

Gene expression analysis in children with complex seizures due to influenza A(H1N1)pdm09 or rotavirus gastroenteritis

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Abstract Viral infections have been implicated as a cause of complex seizures in children. The pathogenic differences in complex seizures due to influenza A(H1N1)pdm09 or rotavirus gastroenteritis remain unclear. This study analyzed the gene expression profiles in the peripheral whole blood from pediatric patients with complex seizures due to influenza A(H1N1)pdm09 or rotavirus gastroenteritis. The gene expression profiles of ten patients (five with seizures and five without) with influenza A(H1N1)pdm09 and six patients (three with seizures and three without) with rotavirus gastroenteritis were examined. Gene expression profiles in the whole blood were different in complex seizures due to influenza A(H1N1)pdm09 or rotavirus gastroenteritis. Transcripts related to the immune response were significantly differentially expressed in complex seizures with influenza A(H1N1)pdm09, and transcripts related to the stress response were significantly differentially expressed in complex seizures with rotavirus gastroenteritis. Pathway analysis showed that the mitogen-activated protein kinases in the T cell receptor signaling pathway were activated in complex seizures due to influenza A(H1N1)pdm09. Dysregulation of the genes related to immune response or stress response could contribute to the pathogenic differences of the complex seizures due to influenza A(H1N1)pdm09 or rotavirus gastroenteritis.

Keywords Influenza A(H1N1)pdm09 · Rotavirus · Oligonucleotide microarray · Complex seizure · Immune response

Introduction

Seizures are the most frequently reported neurological complication, and most of these manifest as single uncomplicated febrile convulsions in young patients. However, some patients exhibit complex seizures, such as partial, prolonged, and repeated seizures. Viral infections have been implicated as a cause of seizures (Millichap and Millichap 2006), and certain viruses, such as human herpes virus type 6, influenza virus, rotavirus, enterovirus, and adenovirus, are more frequently involved than others in pediatric seizures. Each viral infection appears to have characteristics in terms of the frequency, appearance, and outcome of seizures (Hoshino et al. 2012; Kolski et al. 1998; Lloyd et al. 2010; Newland et al. 2007).

In 2009, influenza A(H1N1)pdm09 virus spread globally to become a pandemic infection. This virus caused severe illness, with some patients developing severe acute respiratory distress syndrome (Davies et al. 2009). In addition, neurological manifestations were also reported in many cases worldwide, and seizures were frequently identified as neurological complications (Frobert et al. 2011; Glaser et al. 2012; Kedia et al. 2011; Surana et al. 2011). The majority of cases were simple febrile seizures with no sequelae, but some cases with high morbidity and mortality were described to progress rapidly with a high fever, alteration of cognition, and prolonged complex seizures, which was called influenza-associated encephalopathy (Morishima et al. 2002; Kawashima et al. 2012). The underlying pathophysiological mechanism of the influenza-associated encephalopathy is thought to be production of various inflammatory cytokines (Ichiyama et al. 2003; Ito et al. 2011).

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Rotavirus is the most common cause of severe gastroenteritis diarrhea in young children, causing severe dehydration and electrolyte imbalances. Rotavirus gastroenteritis is accompanied by neurological manifestations, such as seizures, aseptic meningitis, and encephalitis. Afebrile seizures in the absence of dehydration, electrolyte imbalance, or hypoglycemia have also been described (DiFazio et al. 2007; Lynch et al. 2001). Despite having a relatively good prognosis, seizures often occur in clusters and are difficult to treat effectively. To date, the mechanism of central nervous system (CNS) involvement due to rotavirus gastroenteritis is unclear.

To reveal the pathogenic differences in the complex seizures of pediatric patients with influenza A(H1N1)pdm09 or rotavirus gastroenteritis, we analyzed the gene expression profiles in isolated peripheral whole blood cells using an oligonucleotide microarray.

Methods

Clinical characteristics of the patient group

Ten patients with influenza A(H1N1)pdm09 and six with rotavirus gastroenteritis were recruited between January 2009 and January 2010. Among the ten patients with influenza A(H1N1)pdm09, five were hospitalized for complex seizures (Flu-Seizure group; age 4.5–9.7 years, one boy and four girls) and five were uncomplicated (Flu-Control group; age 8.7–11.2 years, four boys and one girl). Among the six patients with rotavirus gastroenteritis, three were hospitalized for complex seizures (Rota-Seizure group; age 0.8–1.7 years, one boy and two girls) and three were uncomplicated (Rota-Control group; age 0.8–2.9 years, three boys). Influenza A(H1N1)pdm09 infection was confirmed using a commercial rapid diagnostic kit that can identify influenza A and B viruses using an immunochromatographic method and by A(H1N1)pdm09-specific polymerase chain reaction (PCR). Rotavirus antigen was confirmed in stool specimens with a commercial rapid diagnostic kit that can identify type A rotavirus. Complex seizures were defined as prolonged seizures of >15 min duration, multiple seizures within 24 h, or prolonged postictal impairment of consciousness for >30 min. None of the patients had underlying disease or a family history of the disease, and none had been vaccinated against influenza A(H1N1)pdm09 or rotavirus. All of the Flu-Seizure patients showed febrile complex seizures, and all of the Rota-Seizure patients showed afebrile complex seizures. Rotavirus RNA was not detected in any of the blood samples in the Rota-Seizure group. All patients, including hospitalized patients, recovered completely. Blood tests in the acute phase revealed that leukocytosis without highly increased C-reactive protein, which was supposed to result from seizure stress, was observed in three cases in the Flu-Seizure group and one case in the Rota-Seizure group. Case 1 in the Rota-

Control group had hyponatremia due to hypotonic dehydration, and case 3 in the Rota-Control group had hypernatremia and increased blood urea nitrogen due to hypertonic dehydration. No electrolyte imbalance was detected in the Rota-Seizure group. No abnormalities of other tests including blood sugar and ammonia were observed in any of the patients (Table 1).

The study was conducted in accordance with the appropriate clinical and experimental ethical guidelines and was approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Science. Informed consent was obtained from all patients and/or their parents for their participation in this study.

