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Prospective cohort study for identification of underlying genetic causes in neonatal encephalopathy using whole-exome sequencing

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Purpose: Neonatal encephalopathy, which is characterized by a decreased level of consciousness, occurs in 1–7/1,000 live-term births. In more than half of term newborns, there is no identifiable etiological factor. To identify underlying genetic defects, we applied whole-exome sequencing (WES) in term newborns with neonatal encephalopathy as a prospective cohort study.

Methods: Term newborns with neonatal encephalopathy and no history of perinatal asphyxia were included. WES was performed using patient and both parents' DNA.

Results: Nineteen patients fulfilling inclusion criteria were enrolled. Five patients were excluded owing to withdrawal of consent, no parental DNA samples, or a genetic diagnosis prior to WES. Fourteen patients underwent WES. We confirmed a genetic diagnosis in five patients (36%): epileptic encephalopathy associated with autosomal dominant de novo variants in *SCN2A* (p.Met1545Val), *KCNQ2* (p.Asp212Tyr), and *GNAO1* (p.Gly40Arg); lipoic acid synthetase deficiency due to compound heterozygous variants in *LIAS* (p.Ala253Pro and p.His236Gln); and encephalopathy associated with an X-linked variant in *CUL4B* (p.Asn211Ser).

Conclusion: WES is helpful at arriving genetic diagnoses in neonatal encephalopathy and/or seizures and brain damage. It will increase our understanding and probably enable us to develop targeted neuroprotective treatment strategies.

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Key Words: genetic; hypoxic-ischemic encephalopathy; neonatal encephalopathy; neonatal seizures; whole-exome sequencing

INTRODUCTION

Neonatal encephalopathy results from a dysfunction of the central nervous system in term newborns and is characterized by a decreased level of consciousness, seizures, respiratory insufficiency, and depression of tone and reflexes. Neonatal encephalopathy occurs in 1–7 infants per 1,000 live-term births.[1] Its etiologies and associated risk factors include (i) antepartum events (reported in about 25% of affected term newborns), such as maternal hypotension, maternal thyroid disease, infertility treatment; (ii) intrapartum events (reported in less than 10% of cases), such as abruption of placenta, forceps delivery, breech presentation, maternal fever; and (iii) postnatal events (reported in less than 10% of cases), such as severe respiratory distress, sepsis, or shock. Despite the heterogeneous etiologies, hypoxic-ischemic encephalopathy (HIE) is considered causative in 50–75% of term newborns with neonatal encephalopathy. However, the majority of term newborns with neonatal encephalopathy attributed to HIE do not have a history of hypoxia or ischemia during the perinatal period.[1,2] In fact, there are no identifiable risk factors in more than half of term newborns with neonatal encephalopathy.[1,2]

The severity of neonatal encephalopathy is on a spectrum with death in 15–20% of affected term newborns. Between 65 to 98% of the newborns with moderate to severe neonatal encephalopathy develop cerebral palsy, characterized by impaired motor function.[1–3]

To date, the genetic causes of neonatal encephalopathy have not been investigated. However, characterizing the genetic contributors of neonatal encephalopathy will probably increase our understanding of neonatal brain injury in the term newborn and also, in time, enable us to develop new treatment modalities using large-scale drug screening based on the underlying genetic defect. To identify underlying genetic defects, we applied whole-exome sequencing (WES) in

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term newborns with neonatal encephalopathy as an exploratory prospective cohort study.

MATERIALS AND METHODS

Patients

This study is approved by the institutional research ethics board (Approval #100032244). Inclusion criteria were neonatal encephalopathy and/or prenatal or neonatal onset seizures or abnormal brain magnetic resonance imaging (MRI) features (such as edema, white matter and/or gray matter signal abnormalities, or hemorrhage) without history of perinatal asphyxia. Exclusion criteria were preterm delivery (<37 weeks gestation) and a sentinel hypoxic event occurring immediately before or during labor. Two of our coauthors and study team members (D.W. and V.C.), as well as Steven Miller (expert in neonatal encephalopathy and HIE), were in the circle of clinical care for all the patients with neonatal encephalopathy. They screened patients according to our inclusion and exclusion criteria and informed the research team of their selections for enrollment in the research study. Term newborns with neonatal encephalopathy meeting the inclusion criteria were enrolled after informed consent was obtained.

