Severe Leukoencephalopathy with Clinical Recovery Caused by Recessive *BOLA3* Mutations

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Abstract *Aim:* To identify the genetic aetiology of a distinct leukoencephalopathy causing acute neurological regression in infancy with apparently complete clinical recovery. *Methods:* We performed trio whole genome sequencing (WGS) to determine the genetic basis of the disorder. Mitochondrial function analysis in cultured patient

fibroblasts was undertaken to confirm the pathogenicity of candidate variants. *Results:* The patient presented at 18 months with acute hemiplegia and cognitive regression without obvious trigger. This was followed by clinical recovery over 4 years. MRI at disease onset revealed bilateral T2 hyperintensity involving the periventricular and deep white matter and MR spectroscopy of frontal white matter demonstrated a lactate doublet. Lactate levels and mitochondrial respiratory chain enzyme activity in muscle, liver and fibroblasts were normal. Plasma glycine was elevated. The MRI abnormalities improved. WGS identified compound heterozygous variants in *BOLA3*: one previously reported (c.136C>T, p.Arg46*) and one novel variant (c.176G>A, p.Cys59Tyr). Analysis of cultured

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patient fibroblasts demonstrated deficient pyruvate dehydrogenase (PDH) activity and reduced quantity of protein subunits of mitochondrial complexes I and II, consistent with *BOLA3* dysfunction. Previously reported cases of multiple mitochondrial dysfunctions syndrome 2 (MMDS2) with hyperglycinaemia caused by *BOLA3* mutations have leukodystrophy with severe, progressive neurological and multisystem disease. **Conclusions:** We report a novel phenotype for MMDS2 associated with apparently complete clinical recovery and partial resolution of MRI abnormalities. We have identified a novel disease-causing variant in *BOLA3* validated by functional cellular studies. Our patient's clinical course broadens the phenotypic spectrum of MMDS2 and highlights the potential for some genetic leukoencephalopathies to spontaneously improve.

Introduction

Bola3 is a mitochondrial protein involved in iron–sulfur cluster (ISC) biogenesis, essential for the maturation of lipoate-containing 2-oxoacid dehydrogenases, the glycine-cleavage system and the biogenesis of complexes I, II and III of the mitochondrial respiratory chain and pyruvate dehydrogenase (PDH) (Uzarska et al. 2016). Bi-allelic variants in *BOLA3* cause multiple mitochondrial dysfunctions syndrome type 2 with hyperglycinaemia [MMDS2; MIM#614299], a severe disorder of mitochondrial energy metabolism, typically characterised by infantile encephalopathy, leukodystrophy, lactic acidosis, non-ketotic hyperglycinaemia and death in early childhood. The longest lifespan associated with MMDS2 has been 11 years and clinical recovery has not been reported (Seyda et al. 2001; Cameron et al. 2011; Baker et al. 2014). There are five genes associated with multiple mitochondrial dysfunctions syndrome types 1–5: *NFU1*, *BOLA3*, *IBA57*, *ISCA1* and *ISCA2*, all of which encode essential proteins involved in the assembly of mitochondrial ISC (Cameron et al. 2011; Ajit Bolar et al. 2013; Al-Hassnan et al. 2015; Shukla et al. 2017). Recessive pathogenic variants in these genes result in multiple defects in the respiratory chain complexes and impaired glycine cleavage causing severe neurological disease, elevated plasma and cerebrospinal fluid (CSF) glycine and combined deficiency of complexes I, II and III, and PDH (Torraco et al. 2017). Five disease-causing variants in *BOLA3* have been identified in patients with MMDS2, one of which may be a founder mutation in the Japanese population (Cameron et al. 2011; Haack et al. 2013; Baker et al. 2014; Kohda et al. 2016).

Methods

Patient

The patient was referred from the Royal Children's Hospital Neurology clinic to a leukodystrophy gene discovery project. This project was approved by the Royal Children's Hospital Human Research Ethics Committee (HREC Project Number 28097).