Whole blood collection and RNA isolation

Blood samples were collected in PAXgene Blood RNA tubes (Qiagen, Valencia, CA, USA) from patients in the acute and recovery phases. For patients with complex seizures, the blood samples were collected on the day of admission (acute phase: Flu-Seizure, 1–2 days from disease onset; Rota-Seizure, 2–4 days from disease onset) and on the day of discharge (recovery phase: Flu-Seizure, 4–6 days from disease onset; Rota-Seizure, 7–9 days from disease onset). For the control group, samples were collected at the initial hospital visit (acute phase: Flu-Control, 1–3 days from disease onset; Rota-Control, 2–4 days from disease onset) and ~1 week later (recovery phase: Flu-Control, 6–9 days from disease onset; Rota-Control, 9–11 days from disease onset). After sample collection, the PAXgene tubes were incubated at room temperature for 2 h and then stored at -80°C until RNA extraction. Total RNA was isolated using the PAXgene Blood RNA Kit (Qiagen). The RNA was treated with DNase for the removal of contaminating genomic DNA. The quality of the RNA was verified using a RNA 6000 Nano LabChip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and the quantity of RNA was determined by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The total RNA samples from the blood had RNA integrity number values >8. The ratio of OD₂₆₀ and OD₂₈₀ was calculated for all samples, and was between 1.8 and 2.1.

Microarray procedure

Synthesis of cDNA, cRNA labeling, and hybridization were performed according to established protocols (Affymetrix, Santa Clara, CA, USA). One microgram of total RNA sample was converted to double-stranded cDNA with a T7-oligo(dT) primer, and cDNA was transcribed in vitro to biotinylated cRNA. Fragmented biotin-labeled cRNA was hybridized to Affymetrix Human Genome U133 Plus 2.0 arrays in a hybridization oven for 16 h. After washing, the chips were stained with streptavidin-phycoerythrin and scanned. The raw and processed data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE50628).

Table 1 Clinical characteristics of cases with complex seizures due to influenza A(H1N1)pdm09 or rotavirus and controls

Group	Case	Sex	Age	Blood tests in acute phase												
				WBC (/ μ l)	Hgb (g/dl)	Plt ($\times 10^4$ / μ l)	BUN (mg/dl)	Cre (mg/dl)	Na (mEq/l)	K (mEq/l)	Cl (mEq/l)	CK (IU/l)	BS (mg/dl)	NH ₃ (μ g/dl)	CRP (mg/dl)	
Flu-Seizure	1	F	9y 9m	13,690	12.8	27.8	12.4	0.39	135	3.7	104	125	104	35	0.3	
	2	F	8y 9m	10,260	13.0	21.0	10.3	0.37	136	3.6	102	94	93	42	0.1	
	3	M	7y 8m	4,930	14.0	28.1	11.7	0.33	138	4.5	103	89	137	37	1.8	
	4	F	5y 10m	11,020	12.3	20.3	11.7	0.39	134	4.2	102	80	121	38	0.2	
	5	F	4y 5m	7,990	11.2	28.9	12.8	0.40	133	4.2	101	87	138	39	0.3	
Flu-Control	1	M	10y 5m	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	2	M	11y 2m	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	3	M	10y 1m	6,400	11.5	52.9	8.8	0.23	142	4.9	106	ND	ND	ND	0.2	
	4	M	10y 11m	3,800	13.0	16.4	15.3	0.37	136	4.4	96	ND	ND	ND	0.4	
	5	F	8y 7m	8,660	14.4	42.2	10.0	0.43	136	4.2	99	ND	ND	ND	0.6	
Rota-Seizure	1	F	1y 9m	6,420	12.2	30.2	4.0	0.21	139	3.8	109	84	77	34	0.2	
	2	F	1y 4m	8,940	12.8	28.5	10.5	0.22	139	4.7	102	119	77	ND	0.2	
	3	M	10m	10,960	13.2	46.8	6.3	0.23	138	4.3	107	ND	97	ND	0.1	
Rota-Control	1	M	2y 11m	9,400	13.4	18.7	16.8	0.40	132	4.9	96	ND	91	ND	1.9	
	2	M	11m	8,980	11.9	21.7	7.1	0.22	140	4.5	105	ND	ND	ND	0.2	
	3	M	10m	8,200	14.5	58.5	18.0	0.29	157	5.0	130	70	128	ND	0.4	

BS blood sugar, BUN blood urea nitrogen, Cre creatinine, CK creatinine kinase, CRP C-reactive protein, F female, Hgb hemoglobin, M male, m months, ND not determined, Plt platelet count, WBC white blood cell count, y years

Microarray data analysis

The Affymetrix Microarray Suite 5.0 Statistical Algorithm was used to calculate the signal intensity and the detection calls for each gene. The low expression probes were excluded, and the probes classified with absent detection calls in all samples were also excluded. Probe datasets were normalized through the following steps using the Subio Platform software (Subio, Nagoya, Japan): shifting of low signals <1.0 to 1.0 , log-based transformation, and global normalization to the 75th percentile. To eliminate any individual differences, the ratio of the signal of the acute phase to that of the recovery phase (acute/recovery: processed signal) in each patient was calculated for further statistical analysis (Yamashita et al. 2013). To analyze the distribution of the gene expression patterns, principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed using the differentially expressed probe sets between the four groups identified by one-way analysis of variance (ANOVA) ($p < 0.05$). Differentially expressed genes between the seizure and control groups were defined by the unpaired Student's *t* test with values of $p < 0.05$ and a fold change < 0.5 or > 2 . Differentially expressed genes were subjected to gene ontology (GO) analysis using the Database for Annotation, Visualization and Integrated Discovery (<http://david.abcc.ncifcrf.gov/>). A pathway-specific analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database.

Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (qRT-PCR) was used to assess and confirm expression levels of selected genes. Total RNA was reverse transcribed using an Omniscript kit (Qiagen) to generate cDNA, which was quantified using Power SYBR Green (Applied Biosystems, Foster City, CA, USA) on the Applied Biosystems 7500 Real-Time PCR system. The PCR conditions comprised 10 min at 95°C and 40 cycles of 95°C for 15 s and 60°C for 1 min. Levels of gene expression were calculated using the comparative cycle threshold method of relative quantification with the GAPDH gene as an endogenous control. Primer sequences are listed in Supplementary material 1.