WES

DNA for WES was extracted from peripheral blood from index cases and their parents and stored at −80°C. WES was performed on patients and parents at the Centre for Applied Genomics, using the Illumina HiSeq 2000/2500 (Illumina, San Diego, CA, USA) following enrichment with the Agilent SureSelect Human All Exon kit V4 50-Mb (Agilent, Santa Clara, CA, USA). CASAVA (Illumina) was used for base calling and raw reads were mapped to the reference human genome (UCSC Genome Browser-hg19; University of California, Santa Cruz, CA, USA) using BWA version 0.6.5a. Duplicate pair end reads were removed using MarkDuplicates (Picard tools version 1.35; Broad Institute, Cambridge, MA, USA). The duplicate-free alignments were refined using local realignment and base quality score recalibration with GATK version1.0.5506 (Broad Institute). Variant calling was performed using the recommended best practices of GATK.[4] Variant annotation and prioritization was performed using a previously described pipeline.[5] Briefly, rare sequence variants, with frequency of occurrence <1% in the National Heart, Lung, and Blood Institute exome server and 1000 Genomes control databases, the Exome Variant Server (EVS) database (http://evs.gs.washington.edu/EVS/PopStatsServlet?searchBy=Gene+Hugotarget=GATMx=0y=0), and the Exome Aggregation Consortium (ExAC) Broswer Beta dabase (Broad Institute) (URL: http://exac.broadinstitute.org/), were filtered for further analysis. Further prioritization was focused on rare variants including loss of function (frameshift, nonsense, and splice-site mutations), homozygous missense, and/or relevant known disease genes from the Online Mendelian Inheritance in Man database (OMIM). To determine whether missense variants were predicted to be deleterious, the predictors Sorting Intolerant From Tolerant (SIFT) (http://sift.jcvi.org),[6] Polymorphism Phenotyping 2 (PolyPhen-2)[7] (http://genetics.bwh.harvard.edu/pph2/), and the Variant Effect Predictor were used.[8] Candidates were chosen based on the following criteria: SIFT score, PolyPhen-2 score, QualByDepth (QD: variant confidence from the QUAL field/unfiltered depth) >5, phylopMam >2, and phylopVert >4 (conservation in mammals and vertebrates respectively); CADD_phred score >10. Genes localized to the brain, with known neurological function, or clinically reported genes were chosen for validation and segregation in the patients and parents.

Sanger sequencing of candidate variants

Primers were designed to span a minimum of 100 bp upstream and downstream of the variant of interest in the candidate gene. Genomic DNA from patients and parents was sequenced using *Taq* DNA Polymerase (ThermoFisher Scientific, #18038067; Waltham, MA, USA), standard buffer and protocols.[9] The polymerase chain reaction (PCR) product was purified using Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel, Duren, Germany). Sequencing was performed according to our institution's sequencing methods. Sanger sequencing further confirmed nonsynonymous, non-SNP changes in patient and parents. All candidates with a known OMIM disease were confirmed in a clinical molecular diagnostic laboratory.

Functional analysis

The effects of identified variants on protein function for two genes, *CUL4B* and *AACS*, were characterized and are briefly described below.

CUL4B variant functional analysis

Lymphoblasts were lysed in a radioimmunoprecipitation buffer (50 mM Tris-HCl (pH 8.0), 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, protease and phosphatase inhibitor cocktail) at 4 °C for 10 min.[10–13] Crude lysates were cleared by centrifugation at 20,000*g* at 4 °C for 15 min, and the resulting supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblot analysis. The relative band intensity of CUL4B (normalized with Tubulin) was calculated with Image J software. Anti-CUL4B (HPA011880) and anti-Tubulin (T6074) antibodies were purchased from Sigma (Seattle, WA, USA). Anti-CUL4A (ab34897) antibody was from Abcam (Cambridge, UK). Anti-DDB1 antibody was a gift from Yue Xiong (University of North Carolina at Chapel Hill).

HEK293T cells transfected with an expression vector encoding myc-tagged either wild-type or mutant CUL4B (N211S or T231I) were treated with cycloheximide (25 μg/ml) for 0 to 6 h, and then lysed with radioimmunoprecipitation assay buffer (50 mM Tris (pH 8.0), 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor cocktail) at 4 °C for 10 min. Crude lysates were cleared by centrifugation at 20,000*g* at 4 °C for 15 min, and

supernatants were subjected to immunoblot analysis. The relative band intensity of CUL4B (normalized with Tubulin) was calculated with Image J software. Anti-Myc (sc-40) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Tubulin (T6074) antibody was from Sigma.

HEK293T cells transfected with an expression vector encoding myc-tagged either wild-type or mutant CUL4B (or the vector as a control) were washed with phosphate-buffered saline and lysed in an NP-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol and protease inhibitor cocktail) at 4 °C for 5 min. Crude lysates were cleared by centrifugation at 20,000*g* at 4 °C for 15 min, and supernatants were incubated with Dynabeads protein G (Thermo Fisher Scientific) which was preconjugated with Myc antibody. The immunocomplexes were washed three times with a wash buffer (0.1% Triton X-100, 10% glycerol in phosphate-buffered saline) and then subjected to SDS-PAGE, followed by immunoblot. Anti-Myc (sc-40) and anti-CSN5 (sc-13157) antibodies were purchased from Santa Cruz Biotechnology. Anti-DDB1 and anti-ROC1 antibodies were gifts from Yue Xiong (University of North Carolina at Chapel Hill, NC, USA).

