Seizure: European Journal of Epilepsy 81 (2020) 47–52

Contents lists available at ScienceDirect

Seizure: European Journal of Epilepsy

journal homepage: www.elsevier.com/locate/seizure

Exosomal microRNA expression profiles of cerebrospinal fluid in febrile seizure patients

Seh Hyun Kim[a], Sin-Weon Yun[a], Hye Ryoun Kim[b], Soo Ahn Chae[a,*]

[a] Department of Pediatrics, Chung-Ang University Hospital, Seoul, Republic of Korea
[b] Department of Laboratory Medicine, Chung-Ang University Hospital, Seoul, Republic of Korea

ARTICLE INFO

Keywords:
febrile seizure
microRNA
cerebrospinal fluid
exosome

ABSTRACT

Purpose: Febrile seizures (FS) are the most common seizures found in pediatric patients. Recently, microRNA (miRNA) have been used as a novel biomarker for the diagnosis of various diseases. This study aims to explore the exosomal miRNA expression profile of the cerebrospinal fluid (CSF) in atypical FS patients.
Methods: This is a case-control study including CSF specimens of 41 pediatric patients. The CSF specimens were categorized into FS and a control group. Microarray assays were performed to evaluate the CSF exosomal miRNA expression profile. Quantitative PCR (qPCR) assays were conducted to validate the microarray assay result. Bioinformatic analysis was performed to analyze the result.
Results: Thirteen (62%) patients in the FS group experienced complex FS. A total of 96 miRNAs were significantly expressed in the CSF study samples and 95 amongst them, exhibited higher expression in FS than in the control group. Further validation qPCR test indicated that the top 5 highly expressed miRNA (miR-4486, miR-6850-5p, miR-642b-3p, miR-7107-5p, miR-4281) showed same results as in the microarray assay. Bioinformatic analysis identified 455 target genes in the FS group.
Conclusion: FS patients displayed higher CSF exosomal miRNA profiles than the control. These altered miRNA profiles appeared to be related to complex FS.

1. Introduction

Febrile seizures (FS), the most common seizures observed in pediatric patients, affect 2-5% of children below 5 years of age [1,2]. These are characterized by fever episodes without any history of neurological insults or spontaneous seizures [3]. Many studies on family and twins have demonstrated that genetic factors play an important role in FS [4,5]. Approximately one-third of children with FS show a positive family history [4]. Possible modes of inheritance for genetic predisposition to FS are autosomal dominance with reduced penetrance and polygenic or multifactorial inheritance [6,7]. In one cytokine genotyping study, certain alleles, genotypes, and haplotypes in transforming growth factor beta genes were overrepresented in patients with FS [8]. FS are accompanied with good long-term outcomes; however, complex FS are associated with increased risk of death [9,10].

RNA interference is one of the gene regulatory mechanisms in eukaryotes that neutralizes targeted messenger RNA (mRNA) molecules. MicroRNAs (miRNAs) and short interfering RNAs are well-known small RNAs that play a major role in RNA interference [11,12]. miRNAs are endogenous, single-stranded, non-coding RNAs with 18-23 nucleotides, capable of playing important roles in targeting mRNAs for cleavage or translational repression [13,14]. They bind to the 3′ untranslated region of mRNA and result in translation repression of the target mRNA [15]. miRNA mostly functions in the intracellular compartment, although cell-free circulating miRNAs have been detected in most extracellular fluids including cerebrospinal fluid (CSF) [16,17]. Exosomes are spherical cell-derived vesicles that fall under the category of extracellular vesicles, and have been reported as miRNA enriched [18–20]. Exosomes are produced in most cells including neurons, microglia, and dendritic cells of the central nervous system (CNS) [18]. Recently, they have been recognized to play an important role in long and short distance intercellular communication. CSF acts as a good site for diagnostic biomarker identification, including exosome-derived miRNA, associated with neurological disease. Due to the presence of

Abbreviations: FS, febrile seizures; mRNA, messenger RNA; miRNAs, microRNAs; CSF, cerebrospinal fluid; CNS, central nervous system; Hb, hemoglobin; WBC, white blood cell; ANC, absolute neutrophil count; CRP, C-reactive protein; qPCR, quantitative PCR; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; SE, status epilepticus

* Corresponding author at: Department of Pediatrics, Chung-Ang University Hospital, 102 Heukseok-ro, Dongjak-gu, Seoul, 06973, Republic of Korea
E-mail address: kidbrain@cau.ac.kr (S.A. Chae).

https://doi.org/10.1016/j.seizure.2020.07.015
Received 24 February 2020; Received in revised form 16 July 2020; Accepted 18 July 2020

several pieces of evidence exhibiting the relation between infection, seizure, and miRNA, we hypothesize that atypical FS might possess a unique miRNA profile. The aim of this study was to explore the exosomal miRNA expression profiles in atypical FS patients and identify a novel biomarker to predict severe FS.

