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Deficient activity of alanyl-tRNA synthetase underlies an autosomal recessive syndrome of progressive microcephaly, hypomyelination, and epileptic encephalopathy

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Abstract

Aminoacyl-transfer RNA (tRNA) synthetases ligate amino acids to specific tRNAs and are essential for protein synthesis. Although alanyl-tRNA synthetase (AARS) is a synthetase implicated in a wide range of neurological disorders from Charcot-Marie-Tooth (CMT) disease to infantile epileptic encephalopathy, there have been limited data on their pathogenesis. Here we report loss-of-function mutations in *AARS* in two siblings with progressive microcephaly with hypomyelination, intractable epilepsy and spasticity. Whole exome sequencing identified that the

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affected individuals were compound heterozygous for mutations in *AARS* gene, c.2067dupC (p.Tyr690Leufs*3) and c.2738G>A (p.Gly913Asp). A lymphoblastoid cell line developed from one of the affected individuals showed a strong reduction in AARS abundance. The mutations decrease aminoacylation efficiency by 70–90%. The p.Tyr690Leufs*3 mutation also abolished editing activity required for hydrolyzing misacylated tRNAs, thereby increasing errors during aminoacylation. Our study has extended potential mechanisms underlying AARS-related disorders to include destabilization of the protein, aminoacylation dysfunction, and defective editing activity.

Keywords

AARS; transfer RNA; microcephaly; hypomyelination; aminoacylation defect

Aminoacyl-transfer RNA synthetases (ARSs) have recently received increased attention because their dysfunctions are associated with many human disorders. ARSs catalyze the ligation of cognate amino acids to transfer RNAs, a critical step for maintaining the efficiency and fidelity of protein translation [Ibba and Soll 2000; Mascarenhas et al. 2008]. The human genome encodes 37 aminoacyl-tRNA synthetases that are present in the cytoplasm and mitochondria (18 and 17, respectively, and 2 bifunctional). To date, more than 20 ARSs have been found to be associated with human diseases [Antonellis and Green 2008; Yao and Fox 2013]. While mutations in mitochondrial ARSs lead to disorders affecting various organ systems, mutations in cytoplasmic ARSs have mainly been associated with autosomal dominant peripheral neuropathies [Antonellis et al. 2003; He et al. 2015]. Recently, a growing number of mutations in cytoplasmic ARSs genes, including *QARS* (MIM# 603727) [Zhang et al. 2014], *DARS* (MIM# 603084) [Taft et al. 2013; Novarino et al. 2014], *MARS* (MIM# 156560) [Novarino et al. 2014], *KARS* (MIM# 601421) [McMillan et al. 2015], *AARS* (MIM# 601065) [Simons et al. 2015], and *RARS* (MIM# 107820) [Wolf et al. 2014], have been identified in recessive disorders affecting the central nervous system. The shared phenotypes among these recessive conditions, including progressive microcephaly [Zhang et al. 2014; McMillan et al. 2015], hypomyelinating leukodystrophy [Taft et al. 2013; Wolf et al. 2014], and epileptic encephalopathy [Kodera et al. 2015], suggest a possible commonality in the ARSs-related molecular pathology underlying these neurodegenerative disorders.

Alanyl-tRNA synthetase (AARS) is an ARS that was initially implicated in autosomal dominant Charcot-Marie-Tooth (CMT) disease [Latour et al. 2010; McLaughlin et al. 2012; Bansagi et al. 2015; Motley et al. 2015]. Recently, biallelic mutations in *AARS* were identified in three individuals from two families, who presented with early-onset epileptic encephalopathy [Simons et al. 2015]. Similar to cases with mutations in other ARS genes, these affected individuals were reported to have microcephaly and hypomyelination of the cerebral white matter. However, the clinical spectrum of the AARS-related disorders has not been fully elucidated, and it remains unclear why *AARS* mutations cause distinct neurological disorders with different modes of inheritance.

