

## Gene panel analysis for nonsyndromic cryptogenic neonatal/infantile epileptic encephalopathy

<sup>1</sup>Cheuk-Wing Fung, <sup>1</sup>Anna Ka-Yee Kwong, and Virginia Chun-Nei Wong

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**Dr. Cheuk-Wing Fung**  
an honorary clinical assistant professor in the Department of Pediatrics and Adolescent Medicine, the University of Hong Kong.



**Dr. Anna Ka-Yee Kwong**  
a postdoctoral fellow in the Department of Pediatrics and Adolescent Medicine, The University of Hong Kong.

### SUMMARY

**Objective:** Epileptic encephalopathy (EE) is a heterogeneous condition associated with deteriorations of cognitive, sensory and/or motor functions as a consequence of epileptic activity. The phenomenon is the most common and severe in infancy and early childhood. Genetic-based diagnosis in EE patients is challenging owing to genetic and phenotypic heterogeneity of numerous monogenic disorders and the fact that thousands of genes are involved in neurodevelopment. Therefore, high-throughput next-generation sequencing (NGS) was used to investigate the genetic causes of non-syndromic cryptogenic neonatal/infantile EE (NIEE).

**Methods:** We have selected a cohort of 31 patients with seizure cryptogenic NIEE and seizure onset before 24 months. All investigations including metabolic work-up, were negative. Using NGS, we distinguished a panel of 430 epilepsy-associated genes by NGS was utilized to identify possible pathogenic variants in the patients. Segregation analysis and multiple silico analysis prediction tools were used for pathogenicity assessment. The identified variants were classified as “pathogenic,” “likely pathogenic” and “uncertain significance,” according to the American College of Medical Genetics (ACMG) guidelines.

**Results:** Pathogenic or likely pathogenic variants were identified in six genes (*ALG13* [1], *CDKL5* [2], *KCNQ2* [2], *PNPO* [1], *SCN8A* [1], *SLC9A6* [2]) in 9 NIEE patients (9/31; 29%). Variants of uncertain significance (VUS) were found in *DNMI* and *TUBA8* in 2 NIEE patients (2/31; 6%). Most phenotypes in our cohort matched with those reported cases.

**Significance:** The diagnostic rate (29%) of pathogenic and likely pathogenic variants was comparable to the recent studies of early-onset epileptic encephalopathy, indicating that gene panel analysis through NGS is a powerful tool to investigate cryptogenic NIEE in patients. Six percent of patients had neurometabolic disorders. Some of our diagnosed cases illustrated that successful molecular investigation may allow a better treatment strategy and avoid unnecessary and even invasive investigations. Functional analysis could be performed to further study the pathogenicity of the VUS identified in *DNMI* and *TUBA8*.

**KEY WORDS:** Epilepsy, Epileptic encephalopathy, Next-generation sequencing, Neurodevelopment, Seizure.

Epileptic Encephalopathies (EEs) are heterogeneous conditions in which motor, sensory, and/or cognitive function deterioration results mainly from epileptic activity, which consists of frequent seizures and/or major interictal

paroxysmal activity.<sup>1</sup> EE may occur at any age, but the phenomenon is most common and severe in infancy and early childhood, which is the most critical period of brain maturation.<sup>2</sup> Although well-known EE such as Dravet syndrome,

## KEY POINTS

- Pathogenic or likely pathogenic variants were identified in six genes (*ALG13*, *CDKL5*, *KCNQ2*, *PNPO*, *SCN8A*, and *SLC9A6*) in 9 non-syndromic cryptogenic NIEE patients
- Variant of uncertain significance (VUS) was found in *DNMI* and *TUBA8* in another 2 NIEE patients
- The diagnostic yield of pathogenic or likely pathogenic variants was 29% (9/31)
- Next-generation sequencing with gene panel analysis is a powerful tool to investigate NIEE patients with unexplained etiologies

Ohtahara syndrome, West syndrome, and migrating focal epilepsy of infancy are recognized by the International League Against Epilepsy (ILAE), the majority of infants are non-syndromic and do not strictly fit into the defined electroclinical phenotypes of these syndromes.<sup>3</sup>