2. Methods

2.1. Subjects

CSF samples were consecutively obtained from pediatric patients that underwent CSF analysis at the Chung-Ang University Hospital, Korea, between May 2017 and January 2019. The samples were further categorized into 2 groups, FS and the control group. The FS group was constituted by pediatric patients that underwent CSF analysis due to atypical presentation of FS, including complex type or atypical age onset. Due to the impossibility of acquiring healthy children's CSF, samples from other patients (other than FS) constituted the control group. The patients of the control group were subjected to CSF analysis because of symptoms suggesting meningeal irritation, including neck stiffness and headache. CSF samples from patients without FS and meningeal irritation symptoms were excluded, as those patients were under one year of age and the age difference between them and the FS group could have influenced the miRNA profile result. The medical record data including age, seizure profiles, fever (up to 38.0 °C), serum hemoglobin (Hb), serum white blood cell (WBC), absolute neutrophil count (ANC), C-reactive protein (CRP), and CSF WBC of the pediatric patients were collected.

2.2. CSF sample collection

CSF samples (1 mL) were additionally collected from each patient during the routine CSF tapping procedure for diagnosis and were stored at -80 °C following centrifugation (500 × *g*, 10 min, room temperature). The CSF samples were pooled into 2 groups which were then subjected to the miRNA microarray assay and quantitative PCR (qPCR).

2.3. Exosomal RNA Purification and Quantification

Exosomes were extracted from 0.14 mL of serum using the miRCURY Exosome Cell/Urine/CSF Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Total exosomal RNA was purified with TRI Reagent LS (Molecular Research Center, Cincinnati, OH, USA). RNA quantity and quality were assessed by Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) and Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

2.4. miRNA Microarray Hybridization

The microarray assay of miRNAs was performed using SurePrint Human miRNA Microarrays Release 21.0 8 × 60k (Agilent, Santa Clara, California). The microarray comprised 2,549 miRNA groups retrieved from miRBase v21 (http://www.mirbase.org).

RNA (100 ng) was incubated with 10× calf intestinal phosphatase buffer, labeling spike-in, and calf intestinal phosphatase at 37 °C, for 30 min. Following this, the reaction mixture was chilled and denatured with 100% dimethyl sulfoxide at 100 °C (5 min). Ligation was performed with 10 × T4 RNA ligase buffer, T4 RNA ligase, and cyanine3-pCp at 16 °C, for 2 h. Unlabeled cyanine3-pCp was removed from the ligation mixture using a Micro Bio-Spin 6 column (Bio-Rad, Hercules, California), complementary RNA was labeled with cyanin3 and incubated with 10 × GE blocking agent, and 2× Hi-RPM hybridization buffer at 100 °C, for 5 min. The reaction mixtures were later hybridized with the human miRNA array (8 × 60 K) for 20 h at 55 °C, after washing the hybridized array with gene expression wash buffers 1 and 2. The image was scanned with an Agilent Scanner B. The signal density on the array was extracted by Feature Extraction Software (Agilent, Santa Clara, California) where the signal density was normalized using Genespring v 13.0 (Agilent, Santa Clara, California). An miRNA was designated as over-expressed if its expression in one of the groups was two-fold higher than that of the other group.

2.5. miRNA qPCR

To validate the microarray assays, qPCR assays for the top 5 highly expressed miRNA were performed. The bovine mature miRNA sequence was determined using the miRBase v21 (http://www.mirbase.org). The bovine miRNA primers were designed using the miRprimer2 program (Busk PK, 2014). cDNA was synthesized from exosomal RNA using a miScript II RT Kit (Qiagen, Hilden, Germany). miRNA expression was determined with a miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) and RotorgeneQ (Qiagen, Hilden, Germany). RNU6 was used as the reference miRNA. The relative miRNA expression level was calculated using the $2-\Delta\Delta Ct$ method.