Results

Multidimensional scaling and clustering analysis of microarray data

To analyze the distribution of the gene expression patterns of the four groups, we first performed unsupervised HCA with 4250 significantly differentially expressed probe sets after one-way ANOVA across these groups. The HCA identified four dominant expression clusters that corresponded to samples belonging to each group, except for the sample from Rota-Control 1. We

also performed a multidimensional scaling to display the position of each sample in a three-dimensional space using the PCA method. Each patient appeared to be distributed into one of four groups, suggesting that the global gene expression patterns were different between each of the groups (Fig. 1).

Identification of differentially expressed transcripts in the seizure groups

A total of 1,111 transcripts were upregulated and 488 were downregulated in the Flu-Seizure group compared with the Flu-Control group. In contrast, 558 transcripts were upregulated and 449 were downregulated in the Rota-Seizure group

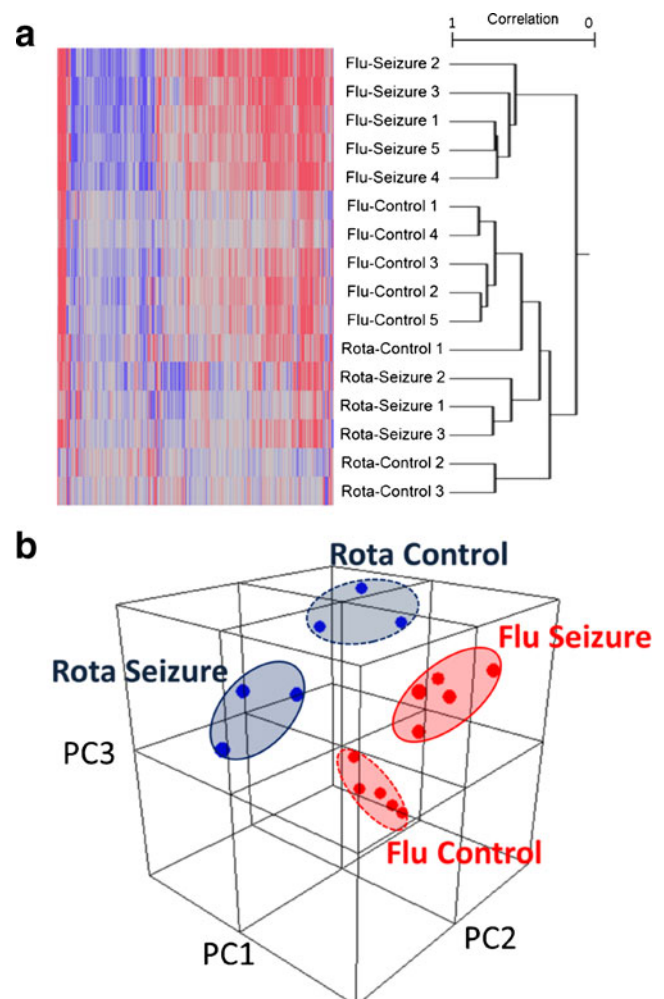


Fig. 1 **a** HCA of gene expression in whole blood from each patient. The dendrogram originated from the clustering of values with Euclidean distances. The distance of the cluster branches was proportional to the differences in gene expression profiles between the patients. The *color* indicates the ratio of the signal of the acute phase to that of the recovery phase (acute/recovery: processed signal) in each patient. *Red* and *blue* indicate the relative upregulated and downregulated processed signals, respectively. **b** Multidimensional scaling analysis of the gene expression in the whole blood samples from each patient. Samples are displayed in a three-dimensional space consisting of the first three principal components (PC1, PC2, and PC3)

compared with the Rota-Control group. The top 100 upregulated and top 100 downregulated genes in the Flu-Seizure or Rota-Seizure group ranked by the fold changes are listed in Supplementary material 2 and 3. The Venn diagram shows 11 transcripts that were upregulated and 8 that were downregulated in the Flu-Seizure and Rota-Seizure groups (Fig. 2).

Functional analysis of the differentially expressed genes in the seizure groups

GO analysis revealed 150 significantly enriched GO terms in biological processes for the Flu-Seizure group and 33 for the Rota-Seizure group. Figure 3 shows the top 15 enriched GO term categories for each group. The Flu-Seizure and Rota-Seizure groups showed common GO terms related to the positive regulation of apoptosis, cell death, and programmed cell death. Enriched GO terms related to the immune response were found in the Flu-Seizure group ($p=0.004$), but not in the Rota-Seizure group. Of 45 significantly differentially expressed transcripts related to the immune response in the Flu-Seizure group, the transcripts of the interleukin-1 receptor (IL-1R)1/Toll-like receptor (TLR) superfamily (IL-18R1, IL-

1R1, IL-1R2, IL-1R accessory protein, TLR4, and TLR8) were increased (Table 2). In contrast, the most significant enriched GO term in the Rota-Seizure group besides apoptosis-related GO terms was the cellular response to stress ($p=0.004$), and this GO term was not significantly enriched in the Flu-Seizure group. Of the GO term related to cellular response to stress, eight genes were up- or downregulated in the Rota-Seizure group (Table 3).

Nine KEGG pathways were significantly enriched in the transcripts associated with the Flu-Seizure group, and two were significantly enriched in the transcripts associated with the Rota-Seizure group (Fig. 4). Among these pathways, the Fc γ -receptor-mediated phagocytosis pathway showed the most significant differences in the Flu-Seizure and Rota-Seizure groups (Flu-Seizure, $p=0.004$; Rota-Seizure, $p=0.005$). The T cell receptor (TCR) signaling pathway was significantly involved in the Flu-Seizure group ($p=0.013$), but not the Rota-Seizure group. Of the transcripts that were involved in the TCR signaling pathway, mitogen-activated protein kinase (MAPK) family members [p38 MAPK and extracellular signal-regulated kinase (ERK)] were upregulated significantly in the Flu-Seizure group (Fig. 5).

