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Research Article

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Neonates with the KCNQ2 Y755C Variants: Not Associated with Neonatal Epileptic Encephalopathy

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Abstract

Background: *Pediatric epilepsy caused by a KCNQ2 gene mutation usually manifests the phenotype of a neonatal seizure. KCNQ2 encephalopathy in newborns continues to be reported on.*

Objectives: *The exact mechanism and phenotype of the KCNQ2 mutation still require investigation.*

Methods: *One hundred twenty-one patients with childhood epilepsy without an identified cause underwent KCNQ2 sequencing. KCNQ2 mutation variants were transfected into human embryonic kidney 293 (HEK293) cells to investigate functional changes.*

Results: *Two patients with the c.2264G>G/A (p.Y755C) variant had neonatal epileptic encephalopathy: one had electroencephalography (EEG) burst suppression and the other had multiple focal spikes. However, the mutation was not found in the 80 healthy adult claiming without ever seizures before. A functional study showed that p.Y755C currents were not different from those in the wild-type and from those in the benign (p.N780T) polymorphism in homomeric and heteromeric (wild-type KCNQ2: mutant = 1:1) transfected HEK293 cells. Electrical current differences between HEK293 cells with wild-type mutations and cells transfected with the wild-type KCNQ2, KCNQ3, and p.Y755C mutations in a 1:2:1 ratio were not significant. Their seizures remitted after they turned 1 year old.*

Conclusion: *We suggest that patients with the KCNQ2 p.Y755C mutations are not associated with neonatal epileptic encephalopathy.*

Keywords: Kcnq2; Newborn; Seizures; Phenotype; Encephalopathy 46

Introduction

KCNQ2-associated childhood epilepsy is a rare, inherited, autosomal-dominant form of neonatal epileptic syndrome. Seizures usually occur during the first week after birth. Benign familial neonatal convulsions (BFNC), a central nervous system channelopathy (ion channel dysfunction), is an oncogenic, autosomal-dominant, benign familial epilepsy syndrome [1, 2]. The *KCNQ2* mutation also can contribute to benign familial neonatal-infantile seizures (BFNIS) and benign familial infantile seizures (BFIS) [1-6]. Most BFNC seizures will spontaneously disappear during the infant's first 12 months of life [5]... However, at present, the outcomes in these patients cannot be accurately predicted. The *KCNQ2* gene is expressed predominantly in the brain and encoded for voltage-gated potassium channel subunits underlying the M-current, a repolarizing current that limits repetitive firing during long-lasting depolarizing inputs [5, 7-9]. In the *KCNQ2* gene, mutations can cause a haploinsufficiency or a more severe dominant-negative effect [10-12]. The precise genotype-phenotype correlation is not known, but the degree of functional disability caused by *KCNQ2* mutations is important. A *KCNQ2* phenotype of neonatal epileptic encephalopathy has recently been reported [13-15]. Most cases are de novo mutations, and patients present with severe seizures

and grave neurological consequences. Some patients present with burst-suppression or multiple focal spikes in neonatal electroencephalographies (EEGs). Seizures will remit after the patients become older, but the patients will usually have intellectual developmental delays. A loss of function via the dominant-negative effect of the *KCNQ2* gene is presumed to be the major mechanism for *KCNQ2* encephalopathy [16-18]. Because the in vitro functional consequences caused by *KCNQ2* mutations are not fully understood, we investigated the mutation variants of p.Y755C from patients with childhood epilepsy without an identified cause, and surveyed the functional changes in human embryonic kidney 293 (HEK293) cells transfected with *KCNQ2* mutation variants.

Patients and Methods

One hundred twenty-one patients with childhood epilepsy without an identified cause underwent *KCNQ2* sequencing. If the mutation variants were detected using direct Sanger sequences, further genetic tests were done for their relatives. Eighty healthy adults (160 chromosomes) without seizures were enrolled as controls. Next-generation sequencing was used to screen their genomes for *KCNQ2*. The mutation variants were compared between the Patient and Control groups. The functional changes in the mutation variants were analyzed.