2.6. Bioinformatic Analysis of miRNA Target Prediction

Three databases, miRWalk (http://mirwalk.umm.uni-heidelberg.de), miRDB (http://mirdb.org), and Mirtarbase (http://mirtarbase.mbc.nctu.edu.tw), were used to predict the target genes of the expressed miRNA in FS group. This was followed by identifying the enriched pathways with miRWalk functional enrichment analysis tool. The pathways were presented according to GO (Gene Ontology, http://geneontology.org) and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg) databases.

2.7. Statistical Analysis

Statistical analysis was performed with SPSS software v 25.0 (SPSS Inc., Chicago, IL, USA). The Mann-Whitney test for continuous data and Fisher's exact tests for categorical data were applied. A p-value < 0.05 was considered statistically significant for all analyses.

2.8. Ethics Statement

The CSF samples were coded for de-identification during all analysis. Informed written consent for this study was provided by all the enrolled patients and the specimens were processed under approval from the Chung-Ang University Hospital Institutional Review Board (Institutional Review Board number C2014082).

3. Results

3.1. Clinical Characteristics

During the study period, CSF samples from 61 pediatric patients were collected. 20 samples were from patients without FS and meningeal irritation symptoms. These samples were excluded from the study population in order to match the age range. Among the remaining 41 samples, 21 (51.2%) constituted the FS group and 20 (48.8%) formed the control group. The patients in the FS group were younger than the ones in control group (47.7 vs. 111.2 months, $p = 0.001$) (Table 1). All patients in the FS group complained of seizure and fever; however, 13 (62%) of them presented complex FS. For comparison, in the control group 1 (5%) patient presented seizure and 15 (75%) patients complained of fever. The 1 patient who presented seizure in the control group did not have a fever. No significant difference was noted between the 2 groups in terms of serum Hb, WBC, ANC, and CRP, along with CSF WBC. In the FS group, 15 patients were subjected to electroencephalography and 12 of them showed normal findings. However, 1 patient showed focal spike discharges and generalized slow waves, and 2 patients showed generalized slow waves. However, in the last

Table 1
Characteristics of study population between febrile seizure (FS) and control group.

	FS group	Control group	*p* value
Number of patients	21	20	
Male/female	16/5	15/5	
Electroencephalography, no.	15	1	
Normal	12	1	
Focal spike discharges	1	0	
Generalized slow waves	1	0	
Generalized sharp waves	2	0	
Brain magnetic resonance imaging, no.	12	7	
Normal	12	7	
Age (month)	47.7 ± 32.2	111.2 ± 60.9	0.001
Fever	21 (100)	15 (75)	0.021
Seizure	21 (100)	1 (5)	< 0.001
Simple febrile seizure	8 (38)	0 (0)	
Complex febrile seizure	13 (62)	0 (0)	
Multiple seizures in 24 h	7	0	
Prolonged duration (> 15 min)	3	0	
Focality	3	0	
Serum Hb (mg/dL)	12.3 ± 0.8	12.9 ± 1.6	0.105
Serum WBC (per mm^3)	11730.5 ± 5012.3	10122.5 ± 3586.4	0.389
Serum ANC (per mm^3)	7635.4 ± 3560.2	7328.5 ± 3640.7	0.896
Serum CRP (mg/dL)	14.3 ± 15.9	19.4 ± 38.1	0.074
CSF WBC (per mm^3)	1.7 ± 3.7	154.1 ± 444.7	0.154
CSF WBC count < 5 per mm^3, no.	19 (90)	14 (70)	0.130

Values are expressed as N (%) or Mean ± SD

follow-up electroencephalography of 4 patients with previous abnormal electroencephalography findings, all showed normal findings. We speculate that these abnormal electroencephalography findings are secondary transient changes of FS. Brain magnetic resonance imaging was performed in 12 and 7 patients in the FS group and control group, respectively and all of them showed normal findings.

3.2. miRNA Expression Profile

A total of 96 miRNAs of the 2,549 miRNAs (on the microarray chip) were significantly expressed in the CSF samples (Table 2). In the FS group, 95 miRNAs showed higher expression when compared to that of the control group. However, 1 miRNA exhibited higher expression in the control group than in the FS group. A validation qPCR test for the top 5 highly expressed miRNAs in the microarray assays (miR-4486, miR-6850-5p, miR-642b-3p, miR-7107-5p, miR-4281) also showed high expression levels in the FS group (Table 3).