Fig. 2 **a** Volcano plots of the comparison between the complex seizure and control groups, at $p < 0.05$ and a >2 - or <0.5 -fold-change cutoff (represented by lines). The x-axis represents the \log_2 of the fold change and the y-axis represents the p value. Each transcript was represented by a single point, and dark areas indicate significant changes in gene expression. **b** Venn diagrams of the up- or downregulated transcripts in the complex seizure group associated with influenza A(H1N1)pdm09 or rotavirus gastroenteritis. The numbers of transcripts with significant ($p < 0.05$, >2 - or <0.5 -fold change) changes in expression are listed

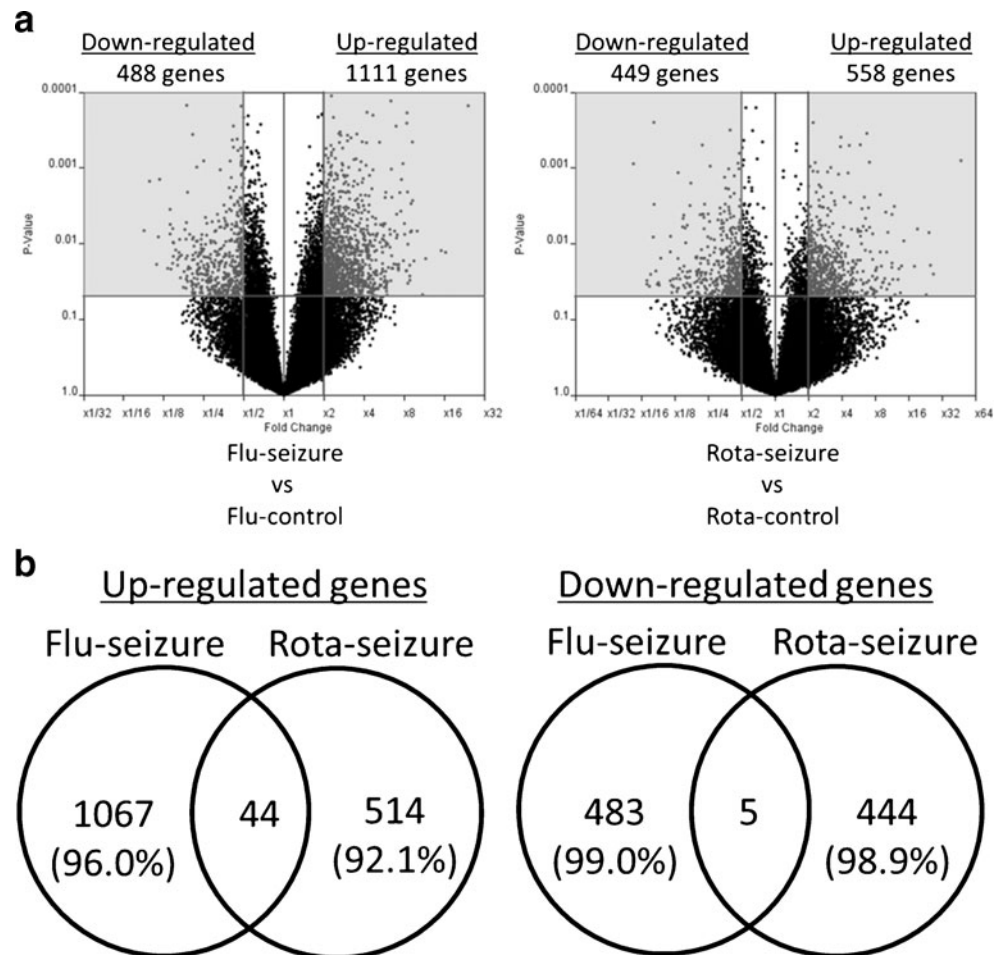
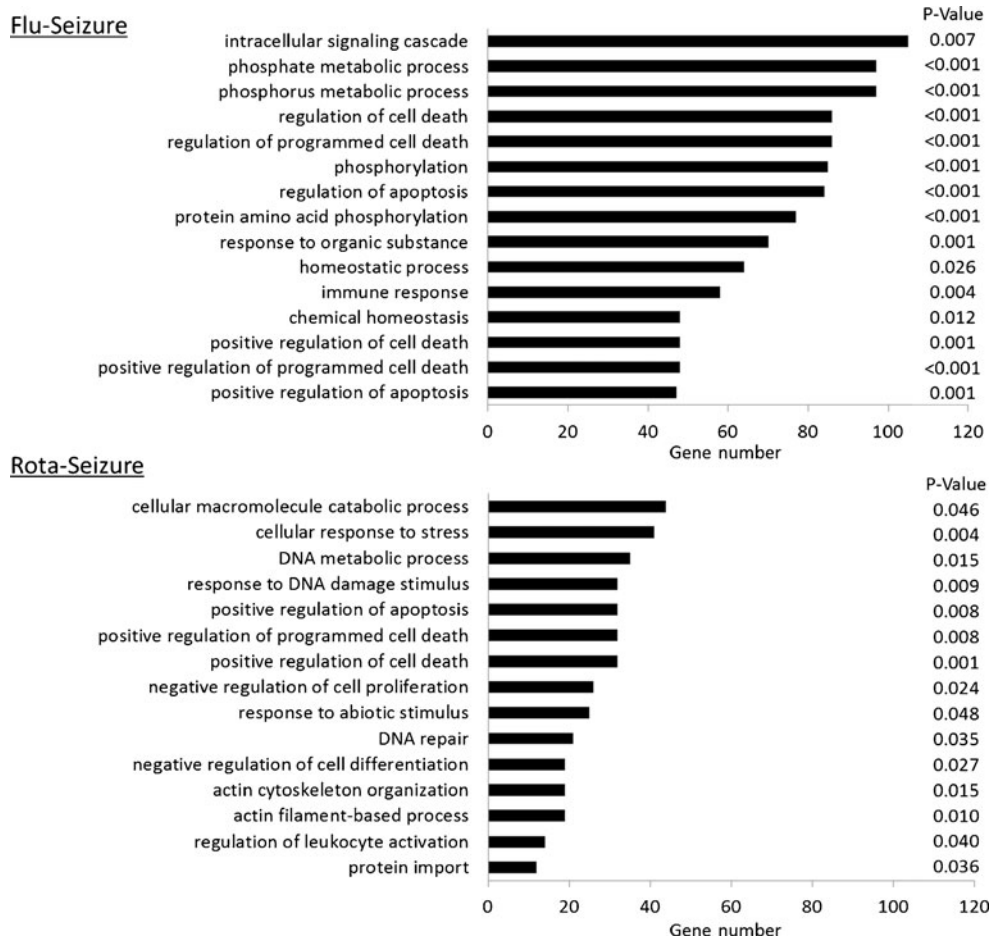