Etiology of EE can be congenital structural brain abnormalities, neurometabolic disorders, recognizable dysmorphic syndromes, or chromosomal, monogenic or environmental causes.<sup>4</sup> Structural brain abnormalities, either congenital (such as cortical malformations) or acquired (such as hypoxic ischemic insults), are the most common cause of early onset EE while neurometabolic disorders are the potentially treatable rare cause.<sup>3</sup> If imaging and metabolic tests fail to identify etiology, genetic-based diagnosis is important so that exhaustive and invasive testing can be avoided. On the other hand, it is challenging because of the genetic and phenotypic heterogeneity associated with particular gene variants.<sup>5</sup> In addition, thousands of genes are involved in neurodevelopment and the number of potential pathogenic variants is too high to be screened by traditional Sanger sequencing. The rapid development and lower cost of high throughput next-generation sequencing (NGS), allows such technology to be used for discovery of causal and predisposing gene variants associated with EE.

In recent studies, we proposed using Sanger sequencing of our selected panel of seven genes (*ARX*, *CDKL5*,

*KCNQ2*, *PCDH19*, *SCN1A*, *SCN2A* and *STXBPI*) as an option for genetic diagnosis in small-scale mutational studies.<sup>6</sup> We have identified 13 variants (46%) within this panel in 28 non-syndromic neonatal/infantile EE (NIEE) patients without clinical signs suggestive of a clear genetic syndrome, such as dysmorphic features or positive findings after extensive metabolic and neuroimaging studies.<sup>6–8</sup> Despite this, the underlying etiology of the remaining patients with negative findings remained unexplained. In the present study, we have applied gene panel analysis of 430 epilepsy-associated genes in 31 non-syndromic cryptogenic NIEE patients for genetic-based diagnosis and subsequent potential treatment strategy.

## METHODS

### Patient samples and clinical diagnosis

The study was conducted in Queen Mary Hospital and Duchess of Kent Children's Hospital, two affiliated hospitals of the University of Hong Kong. The selection criteria and clinical assessments of the nonsyndromic NIEE patients have been described in our previous study.<sup>6</sup> We included patients who satisfied the ILAE definition of EE, with seizure onset before 24 months of age, and who have been actively followed up in our centre. Patients with a definite history of brain insult, malformation of cortical development, neurocutaneous and syndromal disorders, and confirmed or highly suspected neurometabolic disorders based on clinical and biochemical markers were excluded. Patients who fit into distinct electroclinical syndromes proposed by the ILAE when candidate gene approach is straightforward, in particular Dravet syndrome and epilepsy of infancy with migrating focal seizures, were also excluded because the majority of variants are detected in the *SCN1A* (>85%) and *KCNT1* (~50%) genes, respectively.<sup>9</sup>

Data variables collected from the medical charts included demographic information (sex, ethnicity, age at seizure onset and latest follow-up), family history (febrile convulsion, epilepsy, intellectual disability and other neurological diseases), epilepsy details (seizure types at onset and latest follow-up, seizure frequency and evolution, current number of antiepileptic medications), neurological examination findings, and other associated clinical features (such as autism spectrum disorder, visual and hearing impairment). Information regarding the developmental status at the latest follow-up was collected as well. Either formal neuropsychological testing (using Griffiths Mental Developmental Scale or Hong Kong-Wechsler Intelligence Scale for Children) or best clinical assessment (based on developmental milestones recorded in the medical charts) was used to classify development or intelligence as normal, mildly delayed, moderately delayed or severely delayed.

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Division of Paediatric Neurology/Developmental Behavioural Paediatrics/Neurohabilitation, Department of Paediatrics and Adolescent Medicine, Li Ka Shing Faculty of Medicine, the University of Hong Kong, Hong Kong SAR, China

Address correspondence to Virginia Chun-Nei Wong, Department of Paediatrics and Adolescent Medicine, Queen Mary Hospital, Pokfulam Road, Pokfulam, Hong Kong SAR, China. E-mail: vcnwong@hku.hk

<sup>1</sup>These authors contributed equally to the study.

