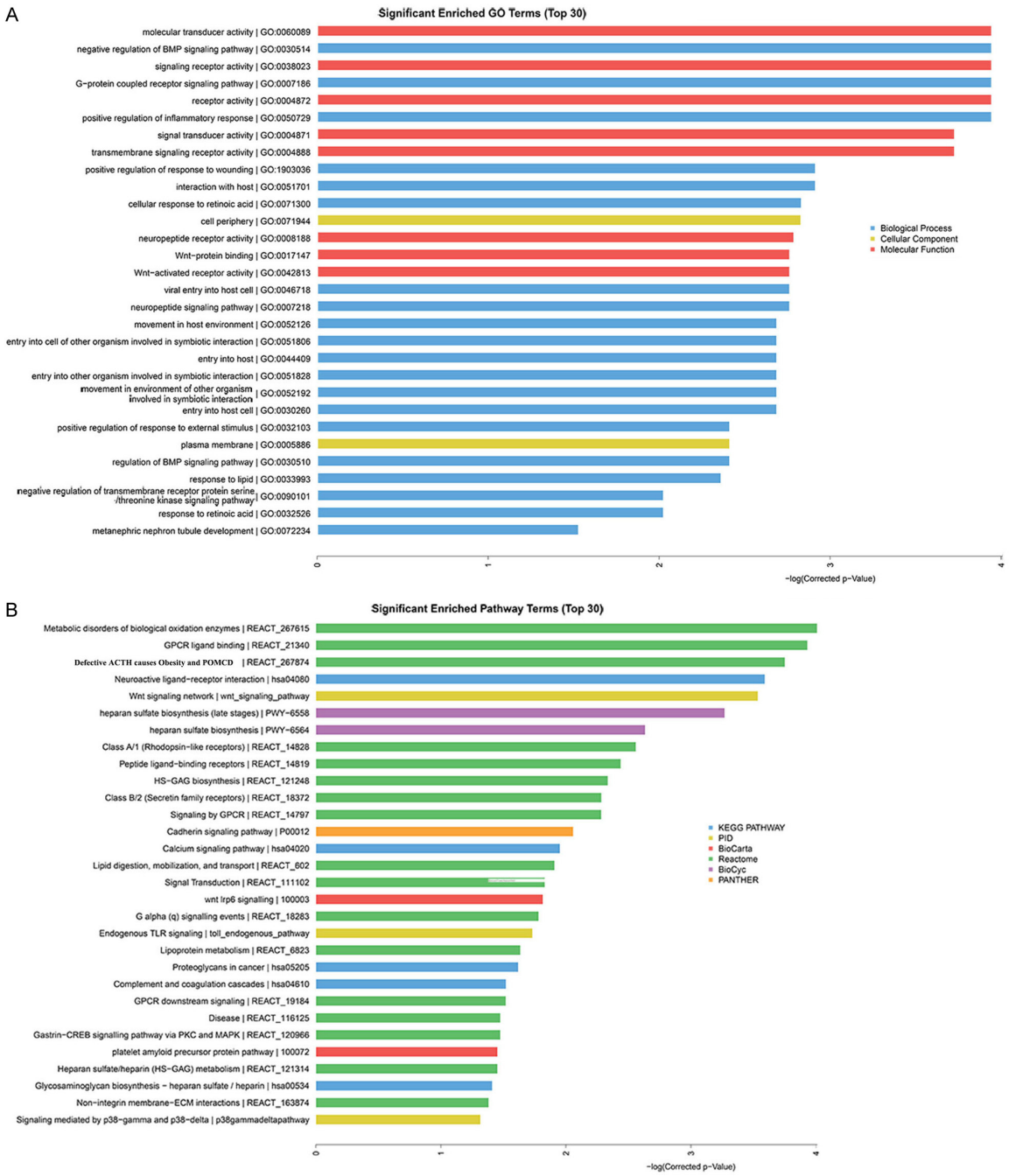

Figure 4. A. The 30 most significantly enriched GO terms. B. The 30 most significantly enriched pathway terms. Results are based on the differential expression of mRNAs in the lncRNA-mRNA co-expression network.

entially expressed genes at the transcriptional level when H19 was overexpressed or knocked down [11]. However, these studies are all animal experiments, and there are no reports investigating lncRNAs in the human hippocampus. In our study, human hippocampi from controls and patients with MTLE were used for microarray analysis. In the HS patients compared with the CH group, 497 lncRNAs were differentially expressed (>2-fold change). These results from human hippocampus samples may provide valuable information for understanding human epilepsy.