Fig. 3 The top 15 GO categories for the differentially expressed transcripts in patients with complex seizures due to influenza A(H1N1)pdm09 or rotavirus gastroenteritis. The black bar represents the numbers of differentially expressed transcripts



Validation of microarray

qRT-PCR revealed that the processed signals of IL-1R/TLR superfamily were upregulated significantly in the complex seizures due to influenza A(H1N1)pdm09, and that the processed signals of four genes related to cellular response to stress (SOD2, OXR1 and HIGD1A) were up- or downregulated significantly in the Rota-Seizure group (Table 4).

Discussion

We analyzed the gene expression profiles associated with complex seizures in pediatric patients with influenza A(H1N1)pdm09 or rotavirus gastroenteritis, using an oligonucleotide microarray. Neither influenza A(H1N1)pdm09 virus nor rotavirus were detected in the brain of patients with complex seizures or encephalopathy in most of previous studies. However, complex seizures were present as clinical neurological manifestations in the patients infected with these viruses as well as other viruses such as human herpes simplex virus, enterovirus, and adenovirus, which can invade the CNS directly. We think it is important to reveal how these viruses can cause

complex seizures despite local infection such as bronchiolitis or gastroenteritis. It is informative to analyze the gene expression of the brain in patients with encephalitis, but we analyzed the gene expression using peripheral blood to compare the complex seizures caused by respiratory and enteric viruses.

By using a commercially available method to stabilize RNA immediately in the blood, we preserved the immunological alterations that were present in vivo, with minimal interference from artifacts that arose from blood handling, storage, and separation. The disadvantage of this approach was that once the whole blood was stabilized, it was not possible to separate subsequently the different cell populations. However, this approach makes sample collection practical in a busy outpatient clinical setting, and data retention is maximized by the immediate stabilization of the RNA at the bedside.

In our study, PCA and HCA showed the apparent cluster of each of the groups. We also even observed different clusters between the Flu-Control and Rota-Control groups. We suppose that this result was caused by the difference in host immune response in the blood against influenza A(H1N1)pdm09 virus or rotavirus. Previous studies have demonstrated that differential expression of several genes is involved in proinflammatory

Table 2 Up- and downregulated genes classified in biological processes of immune response in patients with seizures due to influenza A(H1N1)pdm09 infection

Probe ID	Gene symbol	Gene title	Fold change	<i>p</i> value
Upregulated genes				
206618_at	IL18R1	Interleukin 18 receptor 1	7.562	0.004
220813_at	CYSLTR2	Cysteinyl leukotriene receptor 2	4.949	0.023
206871_at	ELANE	Elastase, neutrophil expressed	4.663	0.008
202948_at	IL1R1	Interleukin 1 receptor, type I	4.557	0.011
1552773_at	CLEC4D	C-type lectin domain family 4, member D	4.349	0.016
205844_at	VNN1	Vanin 1	4.183	0.012
205227_at	IL1RAP	Interleukin 1 receptor accessory protein	3.902	0.016
208250_s_at	DMBT1	Deleted in malignant brain tumors 1	3.782	0.007
202018_s_at	LTF	Lactotransferrin	3.728	0.021
215990_s_at	BCL6	B cell CLL/lymphoma 6	3.621	0.017
228938_at	MBP	Myelin basic protein	3.423	0.016
244313_at	CR1	Complement component receptor 1	3.344	0.019
205403_at	IL1R2	Interleukin 1 receptor, type II	3.255	0.029
213817_at	IRAK3	Interleukin-1 receptor-associated kinase 3	3.250	0.005
221085_at	TNFSF15	Tumor necrosis factor superfamily, member 15	2.929	0.003
219434_at	TREM1	Triggering receptor expressed on myeloid cells 1	2.845	0.028
211574_s_at	CD46	CD46 molecule, complement regulatory protein	2.790	0.034
1555116_s_at	SLC11A1	Solute carrier family 11, member 1	2.731	0.037
221060_s_at	TLR4	Toll-like receptor 4	2.623	0.021
205557_at	BPI	Bactericidal/permeability-increasing protein	2.573	0.005
225330_at	IGF1R	Insulin-like growth factor 1 receptor	2.546	0.008
211305_x_at	FCAR	Fc fragment of IgA, receptor for	2.504	0.007
208451_s_at	C4A	Complement component 4A	2.485	0.009
201925_s_at	CD55	CD55 molecule, decay accelerating factor for complement	2.425	0.011
1555214_a_at	CLEC7A	C-type lectin domain family 7, member A	2.371	0.038
220832_at	TLR8	Toll-like receptor 8	2.314	0.021
1569830_at	PTPRC	Protein tyrosine phosphatase, receptor type, C	2.201	0.010
211395_x_at	FCGR2C	Fc fragment of IgG, low affinity Iic	2.159	0.042
228891_at	SEMA4D	Sema domain, immunoglobulin domain, transmembrane domain and short cytoplasmic domain, 4D	2.150	0.007
217236_x_at	IGHA1	Immunoglobulin heavy constant alpha 1	2.143	0.003
Downregulated genes				
214214_s_at	IL23R	Interleukin 23 receptor	0.089	0.007
231763_at	IL1RAPL2	Interleukin 1 receptor accessory protein-like 2	0.207	0.047
1552912_a_at	EDA	Ectodysplasin A	0.207	0.034
211005_at	SPN	Sialophorin	0.211	0.028
216207_x_at	CNGA1	Cyclic nucleotide gated channel alpha 1	0.387	0.037
206057_x_at	FCER2	Fc fragment of IgE, low affinity II	0.403	0.048
206653_at	IGKC	Immunoglobulin kappa constant	0.419	0.023
217063_x_at	CD28	CD28 molecule	0.420	0.049
204203_at	POLR3A	Polymerase III polypeptide A	0.424	0.003
211127_x_at	TRD	T cell receptor delta locus	0.433	0.032
211583_x_at	NCR3	Natural cytotoxicity triggering receptor 3	0.434	0.035
206417_at	POLR3G	Polymerase III polypeptide G	0.446	0.030
221112_at	C1QBP	Complement component 1, q subcomponent binding protein	0.468	0.002
206759_at	CEBPG	CCAAT/enhancer binding protein, gamma	0.487	0.032
211856_x_at	LAT	Linker for activation of T cells	0.488	0.019