Whole Genome Sequencing

Whole genome sequencing (WGS) was performed for the proband and both parents to identify the causative variant(s). Trio WGS was performed using 2×150 nt paired end reads on an Illumina X (Illumina Cambridge Ltd., Little Chesterford, UK). Read alignment was performed using BWA-mem, variant calling of the nuclear genome was performed using GATK HaplotypeCaller v3.7 and BCFtools was used to call mtDNA variants (Li et al. 2009; McKenna et al. 2010). SnpEff v4.3m was used for variant annotation and a custom script was utilised for variant filtration and prioritisation (Cingolani et al. 2012).

Enzyme Assays

Spectrophotometric assays assessing activities of mitochondrial respiratory chain complexes I–IV and citrate synthase were performed as described previously (Frazier and Thorburn 2012). PDH activity was analysed in fibroblasts using a PDH Enzyme Activity Dipstick Assay Kit (ab109882) as per the manufacturer's protocol.

Immunoblotting

Protein was extracted from cultured fibroblasts and analysed by SDS-PAGE immunoblotting as described previously (Lake et al. 2017). Blots were probed with Total OXPHOS Human WB Antibody Cocktail (ab110411) and VDAC1 (MSA05) primary antibodies, after which they were incubated with anti-mouse secondary antibody and developed as described previously (Lake et al. 2017).

Results

Clinical Details

The proband is the first of three children born to healthy unrelated parents of Anglo-European descent. The proband and his sister have congenital hypothyroidism. He was born

at term after a healthy pregnancy. There were no health or developmental concerns in infancy. He walked at 12 months of age. At 18 months of age, he presented with acute right hemiparesis, ataxia and loss of speech and language skills. No illness or injury was associated with the onset of disease. Over the following 4 years, he gradually regained motor function and speech. At 6 years of age, he had subtle evidence of right arm weakness and otherwise normal neurological and cognitive function for his age. At last assessment at 8 years of age, he had a diagnosis of attention deficit disorder by DSM-V criteria and otherwise normal motor and cognitive function.

MRI

Brain MRI at onset of disease revealed extensive, bilateral, heterogeneous white matter signal abnormality with T2 signal increase and T1 signal decrease sparing subcortical white matter, basal ganglia, brainstem and cerebellum (Fig. 1). The corpus callosum was involved, with significant involvement of the genu and involvement of the

internal leaflet of the splenium. There was restricted diffusion in a multifocal fashion within the white matter change and in the posterior regions, and no contrast enhancement. Magnetic resonance spectroscopy (MRS) of the frontal white matter demonstrated a lactate doublet with decreased N-acetylaspartate (NAA) (not shown). The combination of extensive white matter changes, restricted diffusion and MRI pattern was considered most consistent with a mitochondrial disorder (Schiffmann and van der Knaap 2009). MRI brain at 8 years showed a significant reduction in the superficial extent of abnormal T2 hyperintensity and no restricted diffusion (Fig. 1).

Biochemical Studies and Enzymology

At presentation, the proband had normal lactate and alanine levels in both plasma and CSF. He had elevated urine glycine (only qualitative result available: Gly++; 3–10× cut-off), elevated plasma glycine level (443 $\mu\text{mol/L}$; RR 125–318) and normal CSF glycine level. Respiratory chain enzyme analysis in fibroblasts, muscle and liver were non-

Fig. 1 (a–c) MRI at the onset of disease at 18 months. (a, b) Axial T2 sequences show extensive, bilateral, heterogeneous white matter signal abnormality consistent with dysmyelination sparing subcortical white matter, basal ganglia, brainstem and cerebellum. (c) Apparent diffusion coefficient (ADC) map shows restricted diffusion at the

periphery of the white matter change and in the posteriorly affected regions (arrow). (d–f) MRI at 8 years, axial T2 (d, e) and ADC map (f) show a significant reduction in the extent of abnormal T2 hyperintensity and no restricted diffusion