According to Gene Ontology and Pathway analysis, potential lncRNA function can be predict-

Table 2. Detailed information of nine lncRNA-mRNA pairs

lncRNA	mRNA	lncRNA/mRNA_ chromosome	lncRNA/mRNA_ strand	Cis-regulation
lnc-HSCB-5	LOC102724900	22/22	+/+	Sense
RP11-414J4	CPLX3	15/15	+/+	Sense
RP11-404I7	A_21_P0003507	4/4	-/-	Sense
lnc-CENPQ-2	PTCHD4	6/6	+/-	Intergenic (10k)
lnc-RAB23-1	lnc-KHDRBS2-1	6/6	-/-	Sense
RP11-760H22	DEPTOR	8/8	+/+	Intergenic (10k)
RP11-498J9	lnc-EIF3A-2	10/10	-/-	Sense
AC018799	LOC101927406	2/2	+/+	Sense
AC005592	LOC101926941	5/5	+/+	Sense

ed. In kainic acid and pilocarpine mouse models of temporal lobe epilepsy, 54 and 14 differentially expressed lncRNAs, respectively, had adjacent protein-coding genes, whose significant Gene Ontology terms referred to embryonic appendage morphogenesis and neuron differentiation [10]. Function and pathway analyses showed that H19 has a broad spectrum of roles in epileptogenic processes, such as cell apoptosis, demyelination, inflammatory and immune responses, and activation of MAPK [11]. In our study, the highly enriched GO categories were related to the inflammatory response, neuropeptide receptor activity, and neuropeptide signaling. Many experimental and clinical data support the participation of inflammation in epilepsy, including some specific inflammatory pathways and key mediators, such as interleukin (IL) 1β, IL6, adhesion molecules, tumor necrosis factor-α and component of complement [12]. Moreover uncontrolled inflammation has been suggested to drive the progression of MTLE with HS [13]. Concerning the relationship between neuropeptides and epilepsy, Me et al. found many neuropeptides are highly expressed in normal hippocampal structures, especially neuropeptide Y (NPY) [14]. However, in HS, NPY neuron populations are reduced. NPY is a good histological marker for the epileptogenic hippocampus [15]. It is involved in the reorganization of mossy fiber sprouting and is believed to block the synchronization of granule cells in epilepsy through these recurrent mossy collaterals [15]. Therefore, we suggest that dysregulated lncRNAs in human MTLE with HS facilitates epileptogenesis via the inflammatory response and neuropeptide receptor activity. Further studies are needed to examine the detailed molecular mechanisms of these processes in epilepsy.

Some lncRNAs had been demonstrated to have some function in epilepsy. For example, lncRNA NEAT1 can modulate neuronal excitability through potassium channels and in resected cortical tissue from patients suffering from intractable seizures, NEAT1 expression is increased in high spiking regions compared with low spiking regions [16]. By targeting the lncRNA, NAT (natural antisense transcript), AntagoNATs mediated the upregulation of sodium channel protein *SCN1A* in the epilepsy-related model of Dravet syndrome, which led to improvements in seizure frequency and severity [17]. Furthermore, lncRNA H19 exerted functional effects on epileptogenesis in rats by aggravating status epilepticus-induced neuronal loss, mossy fiber sprouting, glial cell activation, and cognitive impairments [11]. In our study, we focused on lncRNA *RP11-414J4* and the mRNA, *CPLX3*, in MTLE. The relative fold-changes of *RP11-414J4* and *CPLX3* were significantly greater in the HS versus the CH group by qRT-PCR ($P<0.05$). *RP11-414J4* resides on chromosome 15 and has a length of 15,011 nt. Complexins (CPLXs) are a family of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex-binding proteins [18-21] that facilitate or inhibit vesicle exocytosis [22-24]. There are four *CPLX* genes (*CPLX I-IV*) in the mouse genome; *CPLX I*, *II*, and *III* are expressed in the brain and retina, whereas *CPLX IV* is expressed only at retinal ribbon synapses [21]. CPLXs promote neurotransmitter release and do not act simply as inhibitory clamps for the synaptic vesicle fusion machinery in mammalian CNS synapses. There are no

reports relating CPLXs to epileptogenesis; however, because CPLXs are key regulators of synaptic exocytosis, we inferred that aberrant expression of *CPLX3* may be involved in epilepsy. Thus, we speculate that lncRNA *RP11-414J4* contributes to epileptogenesis by targeting *CPLX3*. Further experiments should be designed to confirm this hypothesis, such as measuring changes in neurotransmitter levels and *CPLX3* expression after knock-down of *RP11-414J4 in vivo* and *in vitro*.

Despite these encouraging findings, there are several limitations of the current study. First, the number of samples in each group was low, necessitating validation of our findings in a larger cohort. Second, the patients in the HS group were treated with different antiepileptic drugs prior to surgery and it remains unknown whether this could have influenced the expression of lncRNAs. Third, the samples in the control group were derived from postmortem tissues; a delay in autopsy might have altered the lncRNA profiles from baseline.

To conclude, this microarray genome-wide study is the first to profile the hippocampal expression of lncRNAs in patients with HS and to compare this with that in normal hippocampus. Differential expression of lncRNAs of varying lengths and locations was observed between the HS and CH groups. The highly enriched GO categories of the differentially expressed mRNAs in the non-coding and coding co-expression network were for the inflammatory response and neuropeptide receptor activity. LncRNA *RP11-414J4* and *CPLX3* were the most extensively up-regulated lncRNA and mRNA in the present study.

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Disclosure of conflict of interest

None.

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