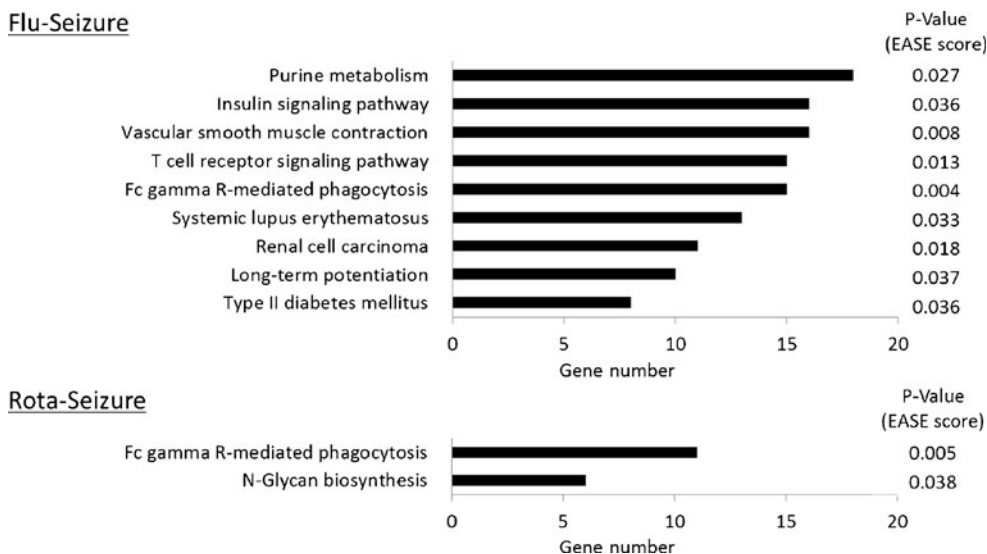
Table 3 Up- and downregulated genes classified in biological processes of cellular response to stress in patients with seizures due to rotavirus infection

Probe ID	Gene symbol	Gene title	Fold change	p value
Upregulated genes				
206252_s_at	AVPR1A	Arginine vasopressin receptor 1A	7.626	0.047
208401_s_at	GLP1R	Glucagon-like peptide 1 receptor	3.624	0.011
215223_s_at	SOD2	Superoxide dismutase 2, mitochondrial	3.466	0.016
207466_at	GAL	Galanin prepropeptide	2.552	0.011
219549_s_at	RTN3	Reticulon 3	2.089	0.023
Downregulated genes				
222553_x_at	OXR1	Oxidation resistance 1	0.371	0.044
217845_x_at	HIGD1A	Hypoxia-inducible domain family, member 1A	0.489	0.017
209476_at	TMX1	Thioredoxin-related transmembrane protein 1	0.480	0.018

cytokine formation, activation of lymphocytes, or antiviral response in the blood cells from children with influenza or rotavirus gastroenteritis (Wang et al. 2007; Xu et al. 2006). The gene expression profile of influenza patients without seizures shows that immune response genes, such as interferon (IFN)-regulated genes, were strongly upregulated in the acute phase (Kawada et al. 2006). We considered that the difference in gene expression between the Flu-Control and Rota-Control groups needed to be eliminated as background, to discuss the proper differentially expressed genes that contributed to progression of complex seizures in each viral infection. By selecting the differentially expressed genes by comparison between each seizure group and the control group, we were able to observe that only a few differentially expressed transcripts overlapped in complex seizure patients with both influenza A(H1N1)pdm09 and rotavirus gastroenteritis. These results strongly indicate that the genes related to progression to complex seizures differ greatly between influenza A(H1N1)pdm09 and rotavirus gastroenteritis.

Our investigations identified a significant dysregulation of IL-1R/TLR signaling in patients with complex seizures due to

influenza A(H1N1)pdm09. TLRs play a crucial role in the early host defense against invading pathogens (Kawai and Akira 2010). TLR7/8 can recognize single-stranded RNA (ssRNA) viruses, such as influenza virus (Heil et al. 2004). TLR8 is predominantly expressed in the lungs and peripheral blood leukocytes, especially monocytes (Bekeredjian-Ding et al. 2006; Hart et al. 2005; Peng et al. 2005). The interaction between the ssRNA and TLR7/8 triggers the activation of nuclear factor- κ B and other transcription factors and the production of proinflammatory cytokines and chemokines (Qin et al. 2006). In contrast, TLR4 can recognize the lipopolysaccharide in most Gram-negative bacteria (Hoshino et al. 1999). Recent studies have reported that TLR4 knockout mice are highly refractory to influenza-induced lethality, and that therapeutic antagonism of TLR4 signaling protects against influenza-induced acute lung injury (Abdul-Careem et al. 2011; Shirey et al. 2013). The increase in TLR4 transcripts in complex seizure patients due to influenza A(H1N1)pdm09 could play an important role in host defense against influenza A(H1N1)pdm09, through activation of innate immunity, but the mechanism by which TLR4 protects

Fig. 4 KEGG pathway analysis of differentially expressed transcripts in patients with complex seizures due to influenza A(H1N1)pdm09 or rotavirus gastroenteritis. The black bar represents the numbers of differentially expressed transcripts