AACS variant functional analysis

Antibody. Rabbit polyclonal antibodies were raised against the keyhole limpet hemocyanin-conjugated polypeptide MSKEERPGREEILEC (amino acids 1–15) of human AACS by Sigma-Aldrich (St Louis, MO, USA). The antibodies were affinity-purified using protein G sepharose and concentrated by ultrafiltration through an Amicon Ultra 30K device (Merck Millipore, Billerica, MA, USA).

Constructs. For subcloning into the Hind III/Kpn I restriction sites of pCMV-(DYKDDDDK)-N (Clontech Laboratories, Mountain View, CA, USA), the cDNA encoding human *AACS* was amplified using the forward primer 5′-AAGC TTATGTCCAAGGAGGAGCGCCC-3′ and the reverse primer 5′-GGTACCTCAGAAGCCCTGCAGCTCAG-3′ from cDNA of Hep G2 cells. The following double-stranded oligonucleotides with mutations were used as primers: 5′- TAC GCTCAACAGCAAGAAAGTGG-3′ for G632S (the underlined nucleotides encode the mutated residues). The insert was sequenced and was identical to human *AACS* (accession number NM_023928). The FLAG (DYKDDDDK)-hAACS (N terminally FLAG-tagged human AACS) vectors (1 μg) were transfected into the HEK293 Cell line. After 2 days of transfection, the cells were harvested in cell lysis buffer (10 mM Tris-HCl pH7.4, 1% NP40, 150 mM NaCl, and 0.5 mM EDTA). AACS activity in 50 μg of protein extracts was assayed by measuring acetyl-CoA formation according to a method previously reported by Hasegawa et al.[14]

RESULTS

Patients

The neonatal intensive care unit in our institution is a tertiary care center for term newborns with neonatal encephalopathy. During our study period of April 2013 to December 2016, there were 2,984 admissions to our neonatal intensive care unit. We do not have any registries giving the exact number of patients admitted with neonatal encephalopathy during the study period. However, between July 2014 and December 2016, using a database generated for Quality and Risk Management for a Neonatal Neurocritical Care Database (maintained by D.W.), we identified 270 patients admitted with neonatal encephalopathy to our neonatal intensive care unit. There were 176 patients with HIE, 15 patients with stroke, 13 patients with congenital brain malformation, 8 patients with meningitis, and two patients with neuromuscular disorders, who did not fulfill our inclusion criteria. Of the remaining 56 patients, 11 died prior to screening for our study. Some of the patients did not have both parents available for our WES trio (patient and both parents) study, and so were not included. Our estimated enrollment rate is 50% of patients with neonatal encephalopathy who fulfilled our inclusion criteria during our study period. Between April 2013 and December 2016, 19 patients and families fulfilling the inclusion criteria signed the consent forms to be included in the study during their admission to the neonatal intensive care unit at the time of their first symptom. Five patients were excluded for the following reasons: (i) one family withdrew their consent after completion of WES and data analysis; (ii) one patient received a clinical diagnosis prior to WES after achieving seizure freedom on pyridoxine therapy and being diagnosed with pyridoxine-dependent epilepsy caused by pathogenic variants in *ALDH7A1* ; (iii) one patient was diagnosed with viral encephalitis based on the response to antiviral therapy; and (iv) two families signed the consent forms but the parents did not provide blood samples for WES. The remaining 14 patients—4 males and 10 females—underwent WES. The average age of onset was 2.1 ± 2.1 SD days (range 10 min to 6 days). One patient passed away at the age of 6 days.

Demographics, clinical features, brain MRI and electroencephalography (EEG) results of all study patients are summarized in **Table 1**. All patients had neonatal encephalopathy and seizures. Pregnancy was remarkable for gestational diabetes mellitus in four mothers and oligohydramnios in two mothers. Three patients had hypoglycemia. Brain MRI features included diffuse symmetrical diffusion restriction, hemorrhages, an increased signal intensity in bilateral basal ganglia. Magnetic resonance spectroscopy showed a lactate peak in four patients. All patients had sharp waves and/or discontinuous background in EEG (**Table 1**).

WES and functional analysis

WES was performed on a trio (patient and both parents) in 14 patients. In seven patients we identified candidate variants and confirmed segregation in patients and parents

Table 1 Clinical features, EEG findings, brain MRI features of patients enrolled into the study and their WES results summarized