Table 1 Biochemical and enzymology results (table)

	Patient	Reference range
Glycine (umol/L)		
Urine	++ (3–10× cut-off)	N/A
Plasma	443	125–318
CSF	7	5–8
Lactate (mmol/L)		
Plasma	1.6–1.8	1.0–1.8
CSF	1.7	1.2–2.3
Respiratory chain enzymes		
Muscle	Normal	
Liver	Normal	
Fibroblasts	Normal	

diagnostic. Complex II activity in liver was lower than other enzymes but the level was normal when expressed relative to protein and citrate synthase (Table 1).

Genetic Results

Chromosome microarray and a targeted panel testing 22 mtDNA point mutations was negative. Trio genome sequencing identified compound heterozygous variants in *BOLA3*: c.136C>T, p.Arg46* (hg19:chr2:74372349G>A; ENST00000327428) (paternally inherited) and c.176G>A, p.Cys59Tyr (hg19:chr2:74369481C>T; ENST00000327428) (maternally inherited) (Supplementary Fig. 1). The p.Arg46* variant creates a premature stop codon in exon 2 of 4 and has been previously reported in the homozygous state in five individuals from four families with MMDS2 (Baker et al. 2014; Lebigot et al. 2017). The p.Cys59Tyr variant has not been reported before in association with MMDS2. It is a rare missense variant with allele count of 6/245984 and no homozygotes in gnomAD (Lek et al. 2016). The tyrosine substitution affects a highly conserved amino acid. It is predicted as damaging by SIFT and PolyPhen2 and has a CADD score of 33 (Supplementary Fig. 2). The p.Cys59 residue has been identified as one of the two possible Fe–S cluster ligands in *BOLA3* (Uzarska et al. 2016; Nasta et al. 2017). The other is the His96 residue which is the site of a putative founder pathogenic missense variant in MMDS2 (Kohda et al. 2016).

Functional Studies

To confirm the pathogenicity of the *BOLA3* variants in the context of this atypical phenotype for MMDS2, PDH activity and the quantity of protein subunits of mitochondrial respiratory chain complexes were analysed in patient

fibroblasts. Three normal control samples plus an affected control sample with a known molecular defect in *NARS2* (MIM #616239) were analysed at the same time. Patient PDH activity, expressed relative to the mitochondrial marker enzyme citrate synthase, was 20% of the mean value for three normal controls while cells from a patient with *PDHX* mutations, which served as a positive control, had undetectable activity (Fig. 2). Fibroblast protein extracts were then analysed by immunoblotting using an antibody cocktail that detects one subunit from each of the OXPHOS complexes. The blot was also probed for VDAC1 as a marker of mitochondrial loading. Western blot showed reduced quantity of protein subunits of respiratory chain complexes I and II in fibroblasts from the proband, as expected for an ISC biogenesis defect (Lebigot et al. 2017). A reduced quantity of protein subunits of complexes I, II and IV was seen in fibroblasts from the patient with *NARS2* mutations, as expected for a mitochondrial translation defect (Lake et al. 2017) (Fig. 2).

Discussion

White matter involvement is a common feature of mitochondrial disorders that affect CNS myelin directly or indirectly via disturbance of axonal, oligodendrocyte, astrocyte or microglia function (Wong 2012; Morato et al. 2014). Mitochondrial leukoencephalopathies are a genetically and clinically heterogeneous group of disorders and diagnosis can be challenging as the clinical presentation may not be accompanied by biochemical or systemic features of mitochondrial disease. MRI pattern recognition can lead to the suspicion of mitochondrial dysfunction as a potential cause of white matter abnormalities. MRI pattern abnormalities suggestive of mitochondrial dysfunction are a diffuse or multifocal T2 hyperintense signal in the periventricular and deep white matter. The changes are often inhomogeneous early in the disease course. There may be cystic change within the affected white matter with cysts that are isolated and well-delineated. Additionally, there may be involvement of the brainstem and spinal cord, dysfunction of the blood–brain barrier demonstrated by contrast enhancement and lactate abnormalities on MR spectroscopy (Van der Knaap and Valk 2005; Parikh et al. 2015). There may also be significant changes on diffusion weighted imaging, thought to be consistent with an energy metabolism defect (Helman et al. 2016).

