Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (LBSL): Novel DARS2 mutation and mitochondrial dysfunction in fibroblasts

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Introduction

LBSL is a mitochondrial disorder caused by mutations in the mitochondrial aspartyl-tRNA synthetase gene DARS2. Clinical presentation varies from severe infantile to chronic, slowly progressive neuronal decline in adolescence (1,2). Rare forms of exercise-induced paroxysmal ataxia have also been reported (3). A distinctive brain MRI shows hyperintensities in periventricular cerebral white matter, brainstem, and spinal cord, and MRS demonstrates increased lactate. Most patients are heterozygous, harboring one splice defect in a hotspot in intron-2, resulting in partial expression of the full-length protein and a second deleterious mutation (2).

Initial studies in DARS2 (1) suggested that the disease was not well expressed in fibroblasts, however, a recent publication demonstrated clear mitochondrial dysfunction in fibroblasts opening the avenue for further investigations in this tissue (4).

Here we present a new family with 2 affected members with LBSL, harboring a previously reported and a new DARS2 mutation. We also report on extensive biochemical defects in mitochondrial metabolism in fibroblasts.

Materials and Methods

Genetic testing was performed in a certified laboratory, while all other studies were done in the Laboratory for Energy Metabolism at CHOC Children's. Primary skin fibroblasts were established from all family members. To assess the splicing mutation in intron 2, PCR was performed using forward primer in intron 2 (5'-GAGGAGAATTCCAGAACAGTAG-3') and reverse primer in intron 4 (5'-CTGGTGGGATTTCGTCTC-3'). mRNA levels were measured using a TaqMan assay spanning DARS2 exon 8–9 junction and ACTB as an internal reference gene. DARS2 antibodies (ab154606) and beta Actin (ab184220) were used for western blot experiments. Oxygen consumption rate (OCR) was measured in a Seahorse Bioscience XFe-24 bioanalyzer. Lactic acid was measured in cultured media with Lactate Reagent Set (Pointe Scientific). Mitochondrial reactive oxygen species (ROS) were detected using MitoSOX™ Red (M36008, ThermoFisher) via flow cytometry. Results were normalized to mitochondrial mass using MitoTracker™ Green (M7514, ThermoFisher), which was also used to assess mitochondrial morphology under a fluorescent microscope.
Patients: Patient 1, was the 28-week product of a triplet pregnancy. He remained in the NICU for 3 months and required PDA ligation. A mild hydrocephalus detected at 2 weeks did not require shunting. After discharge from NICU he had no significant illnesses. At 4.9y a new brain MRI, obtained due to headaches, showed mild prominence of the lateral ventricles with mild T2 hyperintensities in the deep periventricular and subcortical white matter. There was also abnormal T2-hyperintense signals in the dorsal aspect of the proximal cervical spinal cord. At 5.4y patient had transient loss of consciousness following a head trauma. A CT scan was not concerning for acute lesions, and he fully recovered. Patient’s headaches remained mild and intermittent until age 8.4y when headaches became more intense. A new MRI was concerning for leukodystrophy (Fig 1) and patient was referred for further evaluation. He was intellectually normal, attending regular school and had no significant findings in physical exam. A 300-gene leukodystrophy panel revealed a previously reported c.492+2T>C mutation in intron 5 but no second mutation was identified. Family history revealed 3 siblings and non-consanguineous parents, all of them thought to be healthy. Patient 2 is the younger brother of patient 1. He was ascertained at 4y of age after molecular testing was performed in his brother (see below). He was clinically asymptomatic, however MRI of the Brain was already abnormal (Fig 2)

Figure 1, Patient 1: Brain MRI. Axial T2 cuts show extensive patchy and confluent areas of abnormal T2/Flair hyperintensities within the supratentorial white matter with sparing of the subcortical U fibers (arrows). Additionally, there was symmetric involvement of the infratentorial white matter adjacent to the vermis, abnormal hyperintense T2/FLAIR signal along the intraparenchymal course of the trigeminal nerves, and the bilateral pyramid and dorsal column of the medulla and upper cervical spine.