Patient ID/sex/age of onset	Pregnancy/delivery/Apgar scores/clinical features at presentation	Initial EEG	Brain MRI/MRS (age)	Candidate variant, gene, inheritance pattern
NE_NS 001/M/ 2 days	Oligohydramnios/planned C-section/9 and 9/poor feeding, hypotonia, lip smacking, encephalopathy, hypoglycemia	Excessive background discontinuity, bilateral independent sharp transients	Global cortical and bilateral putamen diffusion restriction, diffuse WM edema/prominent lactate peak (4 days)	Hemizygous, novel, c.632A > G (p.Asn211Ser) in *CUL4B*/X-linked, maternal
NE_NS 002/F/2 days	Unremarkable/vaginal forceps/3, 5, and 7/apnea, desaturations at delivery, right-sided seizures	Discontinuous background, multifocal sharp waves	Bilateral diffusion restriction in putamen, thalamus, hippocampi, corticospinal tracts, cerebral peduncles, dorsal pons/large lactate peak (4 days)	None
NE_NS 004/M/3 days (died 6 days)	Unremarkable/not known discharged 24 h/ lethargy, fever, poor feeding, focal seizures, hypoglycemia	Discontinuous background, multiple multifocal subclinical seizures originating from bilateral temporo-occipital head region	Diffuse bilateral symmetrical diffusion restriction of the cerebral cortex and basal ganglia, diffuse WM edema, massive cerebral injury/lactate peak (5 days)	None
NE_NS 005/F/36 h	Unremarkable/C-section for fetal heart rate decelerations/9 and 9/apnea, cyanosis, GTS, lip smacking	Burst suppression, ictal EEG with generalized high-amplitude spike and sharp waves	Inferior basal ganglia hyperintensity in T1 weighted image/ NP (6 days)	Heterozygous novel de novo c.634G > T (p. Asp212Tyr) in *KCNQ2*/AD
NE_NS 007/F/10 min	Unremarkable/vaginal delivery/7 and 7/ hypotonia, apnea, GTS	Discontinuous background, bilateral negative rolandic sharp waves	Bilateral subdural, subarachnoid, and intraventricular hemorrhages and anterolateral temporal ischemic changes, diffuse WM edema/normal (2 days)	None
NE_NS 008/M/12 h	Unremarkable/vaginal vacuum/3 and 6/eye blinking, bicycling, GTS, apnea	Discontinuous background, intermittent sharp waves	Diffuse brain edema with effacement of ventricular lumen and pericerebral space/small lactate peak (1 day)	None
NE_NS 009/F/8 h	Unremarkable/vaginal vacuum/8 and 9/GTCS	Discontinuous background, frequent temporal sharp waves	Bilateral cortical T1 hyperintensity in posterior temporal and perisylvian gyri, T2 hyperintensity in thalami/normal (7 days)	Homozygous c.1894G > A (p.Gly632Ser) in *AACS*/AR/parents heterozygous
NE_NS 010/M/at birth	Gestational DM/vaginal/0, 0, and 8/poor tone and apnea at birth, mild hypoxic-ischemic encephalopathy, GTCS 6 days	Discontinuous background, bilateral t emporal sharp waves	Bilateral increased signal in frontal and peritrigonal WM in T1/normal (8 days)	Heterozygous de novo, novel c.118G > A (p. Gly40Arg) in *GNAO1*/AD
NE_NS 011/F/6 h	Gestational DM/C-section for failure to progress/ 7 and 9/hypoglycemia (1 h) GTCS, apnea	Discontinuous background, burst suppression pattern, central midline sharp waves	Bilateral diffuse cortical and subcortical WM diffusion restriction most prominent in occipital lobes/normal (1 day)	None
NE_NS 015/F/6 days	Unremarkable/vaginal/6 and 8/apnea, focal tonic seizures	Discontinuous background, bilateral central temporal sharp waves	Bilateral frontoparietal subcortical WM T2 hyperintensity/ normal (9 days)	Heterozygous, novel, de novo, c.4633A > G (p.Met1545Val) in *SCN2A*/AD
NE_NS 016/F/21 h	Oligohydramnios/vaginal/8 and 9/focal seizures	Discontinuous background, bilateral central and temporal rolandic sharp waves	Bilateral cortical subcortical, deep WM and basal ganglia diffusion restriction/normal (2 days)	None
NE_NS 017/F/24 h	Gestational DM/vaginal delivery/7 and 8/hypotonia, focal seizures and GTCS	Discontinuous background, bilateral central temporal sharp waves	Bilateral diffuse cortical, corpus callosum splenium, thalamus and posterior limb internal capsules diffusion restriction/normal (2 days)	Heterozygous, novel, de novo, c.1692G > A (p. Met564Ile) in *RAI1*/AD
NE_NS018/F/2 days	Unremarkable/vaginal delivery term/7 and 9/GTCS	Bilateral central temporal sharp waves	Symmetrical diffusion restriction in frontal, perirolandic, and occipital white matter, increased T2 signal in medial thalami/normal (3 days)	None
NE_NS019/F/1 day	Unremarkable/C-section/8 and 9/focal and GTCS	Multifocal frequent sharp waves, discontinuous background	Diffuse symmetrical white matter diffusion restriction (2 days), progressive diffusion restriction in white matter, brainstem, cerebellar white matter (9 days)/increased glycine peak	Compound heterozygous c.757G > C (p. Ala253Pro, maternal)/c.708T > G (p.His236Gln, paternal) in *LIAS*/AR