BOLA3 encodes a protein that is essential in the production of ISC, involved in the maturation of lipoate-containing 2-oxoacid dehydrogenases, the glycine-cleavage system and the assembly of PDH and respiratory chain complexes I, II and III. The specific role of *BOLA3* in ISC assembly is thought to be a coupling with NFU1 to facilitate Fe–S transfer to the recipient protein and

Fig. 2 Identification of pyruvate dehydrogenase (PDH) deficiency and reduced levels of complex I and II subunits in patient fibroblasts. **(a)** PDH was measured in fibroblasts from controls, the patient and a previously reported patient with PDH deficiency (patient 26 in Rahman et al. (1996)) who has a homozygous *PDHX* mutation. PDH activity was normalised to citrate synthase (CS), and results are presented as percent of mean control activity. The data are represented as the mean of triplicate measurements $\pm$ SEM. PDH activity was undetectable in the *PDHX* patient. **(b)** Representative SDS-PAGE

western blot examining the protein levels of one subunit of each of the five OXPHOS complexes (CI–V) in fibroblasts from controls, the patient and a patient with CI, CIII and CIV deficiency who has *NARS2* mutations. VDAC1 was used as a mitochondrial loading control. **(c)** Densitometry analysis of CI subunit NDUFB8, CII subunit SDHB and CIV subunit COXII was performed on western blot data. Results were normalised to VDAC1 and presented as the percent of the control mean. The data represent the mean of duplicate measurements

prevention of oxidative damage to [4Fe–4S] clusters (Melber et al. 2016). Bi-allelic mutations in *BOLA3* are associated with multiple mitochondrial dysfunctions syndrome with hyperglycaemia (MMDS2) (Cameron et al. 2011; Baker et al. 2014). Previously reported cases had severe and progressive multisystem disease with shortened life span (Table 2). A cohort of three unrelated patients reported by Baker et al. (2014) were all homozygous for the c.136C>T (p.Arg46*) mutation identified in our patient and had similar MRI findings to our patient and elevated plasma glycine levels. However, they had more severe and progressive neurological impairment and died at 7 months, 22 months and 11 years, respectively. In this cohort,

respiratory chain enzyme activity was measured in fibroblasts from two patients and was normal, and analysis of liver for the third patient demonstrated low complex II activity. PDH enzyme activity was measured in fibroblasts from two of these cases and was reduced in one and normal in the other (Baker et al. 2014). The patient who lived to 11 years of age was the only one of the three patients with normal respiratory chain activity and PDH activity measured in fibroblasts, no cardiomyopathy, and had only mildly elevated plasma glycine in a similar range to our patient (Baker et al. 2014). This is the only patient reported previously to have survived beyond 2 years. It is interesting that this cohort of patients has the same homozygous

Table 2 Clinical features of previously reported patients with mutations in *BOLA3* and comparison with our patient

	Our patient	Previously reported cases of recessive <i>BOLA3</i> mutation (12 patients) ^a
Age of onset	18 months	Birth–8 months
Age at death or last report	8 years	3 months–11 years
Delay or regression in motor development	Yes	12/12
Acquired independent walking	Yes	1/5
Seizures	No	5/10
Cardiomyopathy	No	7/9
Leukodystrophy	Yes	6/7
Elevated plasma lactate	No	11/12
Elevated plasma glycine	Yes	8/8
Decreased complex I and II enzyme activity	No	10/12
Decreased PDH activity	Yes	6/7
Clinical recovery	Yes	0/10

^aReferences for previously reported cases of recessive *BOLA3* mutation (Cameron et al. 2011; Haack et al. 2013; Baker et al. 2014; Kohda et al. 2016; Lebigot et al. 2017)

mutation but different enzymology results and clinical course. This observation suggests that there may be genetic and/or environmental factors modifying the MMDS2 phenotype.