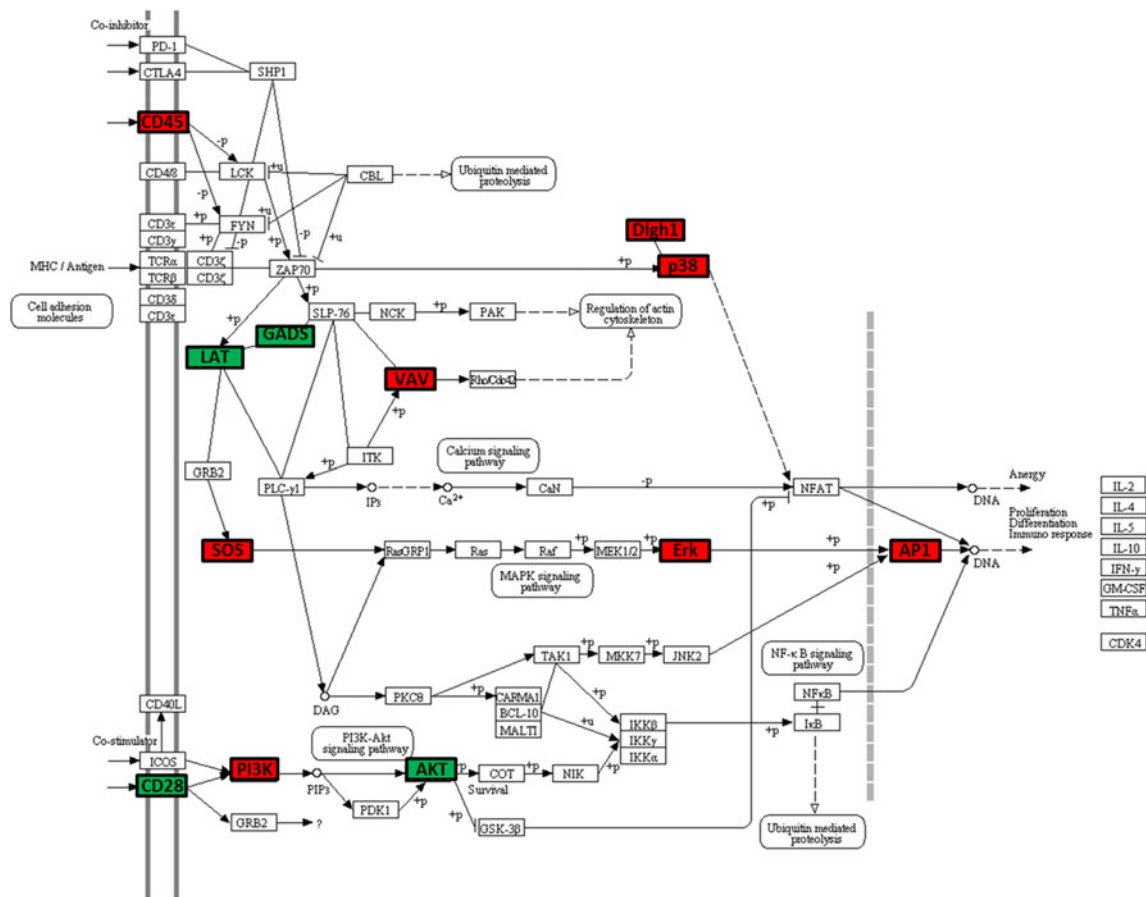


Fig. 5 KEGG pathway of TCR signaling. Gene expression changes were mapped on this pathway. *Colored boxes* indicate significant upregulation (*red*) or downregulation (*green*) of transcripts in patients with complex seizures due to influenza A(H1N1)pdm09 infection

against infection with influenza A(H1N1)pdm09 is not known at present.

During influenza virus infection, IL-1β and IL-18 are mainly produced by macrophages and dendritic cells as part of the innate immune response. IL-1β can bind IL-1R, and IL-1R1 is primarily responsible for transmitting the inflammatory effects of IL-1. IL-1R1 knockout mice infected with influenza A virus show significantly impaired neutrophil recruitment, diminished T cell responses, impaired IgM responses, and increased incidence of virus-induced fatality (Schmitz et al. 2005). In contrast, IL-18R1 specifically binds IL-18, and mice deficient in IL-18 have reduced cellular infiltration into the lungs and decreased levels of IFN-γ in the alveolar space early after influenza A virus infection (Liu et al. 2004). In a separate study, IL-18 knockout mice had a normal number of epitope-specific CD8 T cells but these were less capable of secreting IFN-γ, tumor necrosis factor (TNF)-α, and IL-2 (Denton et al. 2007). Collectively, these previous reports suggest that signaling through IL-1R and IL-18R has an important role in triggering the innate and adaptive immunity to generate a protective anti-influenza response. Our observations of increased expression of IL-18R, IL-1R, and TLRs in influenza A(H1N1)pdm09 patients with complex seizures suggest

intriguing mechanisms by which the innate immune system contributes to the pathogenesis of febrile complex seizures due to influenza A(H1N1)pdm09.

KEGG pathway analysis also showed that the host gene expression in patients with complex seizures due to influenza A(H1N1)pdm09 could be involved in the MAPK-mediated activation of the TCR signaling pathway. MAPK family members, such as Jun N-terminal kinase, p38 MAPK, and ERK1/2, have important functions in antiviral immunity and are crucial for the expression of several proinflammatory cytokines, as well as the regulation of apoptosis (Kujime et al. 2000; Maruoka et al. 2003). Influenza virus infection strongly upregulates the phosphorylation of MAPK family members; thus, MAPK activation induces activator protein-1-dependent gene expression (Ludwig et al. 2001). In particular, influenza virus-mediated MAPK activation also contributes to cytokine production, such as TNF-α, IL-1β, and IL-8. Upregulation of the MAPK pathway of TCR signaling in complex seizures due to influenza virus might therefore lead to the overabundant production of proinflammatory cytokines and chemokines, which is known as the cytokine storm in the influenza-associated encephalopathy.