AD, autosomal dominant; AR, autosomal recessive; CA, cortical atrophy; DB, discontinuous background; EEG, electroencephalography; F, female; GDD, global developmental delay; GER, gastroesophageal reflux; GERD, gastroesophageal reflux disease; GTS, generalized tonic seizures; GTCS, generalized tonic clonic seizures; He, hemizygous; Hmz, homozygous; Htz, heterozygous; M, male; HIE, hypoxic ischemic encephalopathy; Hmz, homozygous; Htz, heterozygous; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NA, not available; NC, no candidate gene; NP, not performed; ShW, sharp waves; WM, white matter.

Table 2 Variants identified by whole-exome sequencing

Patient ID	Variant (NM no.)/parent results	SIFT	PolyPhen-2	gnomAD	EVS	Conservation in species	MutationTaster	Grantham score
NE_NS 001	*CUL4B* c.632A > G (p.Asn211Ser) (NM_003588)/maternal	0.4	0.001	0	0	10/13	Disease-causing	46
NE_NS 005	*KCNQ2* c.634G > T (p.Asp212Tyr) (NM_172109)/de novo	0	0.999	0	0	11/12	Disease-causing	160
NE_NS 009	*AACS* c.1894G > A (p.Gly632Ser) (NM_023928)/both parents heterozygous	0.01	0.959	10[a]	0	10/12	Disease-causing	56
NE_NS 010	*GNAO1* c.118G > A (p.Gly40Arg) (NM_020988)/de novo	0	1	0	0	10/13	Disease-causing	125
NE_NS 015	*SCN2A* c.4633A > G (p.Met1545Val) (NM_001040143)/de novo	0.01	0.43	0	0	11/12	Disease-causing	21
NE_NS 017	*RAI1* c.1692G > A (p.Met564Ile) (NM_030665)/de novo	0.04	0.914	2[b]	0	10/12	Disease-causing	10
NE_NS019	*LIAS* c.757G > C (p.Ala253Pro) (maternal)/c.708T > G (p.His236Gln) (paternal)/(NM_006859.2)	0/0	1/1	1[c]-0	0/0	13/13	Disease-causing/ Disease-causing	27/24

EVS, Exome Variant Server; gnomAD, The Genome Aggregation Database; PolyPhen-2, Polymorphism Phenotyping 2; SIFT, Sorting Intolerant From Tolerant.
[a]Allele number of 246260. [b]Allele number of 245100. [c]Allele number of 244336.

with Sanger sequencing. In silico analyses of likely pathogenic variants in those seven patients are summarized in **Table 2**. We confirmed a clinical genetic diagnosis in four patients in a Clinical Laboratory Improvement Amendments–certified laboratory, including *SCN2A*-, *KCNQ2*-, and *GNAO1*-associated epileptic encephalopathies and lipoic acid synthetase deficiency caused by biallelic likely pathogenic variants in *LIAS*. The *SCN2A* p.Met1545Val, *KCNQ2* p.Asp212Tyr, and *GNAO1* p.Gly40Arg de novo variants were the cause of neonatal encephalopathy and seizures in these patients.

In three patients we identified variants in two known genes, *CUL4B* and *RAI1,* and a novel gene, *AACS*. The functional analysis of the hemizygous p.Asn211Ser variant in *CUL4B* showed a decreased CUL4B protein level, confirming the pathogenicity (**Figure 1a–c**). A homozygous p.Gly632Ser variant in *AACS* encoding acetoacetyl-CoA synthetase (AACS) was identified in one patient as a novel candidate gene. The first cousin parents were heterozygous carriers for the same variant. We found normal AACS activity in the homogenates from cells overexpressing AACS p.Gly632Ser variant, similar to that in wild-type cells (**Figure 2a**). Immunoblot of p.Gly632Ser variant showed the same amount of AACS protein as in wild-type (**Figure 2a and b**). We concluded that this variant is not pathogenic. We were not able to perform functional analysis for the *RAI1* p.Met564Ile variant.

