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ORIGINAL ARTICLE

Mutation analysis of glycine decarboxylase, aminomethyltransferase and glycine cleavage system protein-H genes in 13 unrelated families with glycine encephalopathy

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Glycine encephalopathy (GCE) or nonketotic hyperglycinemia is an inborn error of glycine metabolism, inherited in an autosomal recessive manner due to a defect in any one of the four enzymes aminomethyltransferase (AMT), glycine decarboxylase (GLDC), glycine cleavage system protein-H (GCSH) and dehydrolipoamide dehydrogenase in the glycine cleavage system. This defect leads to glycine accumulation in body tissues, including the brain, and causes various neurological symptoms such as encephalopathy, hypotonia, apnea, intractable seizures and possible death. We screened 14 patients from 13 families with clinical and biochemical features suggestive of GCE for mutation in *AMT*, *GLDC* and *GCSH* genes by direct sequencing and genomic rearrangement of *GLDC* gene using a multiplex ligation-dependant probe amplification. We identified mutations in all 14 patients. Seven patients (50%) have biallelic mutations in *GLDC* gene, six patients (43%) have biallelic mutations in *AMT* gene and one patient (7%) has mutation identified in only one allele in *GLDC* gene. Majority of the mutations in *GLDC* and *AMT* were missense mutations and family specific. Interestingly, two mutations p.Arg265His in *AMT* gene and p.His651Arg in *GLDC* gene occurred in the Penan sub-population. No mutation was found in *GCSH* gene. We concluded that mutations in both *GLDC* and *AMT* genes are the main cause of GCE in Malaysian population.
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INTRODUCTION

Glycine encephalopathy (GCE, MIM #605899, also known as nonketotic hyperglycinemia) is an autosomal recessive disorder of glycine metabolism due to a defect in the glycine cleavage system (EC 2.1.2.10).[1] The glycine cleavage system is a mitochondrial multienzyme system consisting of four protein components: three specific components (P-, T- and H-protein) and one housekeeping enzyme (L-protein).[2] P-protein is encoded by glycine decarboxylase gene (*GLDC*), aminomethyltransferase gene (*AMT*) encodes T-protein, glycine cleavage system protein-H gene (*GCSH*) encodes H-protein and dehydrolipoamide dehydrogenase gene encodes L-protein. Defect in any one of the four enzymes in the glycine cleavage system may lead to glycine accumulation in body tissues, including the brain. Glycine which functions as a neurotransmitter, if present at high levels, in the brain could induce severe neuronal dysfunction.[3]

GCE is a rare disorder in most populations except in Northern Finland (1:12 000 newborns) and British Columbia (1:63 000 newborns).[4] Most commonly GCE presents as the neonatal form of disease, manifests within a few days of birth with progressive lethargy, hypotonia and myoclonic seizures leading to apnea and possible death if not supported by mechanical ventilation. The majority of the surviving infants have profound psychomotor retardation and treatment-resistant epilepsy; although a small number of them may make developmental progress to a variable degree.[5–7] The infantile form of the disease is less common, characterized by early-onset hypotonia, developmental delay, seizures and the outcome may be mild or severe. Atypical form of GCE has been reported, with onset from childhood to adulthood, ranging from milder disease (mild learning difficulties, behavioral problems and choreoiform movement disorders) to severe disease (spastic paraparesis, optic atrophy, pulmonary hypertension and vacuolating leukoencephalopathy).[8,9]

The diagnostic characteristic of GCE is an elevated cerebrospinal fluid (CSF) glycine concentration (normal $<12\,\mu mol\,l^{-1}$) together

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with an increased CSF-to-plasma glycine ratio (<0.02 for normal control ; >0.08 for neonatal form; and 0.04–0.2 for atypical form).[1,4] Confirmatory diagnosis of GCE requires enzymatic and molecular genetics investigation of the glycine cleavage system. GCE can be confirmed by enzymatic assays; however, analysis requires a liver sample that is highly invasive. As such, molecular genetic testing is a much preferred method. The most common defective gene for GCE is the *GLDC* gene accounting for 80% of all GCE patients, and up to 15% have mutated *AMT* gene. Patients with deficient H-protein enzyme activity were less reported and so far, mutations are not detected for *GCSH* gene.[10] Approximately 20% of *GLDC* mutant alleles have exonic/multiexonic deletions or duplications.[11,12] Very rarely, deletions or duplications in *AMT* have been identified.[4] Several recurrent mutations in *GLDC* and *AMT* have been reported in several populations.[4,13] About 5% of patients with enzyme-proven GCE did not have a detectable mutation in any of the above three genes.[4]

The genetic profile for GCE patients in Malaysia has not been reported before. Therefore, this study was aimed to determine mutations in *GLDC*, *AMT* and *GCSH* gene in GCE patients.