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### Whole exome sequencing and gene panel filtering

Blood sample collection from all participants for genetic study was approved by the Institutional Review Board of the Hong Kong West Cluster and the University of Hong Kong (IRB Ref. No.: UW 11-190). Written informed consent was obtained from the parents of the NIEE patients. Genomic DNA samples were extracted from peripheral blood using Flexigene DNA Kit (Qiagen GmbH, Germany). Quality of genomic DNA was evaluated by agarose gel analysis and quantity was measured by Qubit dsDNA assay (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

The genomic DNA sample library was prepared using KAPA Library Preparation Kit, Illumina platforms (KAPA Biosystem, Boston, MA, U.S.A.). DNA target regions were captured by hybridizing the genomic DNA sample library with the SeqCap EZ Human Exome Library v.3.0 (64-Mb exome) (Roche NimbleGen, Basel, Switzerland). The captured and amplified DNA samples were sequenced using Illumina Hiseq 1500 sequencer (Illumina, San Diego, CA, U.S.A.) with 101 base-paired end reads.

The raw sequencing reads were first filtered to remove adaptor sequence and low-quality sequence by retaining only reads with read length  $\geq 40$  bp. The filtered reads were mapped to the human genome using BWA Version 0.6.2 software with default parameters. The mapped reads were then split into on and off target reads and duplicated reads in the on target reads were removed by Picard Version 1.73 tools. Local realignment, base quality recalibration and variants calling (both SNP and Indels) were performed by GATK Version 3.2-2 using default parameters. Raw variants were filtered as recommended by the GATK authors. The final lists of variants were annotated by Annovar for examination of allele frequency, prediction of potential functional impact of the coding variants (missense, nonsense, or frameshift), and reporting the score of pathogenicity assessment. The National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project

(NHLBI GO ESP) with 6,500 exomes and 1,000 genome project datasets was used to assess the allele frequency in the control population; we considered variants with a frequency of  $< 1\%$ .

The variants called from whole exome sequencing (WES) were filtered in a panel of 430 epilepsy-associated genes. The panel (Table 1) included (1) genes associated with early infantile EE (EIEE) and EE in the Online Mendelian Inheritance in Man (OMIM) database (<http://www.ncbi.nlm.nih.gov/omim>), (2) candidate genes identified in exome studies, (3) candidate genes from EE or epilepsy-associated copy number variation (CNV) studies<sup>5,10</sup> and (4) genes related to epilepsy or other neurodevelopmental diseases with most of them selected from a panel suggested by Lemke et al.<sup>11</sup> After selection of variants from the 430-gene panel, the synonymous variants, variants with variant frequency  $< 10\%$ , and variants with allele frequency  $> 1\%$  were removed. The variants were predicted to be pathogenic by the score of pathogenicity assessment that used using in silico bioinformatics tools, including Sorting Intolerant from Tolerant (SIFT) (Genome Institute of Singapore, <http://sift.bii.a-star.edu.sg/>) and Polymorphism Phenotyping v2 (PolyPhen-2) (<http://genetics.bwh.harvard.edu/pph2/index.shtml>). For variants with contradictory predictions in the two pathogenicity assessments, only the 55 genes associated with EIEE and EE genes in OMIM were selected for further analysis. All the variants identified were further confirmed by Sanger sequencing, the corresponding gene contexts were evaluated according to OMIM with the individual phenotypes, and they were further assessed by Mutation Taster (<http://www.mutationtaster.org/>). The Exome Aggregation Consortium (ExAC) database (<http://exac.broadinstitute.org/>) was consulted for the allele frequencies of these variants. Segregation analysis was carried out to select de novo or compound heterozygous variants. The splice site variants were analyzed by another online software tool, Human Splicing Finder (<http://www.umd.be/>)