3.3. Target Prediction using Bioinformatic Analysis

With respect to the differentially expressed miRNAs, miRWalk, miRDB, and Mirtarbase databases were used to predict the target genes of the 95 differentially expressed miRNAs in the FS group. To ensure the veracity of the target genes, only the genes predicted by all 3 databases were selected. A total of 455 target genes were identified in the FS group. The miRWalk functional enrichment analysis tool aided the miRNA target gene prediction in the FS group and resulted in the identification of 267 GO terms, categorized into 142 biological process, 55 cellular components, and 70 molecular functions. In addition, 76 KEGG pathway terms were also recognized. Fig. 1 and 2 display the top 15 biological process with respect to GO enrichment terms (each category) and the KEGG pathway terms, respectively in the form of a bar diagram. Among these pathways/terms, 2 biological process (GO enrichment term) included neuronal projection development and axon guidance. One KEGG pathway term, glioma, was associated with the neurologic system. The top 15 pathways were observed to be related to neurodevelopment, including negative regulation of cell proliferation, the fibroblast growth factor (FGF) receptor signaling pathway, cell cycle arrest, the Wnt signaling pathway, apoptosis, and the PI3K-Akt signaling pathway.

Table 2
miRNA fold change between two groups. Values are expressed as $\log_2$ fold changes.

	FS	Control		FS	Control
miR-6850-5p	10.48	0.00	miR-1225-5p	6.16	0.00
miR-4281	10.10	0.00	miR-4788	6.14	0.00
miR-4486	9.37	0.00	miR-6812-5p	6.07	0.00
miR-7107-5p	8.96	0.00	miR-940	6.06	0.00
miR-642b-3p	8.88	0.00	miR-4443	6.02	0.00
miR-6127	8.84	0.00	miR-6515-3p	5.98	0.00
miR-4507	8.42	0.00	miR-6087	5.98	0.00
miR-5739	8.28	0.00	miR-8071	5.91	0.00
miR-4270	8.21	0.00	miR-939-5p	5.84	0.00
miR-6089	8.11	0.00	miR-4466	5.83	0.00
miR-3656	8.09	0.00	miR-4417	5.82	0.00
miR-188-5p	7.97	0.00	miR-4499	5.81	0.00
miR-6821-5p	7.78	0.00	miR-1268a	5.72	0.00
miR-1915-3p	7.78	0.00	miR-4634	5.70	0.00
miR-642a-3p	7.65	0.00	miR-6808-5p	5.69	0.00
miR-6165	7.59	0.00	miR-4687-3p	5.66	0.00
miR-1249-3p	7.47	0.00	miR-4476	5.61	0.00
miR-3162-5p	7.47	0.00	miR-6819-3p	5.57	0.00
miR-6879-5p	7.41	0.00	miR-6088	5.55	0.00
miR-6800-5p	7.31	0.00	miR-1207-5p	5.54	0.00
miR-6749-5p	7.31	0.00	miR-1914-3p	5.51	0.00
miR-6124	7.28	0.00	miR-1234-3p	5.50	0.00
miR-6803-5p	7.21	0.00	miR-4274	5.49	0.00
miR-6741-5p	7.19	0.00	miR-4538	5.48	0.00
miR-7110-5p	7.13	0.00	miR-3937	5.46	0.00
miR-4271	7.09	0.00	miR-6763-5p	5.45	0.00
miR-6869-5p	7.01	0.00	miR-3131	5.40	0.00
miR-1268b	6.90	0.00	miR-7846-3p	5.38	0.00
miR-1228-3p	6.89	0.00	miR-6743-5p	5.37	0.00
miR-3960	6.88	0.00	miR-1238-3p	5.36	3.32
miR-197-5p	6.86	0.00	miR-4749-3p	5.28	0.00
miR-5787	6.81	0.00	miR-2276-3p	5.23	0.00
miR-6090	6.81	0.00	miR-671-5p	5.11	0.00
miR-3620-5p	6.75	0.00	miR-432-5p	5.09	0.00
miR-3195	6.69	0.00	miR-2861	5.07	0.00
miR-6840-3p	6.62	0.00	miR-4433a-5p	4.98	0.00
miR-1202	6.59	0.00	miR-483-5p	4.78	0.00
miR-296-5p	6.54	0.00	miR-638	4.67	0.00
miR-6826-5p	6.52	0.00	miR-6752-3p	4.56	0.00
miR-211-3p	6.39	0.00	miR-3679-3p	4.51	0.00
miR-4459	6.29	0.00	miR-6865-5p	4.43	0.00
miR-4442	6.27	0.00	miR-6768-5p	4.42	0.00
miR-3665	6.24	0.00	miR-6889-3p	4.41	0.00
miR-5195-3p	6.22	0.00	miR-3610	4.23	0.00
miR-4665-3p	6.21	0.00	miR-191-3p	4.16	0.00
miR-7108-5p	6.20	0.00	miR-6798-3p	4.07	0.00
miR-6766-3p	6.19	0.00	miR-6775-5p	4.02	0.00
miR-6069	6.17	0.00	miR-576-5p	0.00	5.22