Here we report the identification of novel biallelic *AARS* mutations in two siblings presenting with progressive microcephaly with hypomyelination and spastic paraplegia. We

ascertained a non-consanguineous family of mixed European descent with two affected individuals presenting with congenital microcephaly and epilepsy (Fig. 1A). The parents (MC32505 and MC32506) and two siblings (MC32503 and MC32504) were healthy and did not have neurological symptoms, such as peripheral neuropathy. The female proband (MC32502) was born at full term via spontaneous vaginal delivery. Birth weight was 2.98 kg (−0.8 SD) and occipito-frontal circumference (OFC) at birth was 31.5 cm (−2.2 SDs). She had transient hypoglycemia at birth, developed progressive microcephaly and failure to thrive, and presented with infantile spasms at 3 months of age. Her electroencephalogram (EEG) showed discontinuous pattern consisting of bursts of high voltage sharp wave activity followed by spontaneous attenuation, which was reported as modified hypsarrhythmia. The infantile spasms transiently responded to adrenocorticotropic hormone (ACTH) therapy but then she had recurrent spasms and seizures, as well as occasional atypical absence seizures. At 7 years of age she developed recurrent tonic-clonic seizures with persistent slow spike and wave pattern and multifocal epileptiform discharges on EEG. The clinical evolution and multiple types of seizures with the typical EEG findings were consistent with Lennox-Gastaut syndrome.

At 3 months of age, her OFC was 37.5 cm (−1.9 SDs), her height was 58 cm (−0.5 SD), and her weight was 5.16 kg (−0.6 SD). She showed increased tone, with opisthotonic posturing when she was held. At 4 years 4 months of age, her OFC was 43 cm (−4.6 SDs), her height was 82.6 cm (−4.7 SDs), and her weight was 13.6 kg (−1.7 SDs). She had poor head control with increased reflexes in the upper and lower extremities bilaterally. On observation at 8 years 6 months of age, she displayed profound delay of growth and development. She was able to walk with a walker but did not have any speech. She had markedly increased tone in her lower extremities and showed no dysmorphic features.

Magnetic resonance imaging (MRI) at 2 years 5 months of age revealed reduced white matter volume with hypomyelination, suggesting underlying metabolic disorder (Supplmentary Fig. S1). The corpus callosum was fully formed but very thin. A series of tests including liver function, plasma amino acids, urine organic acids, lactic acid, pyruvate, acylcarnitine profile, lysosomal screen and ceruloplasmin was all normal.

The younger sister (MC32501) had a similar clinical course with congenital microcephaly and global developmental delay. She was born via induced vaginal delivery at 38 weeks' gestation. Ultrasound exam at about 36 weeks' gestation revealed poor fetal growth. At birth, her weight was 1.96 kg (−2.8 SDs) and her OFC was 30.1 cm (−3.1 SDs). She had failure to thrive, microcephaly and generalized hypotonia at birth, and spent 6 days in the Neonatal Intensive Care Unit because of apnea and poor feeding. At 2 months of age she developed episodes of seizures that were described as head dropping. EEG showed multiple electrical seizures in the frontal and temporal regions. Possible decrements were noted at the time of the head drops, which are frequently associated with infantile spasms, although there were no high voltage discharges suggestive of hypsarrhythmia. At 6 years of age she presented multiple seizure patterns with tonic seizures and head drops. A follow-up EEG showed frequent slow-spike and wave discharges with bilateral epileptiform discharges (Supplementary Fig. S2), indicating encephalopathy of Lennox-Gastaut syndrome with infantile spasms as a clinical seizure manifestation.

MRI performed at 4 months of age showed relative hypomyelination with diminished white matter volume (Supplmentary Fig. S1). At 5 months of age, her OFC was 35.75 cm (−4.9 SDs), height was 63.5 cm (±0.0 SD), and weight was 4.72 kg (−2.6 SDs). She had bilateral hypertonia and spasticity with increased deep tendon reflexes in the upper and lower extremities, and acquisition of developmental milestones was delayed. Liver function tests were normal. Neither affected individual had nystagmus, tremors, or any congenital abnormalities of their extremities.