DISCUSSION

We utilized WES in cases of neonatal encephalopathy and/or seizures in a prospective cohort study to investigate underlying causes of neonatal encephalopathy. We identified three known genes causing neonatal epileptic encephalopathy: *KCNQ2, SCN2A*, and *GNAO1*. We also identified a new patient with lipoic acid synthetase deficiency caused by biallelic likely pathogenic variants in *LIAS* mimicking neuroimaging features of HIE. We identified a missense variant in *CUL4B* in a male, confirmed to be pathogenic by functional analysis, as the cause of global developmental delay and seizures in this patient. The genetic diagnostic yield of WES was 36% (5 out of 14) for neonatal encephalopathy and/or neonatal seizures in our prospective cohort study. Additionally, we found a de novo likely pathogenic variant in *RAI1* in a 10-month-old girl with microcephaly and global developmental delay with no behavioral problems or sleep disturbances. Likely pathogenic variants in *RAI1* cause Smith-Magenis syndrome, which is a neurodevelopmental disorder characterized by dysmorphic features, intellectual disability, and sleep disturbances. We are not certain if the likely pathogenic variant in *RAI1* in our patient would explain her phenotype, in the absence of typical phenotypic features of Smith-Magenis syndrome. In another patient, we had high suspicion of an AACS deficiency causing a novel disease, based on the prediction tools, using in silico analysis. AACS is an important enzyme for acetyl-CoA production and lipid synthesis. It is required for normal neuronal development, and is expressed strongly in the human and rat brains.[15] However, functional analysis of the patient's *AACS*

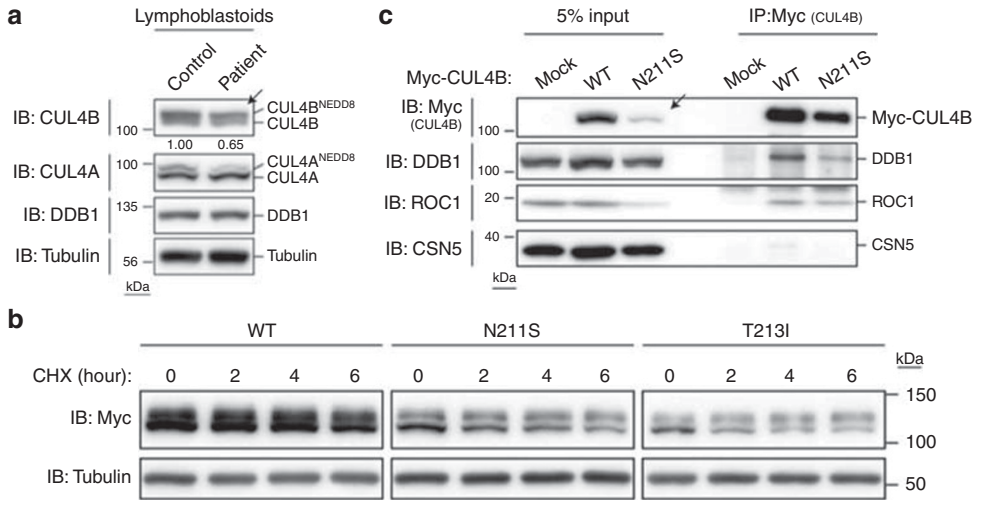

Figure 1 Functional analysis of p.Asn211Ser *CUL4B* variant. (**a**) Reduced protein level of CUL4B in patient lymphoblasts with p.Asn211Ser *CUL4B* variant compared with control lymphoblasts (indicated by the arrow). (**b**) Reduced stability of p.Asn211Ser *CUL4B* variant. (**c**) Formation of ubiquitin ligase complex by p.Asn211Ser *CUL4B* variant. The arrow indicates reduced expression of p.Asn211Ser CUL4B compared with wild-type CUL4B. CHX, cycloheximide (25 μg/ml); CSN5, COP9 signalosome subunit 5; CUL4ANEDD8, NEDD8-modified CUL4A; CUL4BNEDD8, NEDD8-modified CUL4B; DDB1, damage-specific DNA binding protein 1; IP, immunoprecipitation; ROC1, regulator of cullins-1.