**Table 1. The 430 genes selected for further filtering**

Genes associated with EIEE and EE in OMIM (55)	AARS, ARHGEF9, ARX, CDKL5, DNMI1, DOCK7, EEF1A2, FOXG1, GRIN2B, GABRA1, GNAO1, HCNI, ITPA, KCNA2, KCNB1, KCNQ2, KCNT1, MAGI2, MECP2, NECAP1, PCDH19, PIGA, PLCB1, PNKP, PNPO, SCN1A, SIK1, SLC12A5, SLC13A5, SCN2A, SLC25A22, SLC35A2, SCN8A, SPTAN1, ST3GAL3, STXBPI, SZT2, TBC1D24, WWOX, ALG13, ARHGEF15, CACNA2D2, CBL, CHD2, CLCN4, CSNK1G1, GABRG2, GRIN2A, KCNH5, KPNA7, MAPK10, MBD5, PIGQ, SRGAP2, SYNGAP1
Candidate genes identified in exome studies (35)	ADSL, ANK3, CACNA1A, CNTN5, DCX, DNAJC6, FLNA, GABRB1, GRIN1, HDAC4, IQSEC2, MTOR, NEDD4L, PTEN, FASN, GABBR2, RANGAP1, RYR3, TTN, HADHB, KIAA1456, LPHN2, MTMR1, OR10H2, ZMYND8, ZNF182, HNRNP1, MEF2C, UBE3A, ASAH1, FOLR1, SYNJI, CASK, KCND2, KCNV2
Candidate genes from EE or epilepsy-associated CNVs (53)	ADAM22, ADAM23, AKT3, APBA1, APBA2, CACNA2D1, CHD5, CHLI, CLVS2, CPLX1, CRIPT, CRMP1, DLGAP1, DLGAP2, DLGAP3, DLX1, DLX2, EFCAB2, EPHA6, EPHB2, EPN1, ERBB4, FZD9, GABRA5, GABRB3, GABRG3, GAD1, GNAI1, JAKMIP1, KLHL17, MAGEL2, MAPK8, NCAM2, OTX1, RCOR1, PDK1, PPP2R2C, REL, SEMA3A, SEMA3E, SH3GL2, SHROOM2, SLC1A1, SLC1A3, SMARCA2, SNPH, SPI, STX1A, SV2A, SV2B, SYT2, ZNF532, ZNF536
Genes related to epilepsy or other neurodevelopmental diseases (287)	272 genes from NGS panel by Lemke et al., <sup>11</sup> ASNS, BCKDK, CHRNA7, CPA6, DNAJC5, GATM, GOSR2, GRN, KANSL1, LIAS, PRRT2, SLC19A3, ST3GALS, SYNI, UPB1

CNV, copy number variation; EE, epileptic encephalopathy; EIEE, early infantile EE; OMIM, Online Mendelian Inheritance in Man database.

HSF3/HSF.html). The identified variants were classified as “pathogenic,” “likely pathogenic,” or “uncertain significance,” according to the American College of Medical Genetics (ACMG) standards and guidelines.<sup>12</sup>

## RESULTS

Thirty-one non-syndromic cryptogenic NIEE patients were recruited into this study, including 14 patients who had negative findings in our previous 7-gene panel study.<sup>6</sup> We sequenced the exon regions of these patients by WES. At least 49.8 million on-target reads after duplicate read removal were generated per patient. The average on-target coverage was 58×, and the average percentage of coverage ≥8 on-target regions was 96%. The variants called from WES were filtered in a 430-gene panel that included four categories of genes: EE genes from OMIM, candidate genes identified in exome studies, candidate genes from CNV studies, and genes related to other neurodevelopmental disorders. We have identified variants in 11 NIEE patients (35%) in our cohort of 31 cases (Table 2). Eight patients had variants identified in six EE genes (*ALG13*, *CDKL5*, *DNM1*, *KCNQ2*, *PNPO*, and *SCN8A*) defined by OMIM, and the other three had variants in two genes related to other neurodevelopmental disorders, including *SLC9A6* which is associated with Christianson type of syndromic X-linked mental retardation (MRXSCH), and *TUBA8*, related to polymicrogyria with optic nerve hypoplasia. No variant could be found in the other two categories of genes (candidate genes identified in exome and CNV studies) in the panel. Of these 11 patients with variants found, 9 had variants that were classified as pathogenic or likely pathogenic and 2 had variants of uncertain significance (VUS), according to the ACMG standards and guidelines (Table 3).