We performed whole-exome sequencing on the affected individuals (MC32501, MC32502). We screened forvariants that were protein altering and rare (allele frequency less than 0.1% in available population databases and less than 1% in our in-house exome database, consisting of 1466 samples from 512 families, mainly with autism and brain malformations), and fit the autosomal recessive mode of inheritance. Two genes, *AARS* and *FLG* (MIM# 135940), were found to have two variants shared by both siblings, for which they were heterozygous. Another gene, *TAS2R31* (MIM# 612669), was found to have five variants shared by both siblings, for which they were heterozygous. *FLG* is associated with ichthyosis vulgaris, a condition not known to be present in this family, and *TAS2R31* encodes a taste receptor. Therefore, these genes are unlikely to be causative of their neurological phenotype. The two variants in the *AARS* gene were c.2067dupC [p.Tyr690Leufs*3] and c.2738G>A [p.Gly913Asp] (positions of the variants are according to NM_001605.2 for cDNA and NP_001596.2 for protein), and these were confirmed by Sanger sequencing to segregate with the condition in the family (Fig. 1A). The frameshift variant c.2067dupC was inherited from the father (MC32506) and the missense variant c. 2738G>A was inherited from the mother (MC32505). None of unaffected siblings (MC32503 and MC32504) were homozygous for AARS variants. The frameshift variant c. 2067dupC is predicted to produce a truncated AARS (p.Tyr690Leufs*3). The missense variant c.2738G>A leads to a p.Gly913Asp change in AARS and it is predicted to be deleterious (Supplementary Fig. S3). Tyr690 is located in the editing domain of AARS [Beebe et al. 2003] and Gly913 is located in the C-terminal domain (Fig. 1B), which is crucial to its editing activity[Beebe et al. 2003]. Alignment of AARS orthologs shows that both residues are highly conserved (Supplementary Fig. S4).

We established a lymphoblastoid cell line from one of the affected individuals (MC32501) and both her parents (MC32505 and MC32506), and performed immunoblot analysis of AARS. The AARS protein level in the cells from the affected individual was markedly reduced compared to the parents and control (Figure 1C), suggesting that these mutations could impair *AARS* gene functions by reducing protein abundance. No truncated protein was identified in the affected individual (MC32501) or her father (MC32506), who carried the frameshift variant. We performed these experiments using two different AARS antibodies, with epitopes near the N-terminus and C-terminus, and all results were similar (Supplementary Fig. S5). The relative abundance of AARS protein in the affected individuals was estimated to be 3–12% of that in control individuals.

Given that AARS is the only enzyme responsible for alanyl-tRNA aminoacylation reaction, we speculate that a low but functional level of AARS abundance is essential for survival in these affected individuals. Low levels of mischarged transfer alanyl-transfer RNAs due to a

defective tRNA synthetase could lead to an intracellular accumulation of misfolded proteins in neurons (Lee et al., Nature 2006). To examine whether the residual p.Tyr690Leufs*3 and p.Gly913Asp mutants compromise the aminoacylation activity of AARS, we next overexpressed and purified recombinant variants carrying each mutation. The truncation caused by p.Tyr690Leufs*3 was confirmed by the observation that the recombinant variant migrated at a smaller molecular weight than the wild-type AARS on SDS-PAGE (Supplemenary Fig. S6). The aminoacylation activities of both variants were then tested using ^{14}C-labeled alanine with various concentrations of tRNA. The results showed that the p.Tyr690Leufs*3 mutation caused a 86% decrease in catalytic efficiency (k_{cat}/K_m) (Fig. 1D). The p.Gly913Asp mutation led to a 73% decrease in catalytic efficiency. The loss of aminoacylation activity was due to increased K_m and decreased k_{cat}.

tRNA^{Ala} can be mischarged with serine and glycine by AARS to yield Ser-tRNA^{Ala} and Gly-tRNA^{Ala}, respectively [Tsui and Fersht 1981]. To maintain the fidelity of aminoacylation, AARS possesses an editing domain that is capable of hydrolyzing the mischarged tRNA^{Ala} [Beebe et al. 2003; Guo et al. 2009]. As p.Tyr690 is located within the editing domain, we subsequently investigated whether p.Tyr690Leufs*3 impairs this editing activity. An *in vitro* editing activity assay was carried out using [^{3}H] Ser-tRNA^{Ala} as a substrate. AARS p.Cys723Ala, a mutant that has been previously shown to have defective editing activity in mice [Liu et al. 2014a], was included as a positive control. In the presence of AARS p.Tyr690Leufs*3, the deacylation of [^{3}H] Ser-tRNA^{Ala} was diminished to a level comparable to the AARS p.Cys723Ala mutant (Fig. 1E). In contrast, the AARS p.Gly913Asp mutant maintained unaffected deacylation activity compared to the wild-type AARS. This result indicates that the p.Tyr690Leufs*3 mutation drastically decreases the editing activity, whereas the p.Gly913Asp mutation does not impact the editing activity of AARS. Decreased editing activity leads to the production and release of misacylated tRNAs. To further assess whether the defective editing of the p.Tyr690Leufs*3 mutant increases the level of misacylation, we measured production of [^{3}H] Ser-tRNA^{Ala} by wild-type AARS and the p.Tyr690Leufs*3 AARS mutant. Compared with the wild-type, more than 3-fold higher accumulation of [^{3}H] Ser-tRNA^{Ala} was observed in the p.Tyr690Leufs*3 mutant (Fig. 1F), confirming that misacylation resulted from the editing deficiency.