Extracting and Amplifying DNA from *KCNQ2* Exons Using Polymerase Chain Reaction

After we obtained informed consents for all participants, a genomic DNA purification kit was used to extract a genomic DNA sample from a peripheral whole blood sample from each patient. For the patients, all 17 exons of the *KCNQ2* gene were individually amplified using a polymerase chain reaction (PCR). Each mutation was numbered relative to the ATG initiation codon and described according to the Mutation Database Initiative (MDI)/Human Genome Variation Society (HGVS) Mutation Nomenclature Recommendations.

Briefly, genomic DNA (100 ng) was mixed with 10 mM of Tris•HCl (pH 9.0), 1.5 mM of MgCl2, 50 mM of KCl, 0.1% (w/v) gelatin, 1% Triton X-100, 0.2 mM of dNTPs, 0.5 □M of both upstream and downstream primers, and 1 unit of Taq DNA polymerase (ProTech Technology Enterprise Co., Taipei, Taiwan). The PCR was done with thirty-five 30-s cycles at 94□C, annealing at a special temperature for 30 s, and extension at 72□C for 1 min.

Polymerase Chain Reaction (PCR) Product Purification and Sequencing Analysis

The PCR products were then purified (PCR-M™ Clean-Up System; Viogene-Biotek Corp., New Taipei City, Taiwan). The concentrations of these purified PCR products were measured using a spectrophotometer (Ultrospec 3100 Pro; Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK). The products were sequenced using an automated DNA sequencer (3100; Applied Biosystems, Foster City, CA). The patient's sequence data were checked against the published mRNA sequence data of the *KCNQ2* genes (NM_172107.2).

In Vitro Functional Study

Expression in Hek293 Cells, and Whole-Cell Patch-Clamp Analysis

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Biowhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 U/ml of streptomycin, and 2 mM of l-glutamine (Lonza, Walkersville, MD). *KCNQ2* mutations were made using a kit (Quick Change; Stratagene, La Jolla, CA) and verified using sequencing [19].

Whole-cell patch-clamp analysis

For electrophysiological analysis, the cells were bathed in modified Tyrode's solution containing 125 mM of NaCl, 5.4 mM of KCl, 1.8 mM of CaCl2, 1 mM of MgCl2, 6 mM of glucose, and 6 mM of HEPES (pH 7.4). Patch-pipettes had a resistance of 3-4 Ω when filled with pipette solution containing 125 mM of potassium gluconate, 10 mM of KCl, 5 mM of HEPES, 5 mM of ethylene glycol tetraacetic acid (EGTA), 2 mM of MgCl2, 0.6 mM of CaCl2, and 4 mM of adenosine 5′-triphosphate disodium salt hydrate (Na_2ATP) (pH 7.2).

To measure the voltage dependence of activation, the cells were clamped using 3-s conditioning voltage pulses to potentials between □80 mV and +40 mV in 10-mV increments from a holding potential of 0 mV. Data acquisition and analysis were done using electrophysiology data acquisition and analysis software (Clampex 10.0; Molecular Devices, Sunnyvale, CA). *KCNQ2* mutation variants and the wild-type variant were transfected into HEK293 cells to investigate the functional changes that cause cell-current changes. We used p.N780T (rs 1801475, a benign polymorphism) (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1801475) as a negative control

And p.R213Q, which has been proved to cause neonatal epileptic encephalopathy [13], as a positive control.

Statistics

Data are expressed as mean ± standard deviation. Statistically significant differences were evaluated using an independent t test or an analysis of variance (ANOVA) test. Significance was set at $p < 0.05$.

Table 1: Summary of clinical phenotypes for patients 1and 2.