Figure 2, Patient 2: MRI of the Brain obtained at 5 y showed bilateral and symmetric hyperintense T2 signal abnormalities in the supraventricular and periventricular white matter (yellow arrows). A right intraventricular cyst (green arrow) was also uncovered. Abnormal hyperintense signal intensities were also observed in the posterior limbs of the internal capsule, pyramids at the level of the medulla, and dorsal columns of the spinal cord.
Sanger sequencing identified the second mutation as a novel c.228-17C>G variant in the known hotspot in intron-2 in patient 1. Family studies found the same variants in his younger brother (patient 2). Mutation analysis also revealed that an 8y old male sibling (sibling 1) did not carry any of the DARS2 mutations (Table 1) and was therefore selected as a control for all additional studies. RT-PCR results suggested skipping of exon 3 in the father and the two patients harboring the intron 2 mutation (Fig 3).

Table 1. Segregation of the mutations in the family

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<tr>
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<th>Intron 2 (Newly discovered mutation)</th>
<th>Intron 5</th>
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<tbody>
<tr>
<td>Mother</td>
<td>Normal</td>
<td>c.492+2T&gt;C Normal</td>
</tr>
<tr>
<td>Father</td>
<td>c.228-17C&gt;G</td>
<td>Normal</td>
</tr>
<tr>
<td>Sibling 1</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Sibling 2</td>
<td>c.228-17C&gt;G</td>
<td>Normal</td>
</tr>
<tr>
<td>Patient 1</td>
<td>c.228-17C&gt;G</td>
<td>c.492+2T&gt;C Normal</td>
</tr>
<tr>
<td>Patient 2</td>
<td>c.228-17C&gt;G</td>
<td>c.492+2T&gt;C Normal</td>
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Quantitative PCR showed lower DARS2 mRNA expression in the two patients compared to sibling 1 (Fig 4). Western blot also revealed lower DARS2 protein expression in both patients (Fig 5).

Figure 3. Skipping of exon 3 in patients

Figure 4. DARS2 mRNA expression of patient and other family members

Figure 5. DARS2 protein expression of patient and other family members
Oxygen consumption rate demonstrated significantly reduced mitochondrial basal respiration and spare respiratory capacity in patients compared to sibling 1 (Fig 6).

**Figure 6. Abnormal oxygen consumption rate in patient's fibroblasts.** A) showed representative result from triplicate experiments. B) Testing performed in triplicate. All error bars indicate SD.

Lactic acid in media as well as mitochondrial superoxide levels were increased in both patients (Fig 7 & 8).

**Figure 7. Lactic acid level in cultured media.** Testing performed in triplicate. Error bars indicate SD.

**Figure 8. Mitochondrial ROS level in fibroblasts.** Testing performed in triplicate. Error bars indicate SD.
Fluorescent microscopy performed with MitoTracker™ Green, demonstrated fragmented mitochondria in patient’s fibroblasts (Fig 9).

**Figure 9. Fragmented mitochondria in patients’ fibroblast**

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<th>Sibling 1</th>
<th>Patient 1</th>
<th>Patient 2</th>
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**Summary**

We present a new family affected with LBSL and describe a novel mutation in the DARS2 intron-2 hotspot. The proband in this family presented only with headaches, despite findings of extensive white matter disease in the brain and spine. Moreover, a younger asymptomatic sibling was ascertained only due to molecular testing, while he also had extensive white matter lesions. We demonstrate that our patients had lower protein and mRNA expression of DARS2, higher lactic acid and ROS and decreased OCR. Our results confirm expression of the disease in fibroblasts (4) and expand on the nature of mitochondrial dysfunction in LBSL, providing additional metrics for future in vitro therapeutic interventions.

**References**