In this study, we demonstrate that mutations in *AARS* are associated with autosomal recessive progressive microcephaly with hypomyelination, intractable epilepsy and spasticity. We found that the mutations could potentially lead to the loss of function of AARS in multiple ways, including a significant reduction of AARS protein abundance, impairment of aminoacylation activity and impairment of evolutionarily conserved editing function, which maintains fidelity during aminoacylation and overall protein synthesis. Multiple mutations in *AARS* have been implicated in autosomal dominant Charcot-Marie-Tooth disease type 2N (CMT2N), characterized by peripheral neuropathies due to axonal pathology [Latour et al. 2010; McLaughlin et al. 2012; Bansagi et al. 2015] (Fig. 1B). Individuals with CMT2N typically demonstrate distal lower limb-predominant sensorimotor neuropathy with decreased action potentials indicating primary axonal damage [England et al. 2009], though several cases with mild associated myelopathy have been reported [Motley et al. 2015]. Recently, biallelic mutations in *AARS* were reported in three individuals with severe epileptic encephalopathy [Simons et al. 2015]. The affected individuals reported

herein represent additional cases of *AARS* mutations with a phenotype distinct from autosomal dominant CMT2N. Similar to the previously reported autosomal recessive cases, the affected individuals demonstrated congenital and progressive microcephaly, central hypomyelination and severe early-onset epilepsy, including infantile spasms and Lennox-Gastaut syndrome. In contrast to the reported cases, the affected individuals showed retained or exaggerated deep tendon reflexes, suggesting that peripheral neuropathy might not be a major phenotype in these individuals, at least in young ages. Infantile syndromic liver failure has been reported in association with leucyl- and methionyl-tRNA synthetase mutations, but the affected individuals herein did not have abnormalities in their liver function tests. Thus, *AARS* mutations appear to lead to two distinct phenotypes, a peripheral nervous system disorder that is a dominant trait and a central nervous system condition that is a recessive trait.

The difference in manifestation of dominant and recessive AARS-related phenotypes could be explained in part by the residual aminoacylation activities of the enzyme. The mutations identified in this study, p.Tyr690Leufs*3 and p.Gly913Asp, cause an aminoacylation defect but retain 10–30% residual activity compared to wild type (7.4-fold and 3.7-fold decrease in k_{cat}/K_m respectively). This is similar to the previously described missense *AARS* mutations, p.Lys81Thr and p.Arg751Gly [Simons et al. 2015], which possess aminoacylation activity of 10–20% of the wild type (2-fold and 10-fold decrease in k_{cat}/K_m respectively). On the other hand, the *AARS* mutations identified in CMT2N, typified by p.Arg329His and p.Asn71Tyr, result in almost no residual aminoacylation activity [McLaughlin et al. 2012]. In these cases, the wild-type *AARS* allele in the same cell is fully functional and ~50% of overall cellular aminoacylation activity is expected to remain. This suggests a possible genotype-phenotype correlation, where a total residual aminoacylation activity of AARS from both alleles defines the clinical phenotype ranging from CMT2N to progressive microcephaly. It is notable that the parents of the affected siblings described herein (MC32505 and MC32506) are both healthy, with no symptoms suggestive of peripheral neuropathies, even though they are heterozygous for an *AARS* mutation, possibly implicating another threshold of residual aminoacylation activity, above which individuals are asymptomatic. This genotype-phenotype model is in accord with the recently proposed idea that common molecular pathways link CMT neuropathies and hereditary spastic paraplegia [Timmerman et al. 2013; Liu et al. 2014b]. Alternatively, *AARS* mutations might cause CMT in heterozygous individuals through impairment of non-canonical functions outside protein synthesis [Guo and Schimmel 2013; Yao and Fox 2013; He et al. 2015].