The clinical characteristics of these 11 patients with variants are listed in Table 2, and most of the clinical phenotypes of these patients resembled those reported previously with variants identified in the same genes.

Patient 55 was a *PNPO* compound heterozygote consisting of a frameshift variant (p.Pro150Argfs\*27) and a missense variant (p.Arg161Gly) which involves a highly conserved residue Arg161 and was predicted to be an enzyme active site.<sup>13</sup> This patient presented with neonatal onset EE and negative biochemical markers for pyridox (am)ine phosphate oxidase (PNPO) deficiency. He had a transient 2-month complete response to oral vitamin B<sub>6</sub> at 30 mg/kg/day, followed by recurrence of intractable seizures at 3 months. Oral pyridoxal phosphate (PLP) at 30 mg/kg/day was then tried for 4 days without any clinical improvement. After PNPO deficiency was confirmed genetically, PLP was restarted at 40 mg/kg/day, and he became totally seizure-free. He was able to be weaned off anticonvulsants from a total of five to valproate monotherapy. Neurodevelopmental outcome was poor with severe developmental delay, cortical visual impairment and

autistic features. Our patient illustrated the importance of adequate therapeutic trial of PLP up to 50 mg/kg/day.<sup>14</sup>

In addition to the classical phenotype as described by Smith-Packard<sup>15</sup> with early-onset EE and severe psychomotor delay, Patient 75 with a *ALG13* variant (p.Asn107Ser) also had intermittent generalized dystonia and hand stereotypies resembling those of other patients in our cohort with *CDKL5* encephalopathy at a slightly later age of onset at 4 months. Despite having a pathogenic variant in a gene associated with congenital disorder of glycosylation (CDG), there was no clinical evidence of a multi-systemic disorder or positive biochemical markers, including transferrin isoform electrophoresis, similar to the reported cases.

Details of the variants are summarized in Table 3. Variants were located in specific domains, which may be important for the gene functions and we have included them in the table. All variants were not found or were found with extremely low allele frequency in controls of the ExAC database. They were predicted to be pathogenic by all silico prediction tools (SIFT, Polyphen-2 and Mutation Taster) except for p.Tyr284His, identified in *KCNQ2* and p.Asn107Ser, identified in *ALG13*. For Patient 73 with a *CDKL5* variant, segregation analysis could not be performed because the DNA of both parents was not available. However, this is a truncating variant that was not found in controls of the ExAC database and is likely to be pathogenic.

## DISCUSSION

EE is a group of phenotypically and genotypically heterogeneous disorders. In the present study, we recruited a cohort of EE patients with neonatal and infantile onset. In addition to the known EE genes in our gene panel, we included candidate genes from previous exome/CNV studies and genes associated with other neurodevelopmental disorders to identify more genes associated with these disorders. A number of criteria, including allele frequency according to exome sequencing project, pattern of inheritance, and pathogenicity assessed by multiple computational prediction tools, were applied for variant selection.

### Pathogenicity of the variants

Although the pathogenicity assessment of p.Tyr284His of *KCNQ2* was not consistent by different silico prediction tools, a similar variant p.Tyr284Cys reported previously in a family affected by benign familial neonatal seizure (BFNS)<sup>16</sup> was demonstrated to decrease axonal surface expression of *KCNQ2* channels by 50%. In addition, p.Tyr284His is de novo and absent in ExAC controls. Therefore, it is likely to be pathogenic. Instead of BFNS, our patients with p.Tyr284His had EE and severe developmental delay. Some previous studies illustrated that affected family members carrying the same *KCNQ2* variant could present with different phenotypes ranging from benign BFNS to severe epileptic encephalopathy.<sup>17,18</sup> Such



Table 2. Clinical characteristics of 11 patients with cryptogenic Neonatal Infantile Epileptic Encephalopathies (NIEE) in whom variants were detected