Table 3
Expression levels of top 5 highly expressed miRNA in microarray assays and validation qPCR assays.

	Microarray		qPCR	
	FS	Control	FS	Control
hsa-miR-6850-5p	10.5	0	102189141.7	10.8
hsa-miR-4281	10.1	0	996.5	8.9
hsa-miR-4486	9.3	0	33225.4	1
hsa-miR-7107-5p	9	0	79.7	1
hsa-miR-642b-3p	8.9	0	70935279.9	1.6

4. Discussion

The present study revealed that 96 miRNAs were significantly

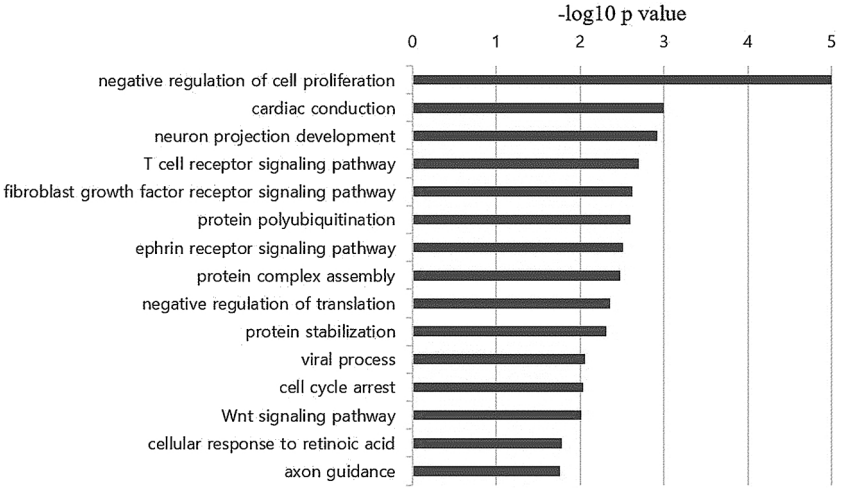

Fig. 1. GO analysis for target genes of expressed 95 miRNAs in FS group. The bar diagram displaying the top 15 GO terms of biological pathway (pop hits > 70 and sort by $-\log10^{p\text{-value}}$ from high to low).

expressed in the 2 groups, with most of them (95 miRNAs) exhibiting overexpression in FS rather than in the control group. The validation qPCR test indicated that the top 5 highly expressed miRNA (miR-4486, miR-6850-5p, miR-642b-3p, miR-7107-5p, and miR-4281) were the same as in the microarray assay. The majority of the patients in the FS group were diagnosed with complex FS and had an atypical FS onset age, usually above 5 years or under 1 year of age. The appropriate control group in our study would be fever without seizure with no evidence of meningitis. However, it is almost impossible to acquire CSF from a child without seizure or evidence of meningitis, so instead we designated the control group as patients subjected to CSF analysis because of symptoms suggesting meningeal irritation. Serum Hb, WBC, ANC, CRP, and CSF WBC displayed no difference between the 2 groups. Additionally, the FS group was younger than the control group. Although the patients without FS and meningeal irritation symptoms were excluded to adjust the age difference between the FS and control groups, the FS group patients were noted to still be younger than the control group. This age difference arose owing to the common age distribution of FS and the control group's indication for CSF analysis, which was meningeal irritation symptoms. All FS group patients displayed seizure events and fever (up to 38.0 °C). However, in the control group, 1 (5%) and 15 (75%) patients complained of seizure and fever, respectively. Thirteen (62%) patients in the FS group possessed complex FS and 8 (38%) patients had simple FS, of which the majority displayed atypical age of onset for FS. Among the complex FS patients, 7 patients showed multiple seizures in 24 hours, 3 patients showed a prolonged duration of more than 15 minutes, and 3 patients showed focal symptoms. Only 1 patient in the control group presented seizure sans fever. Therefore, it can be assumed that the atypical FS type could have influenced the overexpression of several miRNAs (FS group).