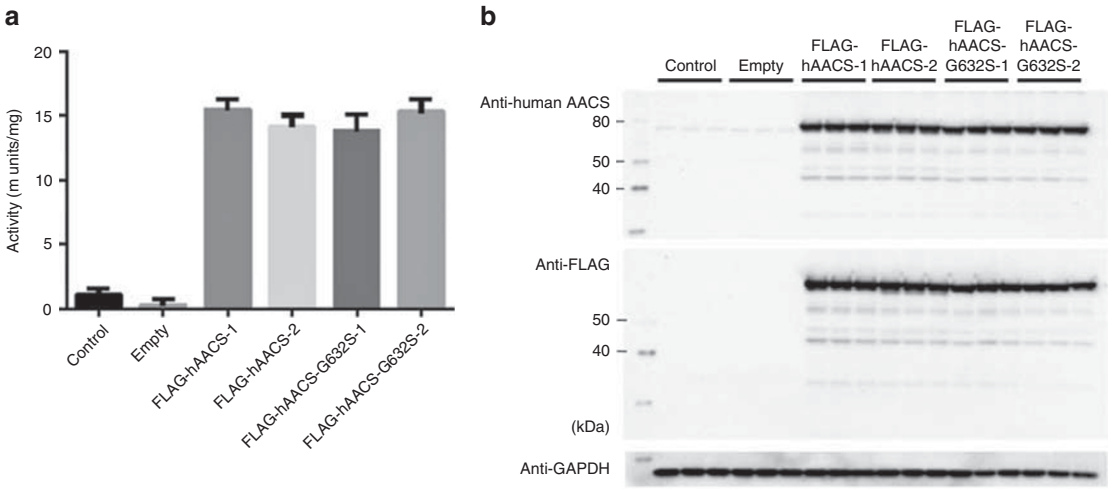

Figure 2 Activity of AACS G632S variant. (**a**) FLAG-hAACS-1 and -2 vectors or FLAG-hAACS-G632S-1 and -2 vectors were isolated from the separate bacterial colonies. The empty FLAG-hAACS or FLAG-hAACS-G632S vectors were transfected into the HEK 293 cell line. After 2 days of culture, the cells were harvested in the TNE buffer. AACS activity in 50 μg of protein extracts was assayed by measuring acetyl-CoA formation, as described in "Materials and Methods." Data are representative of three independent experiments. Error bars indicate the SD (**b**) Each cell lysate (7.5 μg of protein) was subjected to western blotting to detect human AACS, FLAG, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

p.Gly632Ser missense variant revealed normal AACS activity, which excludes the possibility of AACS deficiency. This illustrates the importance of functional studies for missense variants to confirm pathogenicity in the process of identification of novel genetic disorders.

One of the purposes of our study was to investigate whether term newborns with neonatal encephalopathy have genetic defects of energy metabolism pathways that contribute to brain energy failure and neonatal brain injury at delivery, mimicking HIE without any history of hypoxia. We identified lipoic acid synthetase deficiency and severe brain damage without history of hypoxia in a patient. Biallelic likely pathogenic variants in *LIAS,* encoding lipoic acid synthetase, result in defective lipoic acid synthesis. Lipoic acid is a cofactor for pyruvate dehydrogenase, branched-chain keto-acid dehydrogenase, 2-ketoglutarate dehydrogenase, and 2-oxoadipate dehydrogenase. All of these enzymes have key functions in mitochondrial energy metabolism and production of energy. Because of a lack of the cofactor lipoic acid, these enzymes are dysfunctional and cause decrease in energy

production, contributing to lack of energy and hypoxic changes visible in brain MRI, mimicking HIE in our patient. To date, three patients have been reported with lipoic acid synthetase deficiency.[16,17] All patients presented with elevated glycine levels. Our patient had also elevated plasma and cerebrospinal fluid glycine levels, with high cerebrospinal fluid to plasma glycine ratio (0.1; reference range <0.02) with normal blood lactate levels consistent with the biochemical features of previously reported patients. We report the fourth patient with lipoic acid synthetase deficiency identified in this study. A small number of genetic disorders in energy or cofactor metabolism mimic the clinical and neuroimaging features of HIE, including cytochrome C oxidase deficiency,[18] pyridoxine dependent epilepsy,[19] molybdenum cofactor deficiency,[20] glycogen storage disease type IV,[21] and X-linked centronuclear myopathy.[22] Lipoic acid synthetase deficiency should be included in the differential diagnosis of HIE without a history of hypoxia. Application of WES in patients with neonatal encephalopathy will probably identify more genetic disorders involving energy metabolism pathways contributing to the pathogenesis of term neonatal encephalopathy and will increase our understanding of severe neonatal brain damage without history of hypoxia.

A previous study assessed the diagnostic yield of WES in 20 acutely ill newborns, using targeted WES including 4,183 known disease-associated genes.[23] Patients presented with hypotonia (six patients), seizures (four patients), multiple malformations (six patients), family history of the same complex clinical presentation (two patients), and consanguineous parents (three patients).[23] Twelve patients were identified retrospectively and enrolled after discharge from the neonatal intensive care unit and eight patients were enrolled when they were still in the neonatal intensive care unit. A genetic diagnosis was confirmed in eight patients (40%). Seizures were present in four patients (25%) and only one of those received the diagnosis of *SCN1A*-associated epileptic encephalopathy. In our prospective cohort study, we targeted patients with neonatal encephalopathy and/or seizures and enrolled them at the time of their first symptom during their initial admission. We arrived at a genetic diagnosis in 36% of the patients for known disease-associated genes associated with phenotypes in our prospective cohort study. Sixty percent of our patients had one of the epileptic encephalopathy genes, whereas in the previous study only 12.5% of the patients had an epileptic encephalopathy gene.[23] We also identified a patient with lipoic acid synthetase deficiency involving energy metabolism pathways causing neonatal encephalopathy and brain injury. To the best of our knowledge, our study is the first to enroll patients with neonatal encephalopathy at their first presentation to investigate underlying genetic causes.