Case no.	Ethnic origin	Sex/age (years)	Variants with predicted amino acid change	Seizure onset (months)	Type of seizure at onset	Seizure types developed	Number of antiepileptic drugs at most recent follow-up	Seizure evolution	Developmental profile at latest follow-up	Associated clinical features
1	Chinese	F/11	TUBA8 Compound heterozygous p.Arg79Trp & p.Asn186Ser	6	At, M	A, At, F, G, M, S	4	>25% but <50% reduction	Severe ID; developmental regression	UMN signs
7	French/Chinese	M/5	SLC9A6 splice site c.794-2A>G	12	F	F, G, M, O (hypomotor seizures)	2	>50% reduction	Severe ID; developmental slowdown	Autistic, microcephaly
13	Chinese	M/8	SLC9A6 frameshift Leu280Aafs*17	13	G	A, F, G, M, O (ESES)	4	>50% reduction	Severe ID; developmental regression	Autistic, microcephaly
16	Chinese	M/18	SCN8A missense p.Arg1617Gln	10	G	F	4	>50% reduction	Moderate ID; developmental regression	Cerebellar ataxia
52	Chinese	F/3	CDKL5 frameshift p.Arg617Valfs*4	3	G	G, F, S	3	>50% seizure reduction	Severe GDD	CVI, dystonia and chorea, hand-washing stereotypies
55	Chinese	M/3	PNPO Compound Heterozygous p.Arg161Gly & p.Pro150Argfs*27	<1	F	G, F, S	1	Seizure free on regular pyridoxal phosphate at 40 mg/kg/day	Severe GDD	CVI, autistic
68	Chinese	F/4	KCNQ2 missense p.Tyr284His	<1	F	F, M, S, G	1	Seizure free	Severe GDD	CVI, dystonia
72	Chinese	F/3	DNM1 missense p.Pro144Leu	4	G	S, F, G	3	Static	Severe GDD	Marked hypotonia
73	Chinese	F/3	CDKL5 frameshift p.Ile143Aafs*6	2	G	G, A, S	2	>50% seizure reduction	Severe GDD	Hand washing stereotypies
75	Chinese	F/2	ALG13 missense p.Asn107Ser	4	S	S	3	>50% seizure reduction	Severe GDD	Dystonia, CVI, hand washing stereotypies
76	Chinese	F/2	KCNQ2 missense p.Gly315Glu	<1	G	G, M, F	3	Seizure free	Severe GDD	Dystonia

A, absence seizure; At, atonic seizure; CVI, Cortical Visual Impairment; ESES, Electrical Status Epilepticus during Slow-wave sleep; F, focal seizure with or without generalization; G, generalized tonic/clonic/tonic-clonic seizure; GDD, Global Developmental Delay; ID, Intellectual Disability; M, myoclonic seizure; O, other seizure type; S, spasm; UMN, upper motor neuron.