In addition to aminoacylation activity, AARS possesses an editing activity. Here we show that the p.Tyr690Leufs*3 mutation affects the editing activity and causes serine misacylation. To our knowledge, editing deficiency caused by mutations in human aminoacyl-tRNA synthetases has not been experimentally demonstrated prior to our study. In mice, a biallelic missense mutation in the editing domain of Aars (p.Ala734Glu) has been shown to diminish the editing activity by 40–50%, which leads to accumulation of misfolded proteins resulting in neurodegeneration [Lee, et al., 2006]. Another *Aars* mutation, p.Cys723Ala, causes a more severe editing deficiency, leading to cardiomyopathy in mice when placed in *trans* with p.Ala734Glu [Liu, et al., 2014a]. However, the affected individuals reported herein, with the editing-deficient p.Tyr690Leufs*3 mutation, presented

with a very similar phenotype to the individuals with previously reported *AARS* mutations with no editing defects, except for the retained deep tendon reflexes. Therefore, the contribution of editing deficiency to the disease phenotype in humans remains unclear. Even though the recombinant p.Tyr690Leufs*3 AARS was stable, we found a very low level of the AARS protein in the lymphoblast cell line of the affected individual. Thus, we cannot rule out the possibility that the p.Tyr690Leufs*3 mutant may not be present at a high enough level to exert editing defects *in vivo*.

In summary, we have identified loss-of-function mutations in the *AARS* gene as the cause of a human recessive disorder affecting the central nervous system and characterized by progressive microcephaly with hypomyelination and spasticity. We present functional data indicating that both mutations impair stability and aminoacylation activity of AARS, and one mutation, p.Tyr690Leufs*3, disrupts its editing activity. Further investigation at the cellular and organism level would help further delineate the roles of the *AARS* gene in human neurological development and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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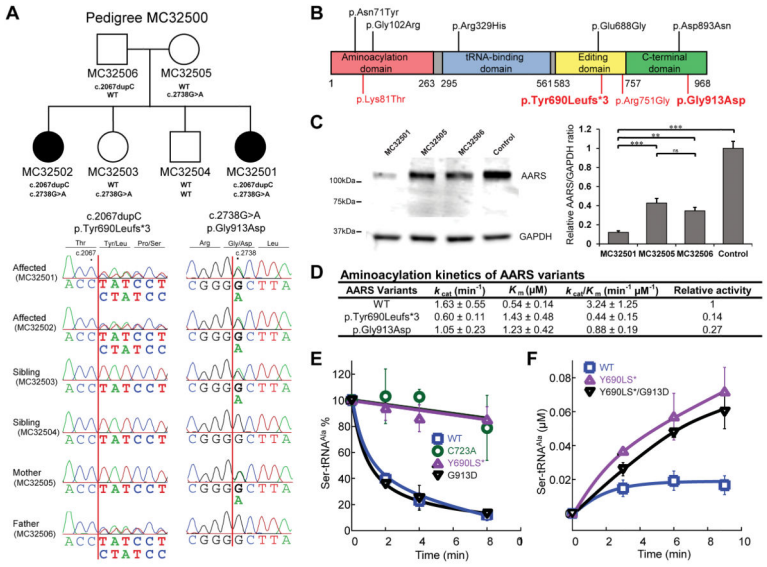

AARS Variants	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})	Relative activity
WT	1.63 ± 0.55	0.54 ± 0.14	3.24 ± 1.25	1
p.Tyr690Leufs*3	0.60 ± 0.11	1.43 ± 0.48	0.44 ± 0.15	0.14
p.Gly913Asp	1.05 ± 0.23	1.23 ± 0.42	0.88 ± 0.19	0.27

Figure 1. *AARS* mutations cause loss-of-fuction defects

A: Identification of *AARS* mutations. Pedigree structure of the family studied is shown. Shaded symbols indicate affected individuals. Sanger sequencing confirmed the segregation of the identified variants in the affected individuals (MC32501 and MC32502). **B:** Schematic representation of the AARS mutations. Variants acting in dominant and recessive manners are shown in black and red, respectively. The variants in this study are in bold. The residue Tyr690 is in the editing domain and the residue Gly913 is in the C-terminal domain. **C:** Immunoblot analysis of AARS abundance in lymphoblastoid cell lines. AARS level in cells available from an affected individual (MC32501) showed a marked reduction of AARS, whereas cells from her parents (MC32505 and MC32506) showed a mild reduction. A representative image by using AARS antibody (A303-473A-T, Bethyl), with an epitope near the N-terminus, is shown. Quantification of AARS abundance was performed Relative AARS/GAPDH intensity ratio was normalized to the control. Values represent the average of three independent experiments. Error bars represent standard deviation. ***p<0.001, **p<0.01, ns; not significant. **D:** Aminoacylation kinetics of AARS protein variants. Relative acitivity is caliculated by ratio to WT activity. **E:** Hydrolytic editing activity of AARS. Deacylation of the incorrectly charged Ser-tRNA^{Ala} was monitored with wild type AARS (WT; blue square), p.Tyr690Leufs*3 (p.T690fs*; purple triangle), and p.Gly913Asp variants (p.G913D; black triangle). p.Cys723Ala (p.C723A; green circle) is served as a positive control of editing defect. **F:** Misacylation of AARS variants. Accumulation of misacylated Ser-tRNA^{Ala} was measured on wild type AARS (WT; blue square) and p.Tyr690Leufs*3 (p.T690fs*; purple triangle). Error bar shows the standard deviation. Values represent the average of at least three independent experiments.