MATERIALS AND METHODS

Study participants

A total of 14 patients referred to Molecular Diagnostics & Protein Unit, Institute for Medical Research in the years 2006–2012 were included in the study. These patients exhibited signs and symptoms of GCE as assessed by the pediatricians from Kuala Lumpur Hospital and other hospitals in Malaysia. Fifty blood samples from normal individuals were collected as control for this study. Ethics approval was obtained from the Medical Research & Ethics Committee, Ministry of Health Malaysia, and written informed consent was obtained from the parents of all patients recruited in the study.

Mutation detection for *AMT*, *GLDC* and *GCSH* gene by direct sequencing

Peripheral blood sample was collected in EDTA tubes. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Mississauga, ON, Canada), and the DNA quantity and quality were measured using Nanodrop spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA). Genomic DNA (100 ng) was subjected to PCR with forward and reverse primers flanking the target sequences. All primers were designed using Primer3 v.0.4.0 software (http://frodo.wi.mit.edu/primer3/). Primers for PCR amplification of all coding exons of the *GLDC* and *GCSH* gene contained two universal M13 primer tails at both forward and reverse primers, as shown in Supplementary Tables 1–3. The use of universal primers for the sequencing reactions reduced the time taken in preparation of cycle sequencing.

PCR was performed in a volume of 50 µl containing 100 ng genomic DNA, 0.1 U *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), 1X of PCR buffer with $(NH_4)_2SO_4$-$MgCl_2$, 1 mM $MgCl_2$, 1 µmol of each primer and 0.2 mM of dNTP mix. Dimethyl sulfoxide 100% (2 µl) was added for *GLDC* and *GCSH* PCRs. Touchdown PCR program was used to amplify all the exons, except exon 4 of *GCSH* gene. Exon 4 of *GCSH* gene was amplified using the normal PCR program with annealing temperature 55 °C. Touchdown PCR was initiated by first denaturing DNA for 3 min at 95 °C, followed by 10 cycles of 1 min denaturing at 95 °C, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. The annealing temperature was reduced by 0.5 °C per cycle for 10 times, followed by 25 cycles of normal PCR conditions starting with denaturation at 95 °C for 1 min, 1 min of annealing at 55 °C and 1 min extension at 72 °C, followed by a final step of complete elongation at 72 °C for 7 min.[20] Bidirectional sequencing was performed using BigDye Terminator Cycle Sequencing v3.1 (Applied Biosystems, Foster city, CA, USA) and was separated on a fluorescent Genetic Analyzer ABI 3130 (Applied Biosystems). DNA sequence was then analyzed for mutation using SeqScape software V2.7 (Applied Biosystems).

Deletion and duplication analysis of *GLDC* gene by MLPA

DNA (50 ng) and SALSA MLPA kit P209-B1 *GLDC* (MRC-Holland, Amsterdam, The Netherlands) assay were used in multiplex ligation-dependent probe amplification (MLPA) for deletion and duplication analysis of *GLDC* gene following the standard protocol. PCR products were separated using the Genetic Analyzer system ABI 3130. The data obtained were analyzed using GeneMarker V1.85 software (SoftGenetics, State College, PA, USA) and compared with the MLPA panel P209_*GLDC*_Vs03 and normal control.

Analysis of mutations detected using HomoloGene database, PolyPhen and SIFT software

All the mutations detected were compared with HomoloGene (NCBI) database to evaluate the percentage of conservation among eukaryote species, whereas PolyPhen and SIFT software were used for protein functions prediction caused by the mutations.

RESULTS

Patient's demographic data

A total of 14 patients from 13 families were recruited consisting of 8 Malays, 2 Chinese and 4 Penan ethnic. Six of them were females and eight were male patients. Their initial clinical presentation, biochemical characteristics of glycine and severity of psychomotor retardation have been summarized in Table 1. At onset, all the 14 patients have clinical features consistent with the classical neonatal form of GCE. In one patient, the symptoms spontaneously remit on subsequent follow up.