FS are common neuropediatric emergencies and are usually benign feature with 30% of the patients exhibiting seizure recurrence [1]. However, children with complex FS have been observed to be younger and are more likely to have delayed development than those with simple FS [21]. Complex FS are characterized by focal features, with a duration more than 15 min, and a recurrence within 24 h [22]. Exosomal miRNAs have been reported to be associated with diseases such as cancer, viral infection, and CNS degenerative diseases [23,24]. Exosomes are known to play a crucial role in normal physiological processes and pathological conditions including infections, which makes them good sources of novel biomarkers [25,26]. In addition, exosomes released by neurons and glial cells play a key role in

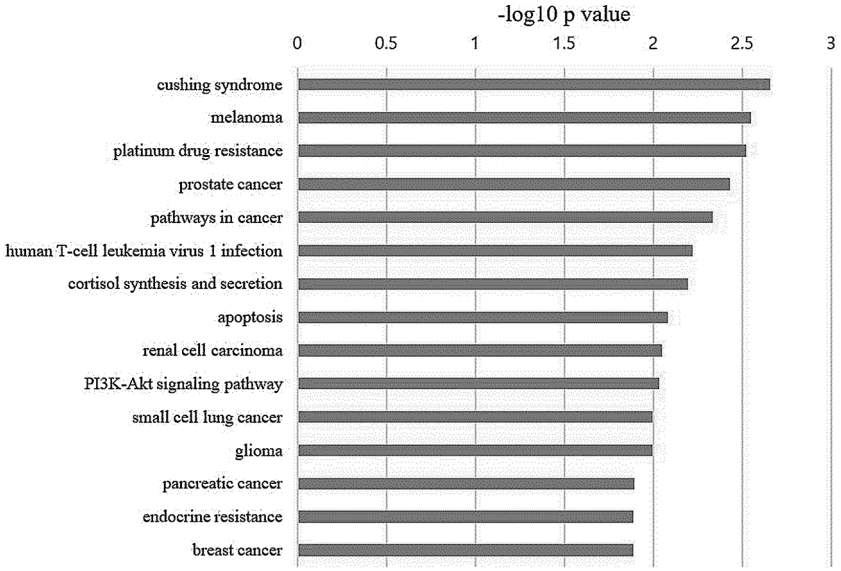

Fig. 2. KEGG pathway analysis for target genes of expressed 95 miRNAs in FS group (pop hits > 70 and sort by $-\log10^{p\text{-value}}$ from high to low).

Table 4
Neurologic system related GO and KEGG terms that are not in the top 15 lists.

Category	Description	-Log10 *p* value
GO biological precesss	Neural tube closure	1.21
GO biological precesss	Neuron differentiation	1.10
GO biological precesss	Brain development	0.84
GO biological precesss	Neuron migration	0.83
GO biological precesss	Nervous system development	0.80
KEGG pathway	Neurotrophin signaling pathway	0.77
KEGG pathway	Alzheimer's disease	0.40
KEGG pathway	Huntington's disease	0.30

neuroglial transmission [27]. In epilepsy, several studies indicated alteration of miRNA expression profiles in animal models and humans [28]. Specific miRNAs in brain tissue have been reported to be related to seizure-induced neuronal death or neuroprotection [29]. Recently, several studies have identified over 100 different miRNAs in epilepsy patients and have provided evidence that relates epilepsy to miRNA expression changes [29–31]. A CSF study described lower levels of miR-19b in temporal lobe epilepsy and higher miR-451a levels in status epilepticus (SE) [32]. In SE animal studies, miR-34a, miR-132, and miR-134 were up-regulated [33–35]. When miR-132 was silenced using antisense oligonucleotides (antagomirs) and then subjected to SE, there was significantly less damage to the CA3 region of the hippocampus [34]. Silencing miR-34a using antagomir infusions into the ventricle of rats resulted in reduced seizure-induced neuronal death and silencing miR-134 also led to reduced seizure severity [33,35]. These animal studies demonstrate that up-regulated miRNAs in SE are important regulators of seizure-induced neuronal death and are coupled to pathogenic brain activity [33–35]. In the current study, the majority of the FS group had complex FS. Complex FS patients had multiple seizures in 24 hours, a prolonged duration more than 15 minutes, or focal symptoms. Similar to SE, these complex FS features are known as harmful seizures compared to simple FS. The risk of developing epilepsy in children with complex FS is 4-6% [36]. It is plausible that harmful complex FS in the FS group resulted in the high miRNA expression profiles, which are likely associated with neuronal death, analogous to the above animal studies. In the present study, known miRNAs related to seizure or inflammatory pathways were not elevated; however, this could be because there is no data on the CSF miRNA profiles of pediatric FS patients and most of the studies on seizure and miRNA were conducted with adult populations.