Table 1

Clinical summary of affected individuals

Study	Current study		Simons et al., 2015		
	MC32501	**MC32502**	**LD_0115.0A**	**LD_0115.B**	**LD_0857.0**
Protein variants	p.Tyr690Leufs*3, p.Gly913Asp		p.Lys81Thr, p.Arg751Gly		p.Arg751Gly, homozygous
Gender	Female	Female	Female	Female	Male
Age at examination	5 months	4 years 4 months	7 years 6 months	3 years 8 months	1 years 4 months
Head circumference at birth	30.1cm (−3.12 SDs)	31.5 cm (−2.17 SDs)	N/A (congenital microcephaly)	N/A (congenital microcephaly)	N/A (congenital microcephaly)
Weight at birth	1.96 kg (−2.75 SDs)	2.98 kg (−0.80 SD)	N/A	N/A	N/A
Intrauterine growth retardation	Yes	Yes	Yes	Yes	Yes
Head circumference at examination	35.75 cm (−4.90 SDs)	43 cm (−4.59 SDs)	44 cm (−6.14 SDs)	41.5 cm (−4.84 SDs)	38 cm (−7.29 SDs)
Weight at examination	4.72 kg (−2.64 SDs)	13.6 kg (−1.67 SDs)	N/A	N/A	N/A
Cognition	Fixating on objects	Tracking objects, no speech	No tracking or fixation	Searching eye movement with no visual tracking	Poor intraction
Motor examination	Increased tone and spasticity	Increased tone and spasticity	Dystonia and spasticity	Dystonia and spasticity	Dystonia and tremulousness
Deep tendon reflexes	1+ bilaterally in the upper and lower extremities, no ankle clonus	2+ bilaterally in the upper and lower extremities, positive ankle clonus	Not elicited	Not elicited	Not elicited
Remarks	6 days of NICU stay due to apnea and poor feeding	Transient hypoglycemia at birth	Severe GI reflux, vertical tali	Severe GI reflux, vertical tali	Poor feeding, vertical tali
Epilepsy/Seizure					
Onset	2 months	3.5 months	2 months	3 months	5 months
Type of seizures	Head dropping	Infantile spasms giving way to LGS	Myoclonic tonic- clonic epilepsy	Myoclonic epilepsy	Myoclonic epilepsy
EEG findings	Multifocal epileptiform discharges	Modified hypsarrhythmia, multifocal discharges with infrequent electrodecrements	Multifocal, posterior-predominant discharges	Multifocal, posterior-predominant discharges	Multifocal, psterior-predominant discharges with background suppression
MRI findings					
Age at MRI	4 months	3 years 4 months	1 year 2 months	1 year 5 months	1 year 2 months
Myelination	Hypomyelination	Hypomyelination	Hypomyelination	Hypomyelination	Hypomyelination

Study	Current study		Simons et al., 2015		
	MC32501	**MC32502**	**LD_0115.0A**	**LD_0115.B**	**LD_0857.0**
White matter volume	Moderately diminished	Moderately diminished	Markedly diminished	Markedly diminished	Markedly diminished
Corpus callosum	Thin	Thin	Very thin	Very thin	Very thin
Cellebellum/brain stem	Normal	Normal	Mild volume loss	Marked volume loss	Hypoplastic

EEG: electroencephalography, GI: Gastrointestinal LGS: Lennox Gastaut syndrome, MRI: Magnetic resornace imaging, N/A: not available, NICU: Neonatal intensive care unit, SD: Standard deviation