Unfortunately, we did not identify any candidate genes in 50% of the patients and there was no confirmed genetic diagnosis in 64% of the patients. This could be associated with the limitations of WES, including (i) targeting only the exonic portion of the genome (approximately 1%); (ii) requiring exon capture and enrichment strategies which introduce bias due to nonhomogeneous capture in all exons; (iii) difficulties with GC-rich or repetitive regions; and (iv) PCR-based enrichment strategies that suffer from general stochastic, template-switch, and polymerase errors.[24] The advantages of WES are that it is less costly, with a higher depth of coverage and a smaller amount of data generation for analysis and diagnosis of known Mendelian diseases in clinical settings.[25] Rapid whole-genome sequencing was applied to 16 newborns and infants in the neonatal (15 patients) or pediatric (1 patient) intensive care units, and 10 of those (62.5%) received a genetic diagnosis.[26] Only four of those patients were enrolled within the first 3 weeks of life. The diagnostic yield of whole-genome sequencing was about 50% higher than that in our study results. This is probably due to advantages of whole-genome sequencing, including (i) coverage of both exonic and intronic regions, (ii) more uniform and complete coverage of the genome, (iii) the ability to identify noncoding pathogenic variants, and (iv) detection of copy-number variants.[27] Applying whole-genome sequencing to our patient population without a genetic diagnosis might increase the diagnostic yield in neonatal encephalopathy.

Likely pathogenic variants in *CUL4B* have been reported to be associated with intellectual disability (OMIM 300354).[28] Ventriculomegaly, cerebral and cerebellar atrophy, white matter volume loss, and polymicrogyria have been reported in patients with likely pathogenic variants in *CUL4B*.[10] The p.Asn211Ser variant in *CUL4B* showed a weak CSN5 immunoprecipitation and was unstable in cycloheximide treatment. Patient lymphoblast lysates with p.Asn211Ser variant showed a decreased CUL4B protein level similar to those found with the previously reported pathogenic missense variants.[10] The p.Asn211Ser variant in CUL4B did not affect complex formation of CUL4B ubiquitin ligase, which was also reported previously for missense pathogenic variants.[10] For these reasons, we conclude that the p.Asn211Ser variant in *CUL4B* results in instable CUL4B protein, confirming the pathogenicity. Our patient with the pathogenic *CUL4B* variant presented with oligohydramnios and neonatal encephalopathy and seizures at the age of 2 days, and had extensive brain damage showing in brain MRI in the neonatal period. The repeat brain MRI revealed cerebral atrophy, encephalomalacia, and diffuse thinning of the corpus callosum. Our patient's brain MRI features are at the severe end of the reported spectrum.[10]

Neuroimaging features of patients with *KCNQ2* associated neonatal epileptic encephalopathy include increased signal intensities in basal ganglia, thalamus, and hippocampus, diffuse hypomyelination and a thin corpus callosum.[29] Our patient had bilateral subdural, subarachnoid, and intraventricular hemorrhages and anterolateral temporal ischemic changes, as well as diffuse white matter edema observed in brain MRI. We think that the neuroimaging features of our patient expand the clinical spectrum of patients with *KCNQ2*-associated neonatal epileptic encephalopathy to include intracerebral bleeding.

Recently the phenotype and genotype of 201 patients with *SCN2A* related disorders have been reported.[30] About 35% of those patients presented with early infantile epileptic encephalopathy, with a seizure onset within the first 3 months of life. A patient with the p.Met1545Val variant was reported in that study, who presented with neonatal onset seizures at the age of 2 days, characterized by tonic and clonic and right and left migrating seizures with normal brain MRI.[30] In our patient seizure onset was at the age of 6 days, characterized by apnea and focal tonic seizures. Our patient had increased T2 signal intensity in the bilateral frontoparietal subcortical white matter in the brain MRI. Neonatal onset epileptic encephalopathy is the unique feature in both patients with the same likely pathogenic missense variant.

Fewer than 30 patients have been reported with *GNAO1*-associated encephalopathy. About half of those patients presented with seizures and the other half presented with movement disorder without seizures.[31] We summarized all patients with *GNAO1*-associated epileptic encephalopathy (**Supplementary Table 1** online).[32–40] Seizure onset was in the neonatal period in approximately half of the patients and only two patients presented with seizures at birth. The main neuroimaging features were cerebral atrophy, delayed myelination, and a thin corpus callosum. Our patient had seizure onset at birth. He presented with abnormal neuroimaging features, including increased signal intensities in frontal and peritrigonal white matter in brain MRI at 8 days of age.

In summary, we report a 36% diagnostic yield of WES in neonatal encephalopathy and/or seizures in a prospective cohort study. This study, although small, represents an important step in improving our understanding of neonatal encephalopathy. Whole-genome sequencing will probably increase the diagnostic yield of genetic disorders and help us to discover novel genetic disorders causing neonatal encephalopathy and brain damage. The identification of novel genetic disorders involving energy metabolism pathways might provide insights into the pathogenesis of neonatal encephalopathy and brain damage and help us to develop targeted neuroprotective treatment strategies that will ultimately improve health outcomes in term newborns with neonatal encephalopathy.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

The authors declare no conflict of interest.

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