Thirteen patients have biallelic mutations identified either in *AMT* (6 patients from 5 families) or *GLDC* (7 patients from 7 families). In one patient (Patient K#1), mutation was identified in only one allele despite sequencing of the entire coding regions of the three genes. There was no mutation in *GCSH* gene being detected in this study. Mutational analyses were performed by direct sequencing followed by MLPA, and the mutation results have been summarized in Table 2.

Mutations in *AMT* gene

Four missense mutations (c.664C>T, c.688G>C, c.794G>A, c.826G>C) and one heterozygous deletion causing frameshift mutation (c.982delG) in *AMT* gene. All these mutations except c.826G>C have not been reported in Human Gene Mutation Database, (http://www.hgmd.org/). Patients J#1 and L#1 of Penan ethnicity have a homozygous c.794G>A mutation detected (Figure 1).

Mutations in *GLDC* gene

Nine missense mutations, one nonsense mutation and one heterozygous deletion of exons 3–25 in *GLDC* gene were reported. Seven of the missense mutations have not yet been reported in Human Gene Mutation Database (http://www.hgmd.org/): c.673T>C, c.883G>T, c.1114C>T, c.1525C>G, c.1607G>A, c.1789G>A and c.2280C>G. The sequencing electropherogram of c.1674C>A mutation in *GLDC* gene resulted in a premature stop codon and a heterozygous large exonic deletion detected by MLPA identified in Patient D#1 (Figure 2).

DISCUSSION

In this study we found biallelic mutations in 13 out of 14 patients with clinical and biochemical features suggestive of GCE; 7 patients have biallelic mutations in *GLDC* gene and 6 patients in *AMT* gene. A high proportion of *GLDC* and *AMT* mutations in these patients are not previously reported. In 12 out of 13 patients, the biallelic mutations are disease-causing based on the prediction using *in silico* bioinformatic tools. In one patient (Patient G#1), the clinical

Table 1 Patient's demography and clinical presentations

				Initial clinical presentation										
Family #Patient	*Gender*	*Ethnicity*	*Parental consanguinity*	*Age*	*Hypotonia*	*Poor sucking*	*Hiccup*	*Apnea*	*Seizure*	*EEG*	*CSF glycine*[a]	*CSF:plasma glycine ratio*	*CNS imaging*	*Outcome—severity of psychomotor retardation*
A#1	Female	Chinese	−	1st day	+	+	−	+	+	BS	439	0.38	HCC	Severe
B#1	Female	Malay	−	2nd day	+	+	−	+	+	BS	130	0.11	HCC	Moderate
B#2	Male	Malay	−	4th day	+	+	−	−	+	NA	122	0.12	NA	Moderate
C#1	Male	Penan	−	3rd day	+	+	−	+	−	NA	651	0.24	NA	Died (6th day)
D#1	Male	Malay	−	1st day	+	+	+	+	−	NA	292	0.13	HCC	Died (1st month)
E#1	Male	Chinese	−	1st day	+	+	+	+	+	BS	200.4	0.17	HCC	Severe
F#1	Female	Malay	−	2nd day	+	+	+	+	+	BS	306	0.17	HCC	Severe (died 5 years)
G#1	Female	Malay	+	1st day	+	+	−	+	+	SP	207	0.37	WM	None
H#1	Female	Malay	−	2nd day	+	+	+	+	+	BS	207	0.24	HCC	Severe
I#1	Female	Penan	−	1st day	+	+	+	+	+	NA	200	0.36	NA	Severe
J#1	Male	Penan	−	1st day	+	+	−	+	+	NA	153	0.17	NA	Severe
K#1	Male	Malay	−	4th day	+	+	−	+	+	NA	103	0.12	CI	Severe
L#1	Male	Penan	−	5th day	+	+	+	+	+	NA	306	0.10	NA	Died
M#1	Male	Malay	−	14th day	+	+	−	+	+	NA	202	0.11	NA	Died

Abbreviations: BS, burst suppression; CI, cerebral infarct; CNS, central nervous system; CSF, cerebrospinal fluid; EEG, electroencephalogram; HCC, hypogenesis of corpus callosum; NA, not available; SP, abnormal spikes; WM, white matter signal abnormalities.
[a]CSF glycine measured in $\mu mol\,l^{-1}$ and normal control is <12.