Table 3. Details of the variants identified in 11 non-syndromic NIEE patients

Gene	Case no.	Type of variants	Exon	mRNA accession no.	Variants			Location in protein	ExAC <sup>c</sup>	Reported/Novel	Inheritance patterns	Silico prediction tools <sup>b</sup>			Classification according to ACMG standards
					Nucleotide	Amino acid	Amino acid					Poly Phen-2	SIFT	Human Splicing Finder	
CDKL5	52	Heterozygous frameshift deletion	12	NM_003159.2	c.1849delC	p.Arg617Valfs*4	—	Upstream of three putative sites at C-terminal	Not found	Novel	De novo	NA	NA	NA	Pathogenic
KCNQ2	73	Heterozygous frameshift deletion	7	c.427_430delATCA	p.Ile143Alafs*6	—	Catalytic domain	Not found	Novel	DNA of parents not available	DNA of parents not available	NA	NA	NA	Likely pathogenic
	76	Heterozygous missense	7	NM_172107.3	c.944G>A	p.Gly315Glu	Calmodulin-binding domain Helix A	Not found	Novel	De novo	De novo	+/I	+/0	NA	Likely pathogenic
SCN8A	68	Heterozygous missense	6	c.850T>C	p.Tyr284His	—	Pore-forming P-loop	Not found	Novel	De novo	De novo	+/0.993	-/0.13	NA	Likely pathogenic
	16	Heterozygous missense	27	NM_014101.3	c.4850G>A	p.Arg1617Gln	S4 of transmembrane domain IV	Not found	Reported <sup>22,31,32</sup>	De novo	De novo	+/I	+/0	NA	Pathogenic
DNM1	72	Heterozygous missense	4	NM_004408.3	c.431C>T	p.Pro144Leu	GTPase domain	Not found	Novel	Mother is not carrier; DNA of father not available	Mother is not carrier; DNA of father not available	+/I	+/0	NA	Uncertain significance
	55	Compound heterozygous missense and frameshift deletion	5	NM_018129.3	c.481C>G	p.Arg161Gly	Between helix 3 and 4	MAF=0.0002313 for East Asian	Reported in ClinVar (Likely pathogenic)	Mother-carrier of p.R161G;	Mother-carrier of p.R161G;	+/I	+/0	NA	Likely pathogenic
SLC9A6	7	Hemizygous splice site	Intron 6	NM_001042537.1	c.794-2A>G	—	Transmembrane domain 7	Not found	Novel	Mother-carrier of p.Pro150Argfs*27	Mother-carrier of p.Pro150Argfs*27	NA	NA	NA	Pathogenic
	13	Hemizygous frameshift deletion and insertion (indels)	6	c.838_839delinsG	Leu280Alafs*17	—	Extracellular region between transmembrane domain 7 and 8	Not found	Novel	Reported in ClinVar (Pathogenic/Likely pathogenic)	Mother-carrier	NA	NA	NA	Likely Pathogenic
ALG13	75	Heterozygous missense	3	NM_001099922.2	c.320A>G	p.Asn107Ser	Conserved domain that may be important for catalytic activity	Not found	Reported <sup>15,33-36</sup>	De novo	De novo	-/0.437	+/0	NA	Pathogenic
	1	Compound heterozygous missense	3	NM_018943.2	c.235C>T	p.Arg79Trp	No information available	MAF=0 for East Asian	Novel	Mother-carrier of p.Arg79Trp;	Mother-carrier of p.Arg79Trp;	+/I	+/0	NA	Uncertain significance
TUBA8	4	Heterozygous missense	4	c.557A>G	p.Asn186Ser	—	Conserved domain	Not found	Reported	De novo	De novo	+/I	+/0	NA	Uncertain significance

<sup>a</sup>Allele frequency from the ExAC database (<http://exac.broadinstitute.org/>).

<sup>b</sup>For Polyphen-2, “+” indicates the variant is probably damaging and “-” indicates that it is benign. For SIFT analysis, “+” indicates the variant affects protein function and “-” indicates that it is tolerated. For Human Splicing Finder, “+” indicates the variant break the acceptor site and most probably affects splicing. For Mutation Taster, “+” indicates that it is disease causing.

phenotypic variation might be caused by genetic modifiers and different environmental backgrounds.

The splice site variant c.794-2A>G of *SLC9A6* was inherited from Patient 7's asymptomatic mother. As reviewed by Pesosoido et al.,<sup>19</sup> a majority of studies report that the female carriers of the *SLC9A6* variants could be asymptomatic or present with different levels of neurodevelopmental or behavioral problems. Most of the pathogenic *SLC9A6* variants identified were truncating.<sup>19</sup> Loss of function of *SLC9A6* may possibly decrease endosomal pH and ion content which may affect the endosomal trafficking required for the growth and maintenance of dendritic spines.<sup>20</sup> Therefore, the *SLC9A6* variant we identified is likely to be pathogenic.

The *DNMI* and *TUBA8* variants were classified as VUS. For the *DNMI* variant, DNA of Patient 72's father was not available for segregation analysis, so the evidence was not enough to show p.Pro144Leu of *DNMI* is pathogenic according to ACMG standards, although it is not found in ExAC controls and is located at the active GTPase domain. For *TUBA8*, a 14-bp *TUBA8* homozygous deletion was previously identified in four children of two Pakistani families and was characterized by generalized polymicrogyria in association with optic nerve hypoplasia (PMGOH), severe developmental delay, hypotonia, and seizures. Alpha-8-tubulin, encoded by *TUBA8*, was demonstrated to participate in the microtubule assembly in mammalian cells.<sup>21</sup> Our patient, however, presented with severe intellectual disability (ID), developmental regression, hypotonia and seizure indistinguishable from the other patients in our cohort. Neuroimaging was normal, without malformation of cortical development or optic nerve hypoplasia. More functional studies should be performed in the future to investigate the association of *TUBA8* defect with NIEE and pathogenicity of the *TUBA8* VUS identified in our study.