Variable	Patient 1	Patient 2
Age	6 years	10 months
KCNQ2	c.2264A>G (p.Y755C),	c.2264A>G (p.Y755C)
KCNQ3	-	-
Other study*	*SCN1A*, Karyotype, urine organic and blood amino acid	*SCN1A*, STXBP1, mitochondrial electron transport chain study, muscle biopsy, karyotype, urine organic and blood amino acid glycine level in CSF to Blood: (004)
Gender	Female	Female
Functional domain	C-terminal	C-terminal
Family mutation	Negative	Mother
Family history	Declared negative	Mother: neonatal seizures
Age at first seizure	Day 2	Day 2
Seizure type	General tonic	General tonic, asymmetrical
Seizure frequency before first week	+++	+++
Seizure frequency before drug control	+++	+++
Drug control	Intravenous PB, PHT, oral SAB, then oral PB, SAB, then LEV after 3 years	Intravenous PB, PHT, LEV, then to oral OXC, LEV, SAB
EEG: neonatal	Multiple focal spikes	BS
EEG: 6 month-1 year old	Central spikes	Normal
EEG: 1-2 years old	Central spikes	Normal
Seizure frequency after drug control	+	+
EEG at age of first seizure	Multiple focal spikes	Burst suppression
Abnormal MRI	Corpus callosum hypoplasia	Corpus callosum hypoplasia
Developmental delay /intellectual disability	Severe, intelligence quoitent:49	Severe, intelligence quoitent:50
Additional features	Apnea	Apnea: dependent on bi-level positive airway pressure (BiPAP)

Other genetic tests in patient 1 and 2 included ABCD1, ADGRV1, ADSL, ALDH7A1, ALG13, ARHGEF9, ARX, ASAH1, CACNB4, CASK, CDKL5,CHD2, CLCN2, CPA6, DEPDC5, DCX, DNM1, DOCK7, EPM2A, EEF1A2, EFHC1, FLNA, GABRA1, GABRD, GABRG2, GFAP, GNAO1, GRIN2A, HCN1, KCNB1, KCNT1, KCNJ10, KCTD7, LBR, LGI1, MBD5, MECP2, MEF2C, MFSD8, NECAP1, NRXN1, PLCB1, PNKP, PNPO, PCDH19, POLG, PTEN, PIGA, PRICKLE2, RS1, SLC13A5, SLC1A1, SLC25A22, ST-3GAL3, ST3GAL5, SYNJ1, SCN1A, SCN1B, SCN2A, SCN8A, SNAP25, SPTAN1, STX1B, STXBP1, SYNGAP1, SCN9A, SLC2A1, SMS, SZT2, SLC35A2, SYN1, TPP1, TBC1D24, TCF4, and WWOX. NA, not available; PHT, phenytoin; OXC, oxcarbazepine; VPA, valproic acid; TOP, topiramate; PB, phenobarbital; LEV, levetiracetam; SAB, vigabatrin; CLN, clonazepam; MRI, magnetic resonance imaging; EEG, electroencephalography; +++, daily attack; ++, weekly attack; +, less than weekly.

Results

The *KCNQ2* c.2264G>G/A (p.Y755C) mutation was detected in 2 cases of neonatal epileptic encephalopathy from 121 patients with childhood epilepsy without an identified cause. However, the mutation was not found in the Control group. The p.Y755C mutation is predicted to be deleterious by the PolyPhen algorithm. The protein in positions 755 is highly conserved from zebrafish (*Danio rerio*) to *Homo sapiens* and other mammals. The p.Y755C causes a protein change in the C-terminal domain. The p.Y755C mutation is classified as a variant of unknown significance (VUS) (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=rs3746366). Two patients Carrying the p.Y755C mutation had neonatal seizures: one had EEG burst-suppression and the other had multiple focal spikes. Their seizures were in remission after they turned 1 year old. The clinical phenotypes are summarized in [Table 1].

Patients 1 and 2 Carried the C.2264a>G (P.Y755c) Mutation

Two patients (1 and 2) had the same mutation, c.2264A>G

(p.Y755C)—one *de novo* in patient 1, and one hereditary in patient 2—identified using next-generation sequencing and confirmed by Sanger sequences. Both patients had similar presentations of frequent neonatal seizures (within the first week of life) and apnea. The mother of patient 3 also had the p.Y755C mutation and a history of neonatal seizures. Magnetic resonance imaging (MRI) of patient 1 and patient 2 showed thin corpus callosum. Patient 1 had a moderate intellectual disability and could not walk until 3 years old. Patient 2 had a severe developmental delay.