Table 2 Mutations of *AMT* and *GLDC* genes of GCE patients

Family/ #Patient	*Gene/exon*	*Nucleotide changes*	*Amino acid changes*	*Type of mutations*	*Mutation state*	*Gene conserved in eukaryota (%)*	*Protein function prediction by PolyPhen*	*Protein function prediction by SIFT*	*Reference*
A#1	*AMT*/7	c.826G>C	p.Asp276His	Missense	Heterozygous	100	Probably damaging	Affect	15
	AMT/8	c.982delG	p.Ala328fs	Frameshift	Heterozygous	44	NA	NA	Novel
B#1	*AMT*/6	c.664C>T	p.Arg222Cys	Missense	Heterozygous	100	Probably damaging	Affect	Novel
	AMT/6	c.688G>C	p.Gly230Arg	Missense	Heterozygous	100	Probably damaging	Affect	Novel
B#2	*AMT*/6	c.664C>T	p.Arg222Cys	Missense	Heterozygous	100	Probably damaging	Affect	Novel
	AMT/6	c.688G>C	p.Gly230Arg	Missense	Heterozygous	100	Probably damaging	Affect	Novel
C#1	*AMT*/7	c.794G>A	p.Arg265His	Missense	Homozygous	100	Probably damaging	Affect	Novel
	GLDC/17	c.1952A>G	p.His651Arg	Missense	Heterozygous	100	Probably damaging	Affect	12
D#1	*GLDC*/14	c.1674C>A	p.Cys558Stop Codon	Nonsense	Heterozygous	100	NA	NA	Novel
	GLDC/3–25	Del one allele *GLDC*	Del one allele *GLDC*	Large deletion	Heterozygous	NA	NA	NA	Novel
E#1	*GLDC*/7	c.883G>T	p.Asp295Tyr	Missense	Heterozygous	94	Probably damaging	Affect	Novel
	GLDC/13	c.1607G>A	p.Arg536Glu	Missense	Heterozygous	100	Probably damaging	Affect	Novel
F#1	*GLDC*/4	c.605C>T	p.Ala202Val	Missense	Heterozygous	100	Probably damaging	Affect	16
	GLDC/19	c.2280C>G	p.His760Gln	Missense	Heterozygous	95	Probably damaging	Affect	Novel
G#1	*GLDC*/12	c.1525C>G	p.Pro509Ala	Missense	Heterozygous	19	Possibly damaging	Tolerate	Novel[a]
	GLDC/15	c.1789G>A	p.Glu597Lys	Missense	Heterozygous	33	Benign	Tolerate	Novel[a]
H#1	*GLDC*/8	c.1114A>T	p.Ile372Phe	Missense	Homozygous	100	Probably damaging	Affect	Novel
I#1	*GLDC*/17	c.1952A>G	p.His651Arg	Missense	Homozygous	100	Probably damaging	Affect	12
J#1	*AMT*/7	c.794G>A	p.Arg265His	Missense	Homozygous	100	Probably damaging	Affect	Novel
K#1	*GLDC/8*	c.1114A>T	p.Ile372Phe	Missense	Heterozygous	100	Probably damaging	Affect	Novel
L#1	*AMT*/7	c.794G>A	p.Arg265His	Missense	Homozygous	100	Probably damaging	Affect	Novel
M#1	*GLDC/5*	c.673T>C	p.Cys225Arg	Missense	Homozygous	67	Probably damaging	Affect	Novel

Abbreviations: AMT, aminomethyltransferase; GCE, glycine encephalopathy; GLDC, glycine decarboxylase; NA, not available.
[a]Clinical significance uncertain.

significance of the biallelic mutations is uncertain. The high mutation rate in the *GLDC* gene resulting in GCE is similar to previous reports.[4,10] Recently, defects of lipoate biosynthesis and related iron sulfur cluster biogenesis genes, including *LIAS*, *BOLA3* and *GLRX5*, have been identified to be causative for some patients with GCE without disease-causing mutations identified in gene encoding a