### Insights into diagnostic and treatment strategies

An accurate and early genetic diagnosis is crucial to avoid unnecessary investigations. This can be exemplified by our Patient 16 with an *SCN8A* variant who presented with intractable NIEE, progressive dementia, and cerebellar ataxia mimicking a neurodegenerative, possibly mitochondrial disorder. Successful identification of this pathogenic variant could avoid further invasive biopsies for investigating a possible mitochondrial cytopathy.

More importantly, a successful molecular diagnosis may allow for a better treatment strategy. For Patient 55, genetic confirmation of PNPO deficiency prompted a retreat of an adequate dose of PLP therapy, rendering the patient seizure-free. Besides, both Patient 16 and 65 with *SCN8A* variants had excellent responses to sodium channel blockers such as carbamazepine and phenytoin. Larsen et al.<sup>22</sup> also observed that a few cases became seizure-free with use of sodium channel blockers, in contrast to the exacerbating effect to seizures in Dravet syndrome. This may be due to the

differences in localizations and roles of Na<sub>v</sub>1.6 and Na<sub>v</sub>1.1 channels in neuronal excitability.<sup>23</sup> In addition to *SCN8A* encephalopathy, a recent study showed that carbamazepine and phenytoin should be considered as first-line treatments in patients with *KCNQ2* encephalopathy because of their modulating effects on voltage-gated sodium and potassium channels for reducing neurodevelopmental impairment.<sup>24</sup> This further supports the importance of genotype identification for a better choice of anticonvulsant in the patients with different channelopathies. Encouragingly, studies on targeted treatments in genetic NIEE show promising results, including the use of potassium channel openers such as ezogabine in patients with pathogenic *KCNQ2* variants for positive effects on seizures and/or developmental status.<sup>25</sup>

Our two patients ultimately diagnosed with PNPO deficiency and CDG illustrated that neurometabolic disorders can present non-specifically with EE and severe intellectual disability. Therefore, absence of multi-systemic involvement and/or biochemical metabolic markers does not rule out inborn errors of metabolism. Diagnosis is essential because patients may have a potentially treatable disorder, just like our cases with PNPO deficiency.

### Diagnostic rate of variants identified by gene panel study

The diagnostic rate of pathogenic or likely pathogenic variants identified in the present study of 31 non-syndromic NIEE patients by the 430-gene panel analysis was 29%. This rate is comparable to several recent NIEE studies by whole exome or gene panel analysis with diagnostic yields ranging from 18% to 61%.<sup>26–30</sup> However, it is not easy to compare these studies with ours because of the variations in patient inclusion criteria, number of genes in the targeted panel, and pipeline applied. Among our 31 patients, 14 patients had negative findings in our previous 7-gene panel study<sup>6</sup> and were subsequently recruited to this study. For these 14 patients, the present study identified four additional variants.

Because most of the variants were identified in 55 EE genes defined by OMIM, the EE gene panel analysis will be useful as a first-line molecular diagnostic tool to identify the genetic etiologies of non-syndromic cryptogenic NIEE patients. It is quicker to analyze gene panels instead of whole exome data. If the result is negative, the remaining WES data can be used to detect variants in rarer NIEE genes or even to discover novel genes.

Our study has several drawbacks. Because of limited resources, we used singletons instead of trio-based exome sequencing, which can narrow down the candidate variants according to the underlying inheritance patterns. WES can only identify the genetic defect in coding regions. Non-coding regulatory defects and structural genomic variants could not be detected. Another limitation of our study is that array comparative genomic hybridization (aCGH) was not performed to identify deletion/duplications. If more resources

are available, aCGH, trio-based studies, WES should be employed in research to detect more causative genetic defects in the NIEE patients. Moreover, detection rates in NGS-based studies will inevitably be improved by the increased knowledge of the genes that underlie a particular disorder.

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## DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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