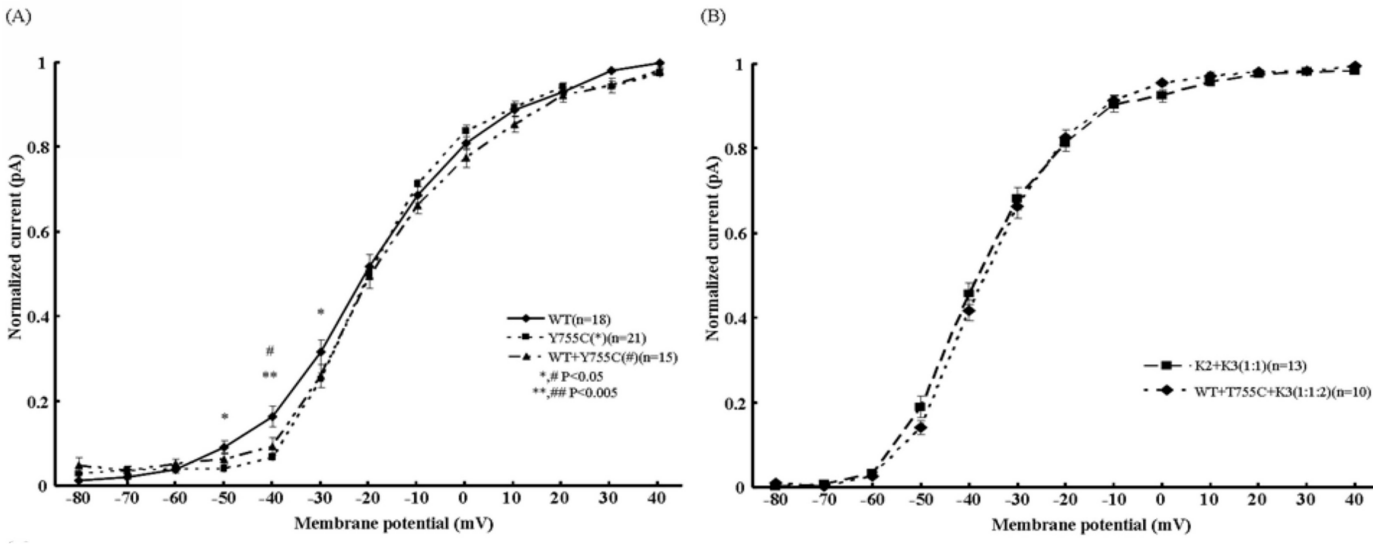

Figure 1: The membrane potential currents induced with conditioning voltage pulses to potentials between −80 mV and +40 mV using whole-cell patch-clamp analysis. (A) The current in the p.Y755C variants was significantly ($p < 0.05$ in homomeric 30 to 50 mV and heteromeric 40 mV; $p<0.005$ in homomeric -50 mV) lower than that in the wild-type ($p < 0.05$). Wild-type+ p.Y755M means wild-type+ p.Y755M in the ratio of 1:1; *$p<0.05$ when KCNQ2 mutation variants compared with wild-type; # $p<0.05$ when wild-type+ KCNQ2 mutation variants (1:1) compared with the wild-type. ## $p<0.005$ when wild type+ KCNQ2 mutation variants (1:1) compared with wild-type. (B) The cells transfected with the KCNQ2 wild-type, p.Y755C, and the wild-type KCNQ3 (1:1:2) showed no significantly different currents compared with the wild-type KCNQ2+ wild-type KCNQ3 (1:1).

Functional Study

To investigate the functional consequences of the p.Y755C mutations, we recorded macroscopic currents with the whole-cell configuration of the patch-clamp technique in HEK293 cells transfected with cDNAs encoded for the wild-type or one of the following mutants: p.Y755C and c.2339A>C (p.N780T) (rs 1801475, a benign polymorphism) [20].

The electrophysiological properties of the human wild-type mutant in KCNQ2's homomeric and heteromeric (wild-type and mutant = 1:1) transfected expressed in HEK293 cells were analyzed. The homomeric transfected cells were clamped between 80 mV and +40 mV in 10-mV increments from a holding potential of 80 mV (wild-type [A], n = 18, p.N780T [B] (n = 13), and p.Y755C [E] (n = 21) [Figure 1]. The membrane potential currents were induced with conditioning voltage pulses to potentials between −80 mV and +40 mV using whole-cell patch-clamp analysis. The data were then fit to a Boltzmann distribution of the following form: G/Gmax = 1/ (1 + exp ((V V½)/*dx*)), where V is the test potential, V½ the half-activation potential, dx the slope, and max the maximal amplitude of the Boltzmann distribution.