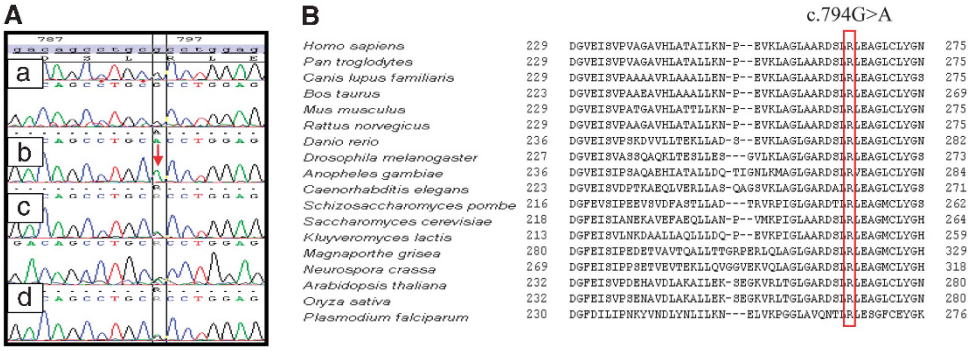

Figure 1 Mutation analysis of Patients J#1 with the sequencing electropherogram showed a homozygous c.794G>A mutation (**A**), and the homologene sequence showed the position of the mutation and the conserved amino acid (**B**); a: normal control, b: Patient J#1, c: mother and d: father.