Additionally, several studies revealed the relationship between infection and miRNAs [37,38]. One such study analyzed miRNA-mRNA expression in A549 cells (influenza A virus infected) [39]. On the other hand, a study reported that miR-671-5p, miR-16-5p, miR-150-3p, and miR-4281 levels differed significantly in exosomes of patients with hand, foot, and mouth disease when compared to those of the control [24]. The evidence highlighting the relationship between seizure, infection, and miRNA is robust. Additionally, low miRNA expression was noted in the control group, which also had low seizures and fever frequency. Therefore, FS (a combination of complex FS and atypical onset of FS) could be speculated to involve a synergistic interaction in miRNA expression. Available evidence based on an animal models and pediatric patients shows that bacterial meningitis such as E. coli meningitis or tuberculous meningitis results in the altered miRNA profiles [40–42]. However, in our study, there were only a few patients with enteroviral meningitis in the control group, and none of them had severe symptoms. There is no evidence to indicate a relationship between exosomal microRNA in the CSF of the pediatric population and enterovirus meningitis. Moreover, enteroviral meningitis is often less severe than bacterial meningitis [43], and thus, we believe that enteroviral meningitis had minimal effects on miRNA expression profiles in our study. The present study used the miRWalk database to analyze the target genes and enriched pathways of 95 expressed miRNAs in the FS group. The miRWalk database, a project of the Medical Faculty Mannheim (University of Heidelberg, Germany), uses the software TarPmiR to predict miRNA target site and integrates results from other databases with predicted and validated miRNA-target interactions [44]. To verify the results, all predicted target genes from miRWalk, miRDB, and Mirtarbase databases were noted and the top 15 enriched pathways (pop hits > 70, sorted by –log10 *p*-value from high to low) were listed. It was observed that annotated targets of 2 GO biological pathways and 1 KEGG pathway were associated with neurologic system. As FS is a neurologic disorder with no associated reference pathways, we could identify the linked pathways that involved terms like neuron projection development, axon guidance, and glioma in the expressed miRNA of the FS group. Additionally, in the top 15 pathways/terms identified by GO and KEGG, the terms related to neurodevelopment could be recognized. Cell proliferation, Wnt signaling, and the FGF receptor pathway are reported to be associated with CNS development and function [45–48]. Additionally, cell cycle, apoptosis, and the PI3K-Akt signaling pathway are associated with autism spectrum disorder, a neurodevelopmental disorder [49,50]. The neurologic system and neurodevelopment related terms could be a risk factor for developmental disorder in complex FS patients. Despite being absent from the top 15 list, many enriched GO biological pathway terms were related to neurologic systems; this includes neural tube closure, neuron differentiation, brain development, neuron migration, and nervous system development. In addition, the results for enriched KEGG pathways revealed terms related to neurologic systems such as the neurotrophin signaling pathway, Alzheimer's disease, and Huntington's disease (Table 4). Owing to the presence of several neurology-related enriched pathway terms in expressed miRNA of the FS group, we can speculate that there is a connection between FS and miRNA.

In the present study, several features posed limitations, such as small sample size and analysis of only pooled samples of the groups. However, to the best of our knowledge, this is the first study to report the CSF exosomal miRNA expression profile in FS pediatric patients, using the most recent microarray assay methods with thorough review and clinical data analysis and bioinformatics tools. However, further studies are required to verify the relationships between miRNAs and FS.

Funding

This work was supported by the Dong-a st, Seoul, Korea. [grant number 20150080] Except that, all authors have no other relevant financial relationships to disclosure or conflicts of interest to solve.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

None

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