The currents were significantly ($p < 0.05$) lower in homomeric p.Y755C when the conditioning voltage potential was from 30 to 50 mV in p.Y755C, and significantly ($p<0.05$) lower in heteromeric (wild-type: mutant *KCNQ2* = 1:1) p.Y755C when the conditioning voltage potential was from 40 mV in p.Y755C (Figure 1). The p.Y755C substitution in *KCNQ2* affected the dx of the conductance-voltage curve in the homomeric and in the heteromeric (wild-type: mutant *KCNQ2* = 1:1) configuration, which suggested that channels carrying *KCNQ2* p.Y755C subunits were less sensitive to voltage and thus required stronger depolarizations to open probabilities than did the homomeric channels formed by Wild-type *KCNQ2* subunits and p.N780T subunits.

[Table 2].

The conductance-voltage curves showed that the current of the wild-type variant was almost equal to the current curve of the homomeric p.N780T variant [Table 2]. A proved benign single nucleotide polymorphism (SNP) transfected with p.N780T was not significantly different in functional degree, as in the wild type (Table 2). However, the currents in the wild-type *KCNQ2*, *KCNQ3*, and the p.Y755C (1:2:1), which were transfected respectively were not significantly different [Figure 1 and Table 2] from the current in the HEK293 cells with wild-type *KCNQ2* and *KCNQ3* (1:1). Taken together, current in the transfected pY755C mutants had activation kinetics that were almost equal to the current in the wild-type *KCNQ2* and p.N780T channels.

Table 2: The membrane potential currents induced with conditioning voltage pulses to potentials between −80 mV and +40 mV using whole-cell patch-clamp analysis.

P# (mV)	Wild type (n = 18)	Wild type KCNQ2 + KCNQ3 (1:1) (n = 13)	p.N780T (n = 13)	p.Y755C (n = 21)	Wild type KCNQ2 + p.Y755C (1:1) (n = 15)	Wild type KCNQ2 + wild type KCNQ3 + p.Y755C (1:1:2) (n = 10)
-80	0.012 ±	0.004 ±	0.02 ±	0.029±	0.049±	0.0118±
	0.005	0.002	0.005	0.008	0.017	0.004
-70	0.020 ±	0.009 ±	0.021±	0.036±	0.036±	0.003±
	0.004	0.003	0.006	0.008	0.01	0.001
-60	0.038 ±	0.034 ±	0.025±	0.041±	0.051±	0.027±
	0.007	0.008	0.007	0.008	0.01	0.005
-50	0.091 ±	0.191 ±	0.071±	0.041±	0.063±	0.143±
	0.016	0.025	0.013	0.008*	0.014	0.016
-40	0.163 ±	0.458 ±	0.117±	0.067±	0.092±	0.419±
	0.024	0.026	0.019	0.009**	0.02*	0.025
-30	0.314 ±	0.682 ±	0.306±	0.251±	0.258±	0.665±
	0.0297	0.026	0.032	0.013*	0.027	0.028
-20	0.516 ±	0.814 ±	0.524±	0.498±	0.492±	0.827±
	0.027	0.019	0.023	0.018	0.028	0.018
-10	0.684 ±	0.905 ±	0.717±	0.71±	0.659±	0.915±
	0.019	0.019	0.019	0.01	0.02	0.012
0	0.805 ±	0.926 ±	0.841±	0.833±	0.771±	0.956±
	0.015	0.015	0.012	0.015	0.023	0.007
10	0.885 ±	0.958 ±	0.912±	0.89±	0.849±	0.973±

P# (mV)	Wild type (n = 18)	Wild type KCNQ2 + KCNQ3 (1:1) (n = 13)	p.N780T (n = 13)	p.Y755C (n = 21)	Wild type KCNQ2 + p.Y755C (1:1) (n = 15)	Wild type KCNQ2 + wild type KCNQ3 + p.Y755C (1:1:2) (n = 10)
	0.014	0.011	0.011	0.014	0.018	0.008
20	0.927 ±	0.976 ±	0.949±	0.937±	0.918±	0.982±
	0.009	0.007	0.007	0.011	0.017	0.005
30	0.976 ±	0.982 ±	0.981±	0.938±	0.941±	0.984±
	0.004	0.006	0.004	0.012	0.018	0.006
40	0.994 ±	0.984 ±	0.992±	0.969±	0.975±	0.996±
	0.004	0.005	0.004	0.01	0.009	0.002
V1/2	-20.635 ±	-38.364 ±	-21.081±	-19.798±	-18.248±	-36.664±
	1.618 (mV)	1.138 (mV)	1.166 (mV)	0.5656(mV)	1.127 (mV)	0.971(mV)