The *BOLA3* variants identified in our patient are consistent with his MRI abnormalities and hyperglycinaemia. However, his clinical presentation is milder than those previously reported and thus expands the phenotype associated with mutations in this gene and the MMDS in general. It is possible that the tyrosine substitution at p. Cys59 results in a protein with partial activity, imparting a milder phenotype. The onset of disease at 18 months may have been precipitated by an increased metabolic demand in the cerebral white matter that exceeded the residual function of *BOLA3* in this patient. As no illness or injury was associated with disease onset, an increase in metabolic demand may have related to a developmental stage in brain development and/or myelination at this age. Alternatively, there may be genetic or environmental factors modifying the impact of these mutations. Mitochondrial disease is known to have a recurrent phenotype with partial clinical neurological recovery in between; whether this is a

recurrent phenotype is yet to be known given the age of the patient.

There are other white matter disorders that can be associated with a clinical and radiological recovery. Two examples are leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL, MIM#614924) and megalencephalic leukoencephalopathy with cysts type 2B (MLC2B, MIM#613926). The clinical phenotype of LTBL caused by bi-allelic variants in *EARS2* can vary from multisystem fatal infantile disease to a childhood-onset leukoencephalopathy with slow improvement of neurological function and MRI abnormalities (Steenweg et al. 2012). MLC2B, caused by dominant mutations in *HEPACAM*, can present similarly to megalencephalic leukoencephalopathy with cysts type 1 (MLC1, MIM# 604004) with postnatal macrocephaly, developmental delay and diffuse signal abnormality and swelling of the brain white matter and subcortical cysts. However, unlike MLC1, the clinical course of MLC2B can improve, with normalisation of head size and resolution of MRI abnormalities (Lopez-Hernandez et al. 2011). The mechanism of clinical recovery and phenotypic variability is not known for either of these diseases.

For our patient, the genetic diagnosis of MMDS2 had implications for his clinical management. The potential for multisystem involvement was considered, prompting baseline cardiac and ophthalmology assessments which were normal. In addition, treatment with a mitochondrial cocktail was initiated as a potentially protective measure. His siblings had genetic testing, in case they were pre-symptomatic and could benefit from protective measures, and both were unaffected. This atypical MMDS2 phenotype has implications for new diagnoses of MMDS2 as there is the potential for a favourable prognosis. The mechanism of clinical and radiological recovery is not known in this case and may be a mutation-specific phenotype or related to genetic/environmental modifiers. Further research is required to investigate biomarkers that may be targeted for therapeutic benefit.

Conclusion

We report a novel phenotype for MMDS2 associated with complete clinical recovery and partial resolution of MRI abnormality. We have identified a novel disease-causing variant in *BOLA3* validated by functional cellular studies. Our patient's unique clinical course provides important information which expands the phenotypic spectrum of MMDS2.

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Synopsis

We report a novel phenotype for MMDS2 associated with apparent clinical recovery and partial resolution of MRI abnormalities, highlighting the potential for some genetic leukoencephalopathies to spontaneously improve.

Conflict of Interest

Chloe Stutterd, Nicole Lake, Heidi Peters, Paul Lockhart, Ryan Taft, Marjo van der Knaap, David Thorburn, Cas Simons and Richard Leventer declare that they have no conflict of interest. Adeline Vanderver receives in kind and research support from Illumina, Shire, Gilead, Eli Lilly and Ionis.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Author Contributions

Conception and design of the study: RL, CAS, NL, DT and PL.

Acquisition and analysis of data: CAS, NL, HP, RT, MV, AV and CS.

Drafting a significant portion of the manuscript or figures: CAS, NL, DT and RL.

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