Table 4 Validation of the expression of selected genes from microarray analysis by quantitative RT-PCR

IL-1R/TLR family-related genes								
Gene	Flu-Control (<i>n</i> =5)			Flu-Seizure (<i>n</i> =5)			Flu-Seizure vs Flu-Control	
	Acute	Recovery	Processed signal	Acute	Recovery	Processed signal	Fold change	<i>p</i> value
IL18R1	0.42±0.12	0.20±0.10	2.48±1.26	3.32±1.48	0.16±0.10	23.37±11.69	9.41	0.016
IL1R1	0.13±0.06	0.11±0.08	1.76±1.24	0.42±0.21	0.08±0.06	7.42±4.02	4.23	0.032
IL1R2	2.36±1.18	1.44±1.32	2.55±1.79	15.73±10.78	1.79±1.79	11.27±4.78	4.42	0.012
IL1RAP	0.53±0.26	0.30±0.14	1.86±1.05	0.67±0.30	0.17±0.11	4.52±1.72	2.42	0.023
TLR4	0.59±0.37	0.18±0.13	4.08±2.02	2.54±1.56	0.25±0.15	11.50±5.76	2.82	0.042
TLR8	3.17±1.04	1.66±1.13	2.57±1.50	4.72±1.45	0.88±0.36	5.63±1.75	2.20	0.018
Response to stress-related genes								
Gene	Rota-Control (<i>n</i> =3)			Rota-Seizure (<i>n</i> =3)			Rota-Seizure vs Rota-Control	
	Acute	Recovery	Processed signal	Acute	Recovery	Processed signal	Fold change	<i>p</i> value
AVPR1A	0.007±0.006	0.008±0.003	0.85±0.38	0.015±0.005	0.010±0.004	1.82±1.10	2.12	0.225
GLP1R	0.005±0.004	0.004±0.001	1.40±1.40	0.013±0.001	0.009±0.005	2.02±1.31	1.45	0.603
SOD2	9.70±5.70	10.98±3.82	0.87±0.33	27.67±15.90	11.49±6.55	2.52±0.80	2.89	0.030
GAL	0.002±0.002	0.002±0.001	1.47±1.88	0.003±0.002	0.002±0.002	2.87±3.09	1.95	0.539
RTN3	6.37±4.22	5.08±1.11	1.18±0.52	6.70±1.10	4.13±0.56	1.62±0.13	1.38	0.223
OXR1	0.68±0.23	0.49±0.23	1.48±0.40	0.41±0.25	0.69±0.34	0.56±0.12	0.38	0.019
HIGD1A	1.52±0.68	1.03±0.40	1.51±0.33	1.24±0.78	1.90±0.99	0.61±0.12	0.40	0.011
TMX1	0.82±0.16	0.59±0.27	1.71±0.99	1.12±0.84	1.10±0.60	0.92±0.36	0.54	0.262

Levels of gene expression were calculated using relative quantification with the GAPDH gene as an endogenous control. Processed signals are the ratios of the signal of the acute phase to that of the recovery phase. Data shown in the table are the mean±standard deviation of gene expression in patients of each group

In contrast, GO analysis revealed there was no significant expressed transcript related to immune response in the blood from the complex seizures due to rotavirus gastroenteritis. Rotavirus infections induce virus-specific T cell and cytokine responses in children (Jaimes et al. 2002; Mäkelä et al. 2004; Rojas et al. 2003); however, there are few reports about the correlation between the cytokine responses and the occurrence of seizures due to rotavirus gastroenteritis. A previous study reported that rotavirus RNA was detected in the heart and liver and, in patients with seizures, in cerebrospinal fluid, suggesting a possible mechanism for extraintestinal complications (Lynch et al. 2003; Pang et al. 1996). Although rotavirus antigen or RNA is frequently detected in the cerebrospinal fluid or serum of rotavirus gastroenteritis patients with neurological manifestations, the antigenemia is also known to be commonly detected in rotavirus-infected children without any complications (Fischer et al. 2005; Sugata et al. 2008). None of the patients showed antigenemia in our study. Recently, the mutation of genes such as PRRT2 or SCN1A was reported to be one of the causes of benign infantile convulsions with mild gastroenteritis, which is a common condition characterized by repetitive brief seizures associated with symptoms of gastroenteritis in infants and young children. These studies suggest that complex seizures due to rotavirus gastroenteritis do not result from exaggerated immune responses different from those due to influenza A(H1N1)pdm09.

GO analysis also revealed that the most significant enriched GO term expressed in the complex seizures due to rotavirus was the cellular response to stress, but not those due to influenza A(H1N1)pdm09. Previous studies revealed that genes involved in stress response were induced by rotavirus in vitro (Bagchi et al. 2012; Gac et al. 2010); however, there has been no study about the gene expression related to the cellular response to stress in the blood of patients with rotavirus gastroenteritis. qRT-PCR validated that SOD2, OXR1, and HIGD1A were differentially expressed in the complex seizures due to rotavirus. The common characteristics of these genes are that their proteins are localized to the mitochondria, and that these genes are induced by oxidative stress or hypoxia. Mitochondria have important functions that include cellular ATP production, control of apoptotic/necrotic cell death, reactive oxygen species (ROS) formation and calcium homeostasis, and the major source of ROS within the cell is the mitochondria (Liu et al. 2002). These genes can function to protect cells against oxidative DNA damage produced by oxidative agents (superoxide anions and ROS) and prolong cell survival. Depletion of these genes results in mitochondrial dysfunction, leading to an increase in sensitivity to oxidative stress or hypoxia, and enhanced apoptosis. Dysregulation of expression of these genes in the blood could change the susceptibility to apoptosis and survival of blood cells. In our study, we were not able to clarify whether the response to oxidative stress was induced as the cause or the consequence

of seizures due to rotavirus. However, we speculate that mitochondrial oxidative stress in the blood could be associated with the difference in pathogenesis in the seizures caused by rotavirus or influenza A(H1N1)pdm09.

In conclusion, our studies highlight the signatures of dysregulation of gene expression in complex seizure patients with influenza A(H1N1)pdm09 or rotavirus gastroenteritis and provide a framework for subsequent investigations into the different disease mechanisms. This study was limited by its small sample size for both the complex seizure and control patients, so the results must be interpreted with caution. Future studies will require careful case ascertainment and matching with healthy controls, as well as disease controls, to minimize the impact of confounding variables, such as age, sex, and pharmacological interventions, with a sufficiently large cohort of patients.

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Conflict of interest The authors declare that they have no conflicts of interest.

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