#P, conditioning voltage potential; *, $P < 0.05$ compared with wild-type; **, $P < 0.005$; V½, half-maximal activation voltage; bold font, significantly different from wild-type.

Data rounded off to the 3rd decimal place.

The currents in the transfected cells were significantly lower in homomeric and heteromeric (mutant + wild-type, 1:1) p.E515D from -30 to -50 mV ($P < 0.05$). All mutations were confirmed to cause functional disabilities. V½ (half-maximal activation voltage) in heteromeric p.E515D and p.R213Q is significantly ($P < 0.05$) higher than in the wild-type.

Discussion

We report two patients with early-onset neonatal epileptic encephalopathy and with the *KCNQ2* p.Y755C mutation. Although their apnea and seizures went into remission after the patients were 1 year old, they had a neurodevelopmental delay or a cognitive disability. The contribution of this study is its examination of the functional change in the p.Y755C variant, which has been classified as a mutation with unknown significance, and its functional evaluation. We found that the voltage levels of the p.Y755C variants caused approximately almost equal to those of the wild-type and a proved benign polymorphism, p.N780T. The electrical current differences between HEK293 cells with wild-type mutations and cells transfected with the wild-type *KCNQ2*, *KCNQ3*, and p.Y755C mutations in a 1:2:1 ratio were not significant. *KCNQ2* expression is more common than *KCNQ3* expression in neonates (Positively stained neurons are > 50% of all neurons in *KCNQ2* protein but < 50% in *KCNQ3* protein), and *KCNQ3* will persist in the hippocampus and temporal lobe until adulthood, but *KCNQ2* expression will decrease in older children [20]. Functional changes in homomeric and heteromeric p.Y755C showed a significant difference in electrical current, but the current was not significantly different after transfecting HEK293 cells with wild-type *KCNQ2*, *KCNQ3*, and p.Y755C mutations in a 1:1:2 ratio. Taken together, family history, the PolyPhen algorithms, and functional studies predict that *KCNQ2* mutations p.Y755C not likely cause functional change, and should be suggested not associated with neonatal epileptic encephalopathy.

Two patients with p.Y755C mutations (one de novo and one hereditary) had associated developmental delay or cognitive disability. Both patients' seizures and apnea gradually disappeared. Patients with neonatal EEG burst-suppression have worse outcomes. Neither patient had seizures after turning 1 year old. A clinical history of seizure remission and EEG burst-suppression is compatible with *KCNQ2*-associated encephalopathy. It is obviously that the etiology in the two cases need further investigation, including whole exon study. The *KCNQ2*-associated burst-suppression pattern in newborns is different from other burst-suppression patterns with other etiologies, such as brain malformation, mutations of the ARX or STXBP1 genes [15, 21], or early myoclonic epileptic encephalopathy. Usually, *KCNQ2*-associated neonatal EEG burst-suppression produces general tonic seizures and, rarely, myoclonic seizures [13, 15]. There are three *KCNQ2*-associated neonatal EEG burst-suppression phenotype differences compared with other etiologies. First, an evolution to West syndrome characterized by epileptic spasms is infrequent. Second, medical control of seizures is relatively good and the seizures will disappear or be otherwise mitigated after the patient is 1 year old, as in the current cases. Third, effective antiepileptic drugs for early-onset KCNQ2-associated epileptic encephalopathy are not unique [15-24].

Conclusions

Neonates with the p.Y755C variants are not associated with neonatal epileptic encephalopathy.

Acknowledgments

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Declaration of Conflicting Interests

The authors declare that they have no conflicts of interest with respect to the authorship or publication of this article.

Author Contributions

ICL and SHW collected and analyzed the data, and drafted and revised the paper. ICL acted as the guarantors of the article.

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