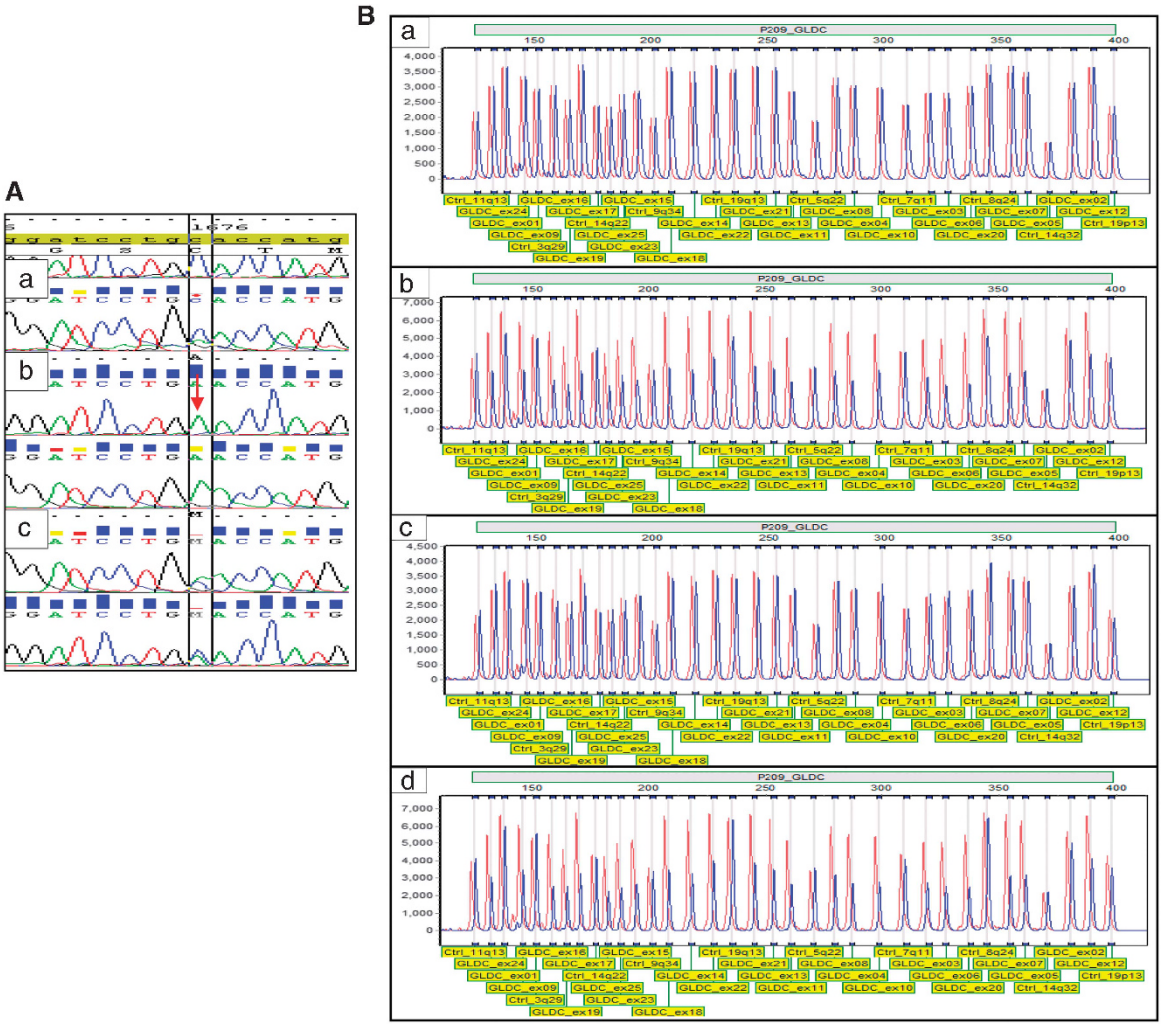

Figure 2 Mutation analysis of Patient D#1 with the sequencing electropherogram showed mutation at c.1674C>A in *GLDC* gene resulting premature stop codon (**A**); a: normal control, b: Patient D#1, c: mother, and the MLPA results showed the heterozygous deletion of exons 3–25 of *GLDC* gene (**B**); a: normal control, b: Patient D#1, c: mother and d: father.

constituent of the glycine cleavage enzyme.[14] We did not study these genes in the single patient without biallelic mutations detected.

Most of the mutations detected are single family mutation except for patients from Penan ethnicity. The Penans are nomadic aboriginal people living in Sarawak, one of the states of East Malaysia located in Borneo Island. There are a total of 16 GCE patients registered in Malaysia, 4 of them are Penan patients. Although the prevalence of GCE in Malaysia is estimated at 1:187 500 newborns, it is possible that the prevalence among the Penans may be higher. Two mutations c.1952A>G (p.His651Arg) in *GLDC* gene and c.794G>A

(p.Arg265His) in *AMT* gene are observed exclusively in the Penan patients. Further study to determine the carrier frequency of the two mutations among the Penans is currently constrained by difficulties to get control subjects among this nomadic population, who mostly stay at geographically remote areas.

All the patients in this study have clinical features of classical neonatal form of GCE. Probably the late-onset and atypical forms of GCE are still unrecognized in our population and may require further study. Almost all the patients in this study have devastating neurological outcome except Patient G#1. This patient was born at full-term gestation to consanguineous parents with birth weight of 2.9 kg. She developed feeding problem and cyanosis after a few hours, fitted on the second day and became apneic needing ventilator support. She regained spontaneous respiration 1 week later. She was treated with dextromethorphan and sodium benzoate. Her developmental milestones were normal. Dextromethorphan and sodium benzoate were taken off subsequently. A repeat CSF glycine analysis at 5 years old was normal (11.4 μM). To date, she remains seizure free and neurologically normal. She attended normal school and academic performance has been good. It is most likely that she is in the category of transient GCE. Genetic analysis revealed two mutations of unknown significance in *GLDC*: compound heterozygous c.1525C>G (p.Pro509Ala) and c.1789G>A (p.Glu597Lys), whereas her parents were found to be heterozygous carriers. For mutation c.1525C>G, a protein function prediction by PolyPhen suggested a possible damaging effect but prediction by SIFT suggested that it is tolerable. Whereas for mutation c.1789G>A, analyses by both software suggested a tolerable effect. Both mutations were not found in 50 control subjects of the same ethnicity. Search on Mutation Taster database (http://www.mutationtaster.org/) showed both mutations are not listed as single-nucleotide polymorphism.[15] Therefore, we are unable to conclude definitely if her clinical symptom was indeed caused by *GLDC* mutations. Transient GCE remains a controversial diagnosis. It is clinically and biochemically indistinguishable from GCE at onset. Heterozygous *GLDC* and *GCSH* gene mutations have been reported in three patients with transient GCE , suggesting that transient GCE may develop in some heterozygous carriers.[16] Although CSF hyperglycinemia and elevated CSF:plasma glycine ratio in an infant with acute encephalopathy are considered highly characteristic of GCE, similar findings can be encountered in a variety of clinical conditions including hypoxic ischemic encephalopathy.[17] Our experience with Patient G#1 illustrated the potential pitfall and difficulties in confirming the diagnosis of transient GCE.

In conclusion, the genetic mutations of Malaysian children with GCE had been studied, and we found that mutations in both the GLDC and *AMT* genes are the main causes of GCE in the population.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)