A new case of SFXN4 (Sideroflexin-4) defect: Novel mutation and further evidence of mitochondrial dysfunction

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Introduction
Sideroflexin4 is a nuclear-encoded mitochondrial protein located at the mitochondrial inner membrane. SFXN4 defects cause autosomal recessive combined OXPHOS deficiency-18. To date, only 3 female European patients with biallelic variants in SFXN4 have been reported, which shared similar clinical presentation including oligohydramnios, intrauterine growth restriction (IUGR), microcephaly, neurodevelopmental delays, visual impairment, and lactic acidosis (1,2). Muscle biopsy studies have shown ultrastructural mitochondrial abnormalities and severe complex-1 deficiency. Fibroblast studies, performed in 2 patients, demonstrated defects in respiratory chain activity, although the mechanism is still unknown (1, 2). Knockout studies in K562 and HEK293 cell lines revealed an important role of SFXN4 in Fe-S clusters biogenesis (3). Herein, we present a new case of SFXN4 deficiency and explore its role in mitochondrial function and iron metabolism.

Methods
Whole exome sequencing (WES) was performed in a certified laboratory. Electron transport chain (ETC) activity was measured in fibroblasts in a reference lab. Primary skin fibroblasts were established from the patient. mtDNA copy number was measured by qPCR using primers targeting B2M and ND1 genes. Western blots were performed with specific antibodies as follows: SFXN4 (PA5-98509), beta Actin (ab184220), TOMM20 (ab186735), Aconitase2 (ab129105), FECH (14466-1-AP) and NUBPL (17393-1-AP). Lactic acid was measured in cultured media with Lactate Reagent Set (Pointe Scientific). Mitochondrial reactive oxygen species (ROS) and iron levels were measured using MitoSOX™ Red (M36008, ThermoFisher) and Mito-FerroGreen (M489-10, Dojindo) via flow cytometry. Results were normalized to mitochondrial mass using MitoTracker™ Green (M7514, ThermoFisher). Oxygen consumption rate (OCR) measurements were performed on a Seahorse Bioscience XFe-24 bioanalyzer.

Results
Patient: A male patient was born to consanguineous Pakistani parents after a pregnancy complicated by poor growth and oligohydramnios. Symmetric IUGR, dysmorphic features and a VSD were noted. At 19 months he was evaluated for FTT, elevated lactic acid (3.2-7.0 mMol/L [<2.1]), elevated alanine (693 umol/L [143-439]), and abnormal organic acids (increased lactate, pyruvate and Krebs cycle intermediates) suspicious for a mitochondrial disease. Brain MRI was unremarkable. WES revealed a novel homozygous mutation, c.649C>T (p.R217*), in SFXN4, predicted to be deleterious. The patient is currently 4.5 years old and has mild delays and persistent lactate elevation.
Patient showed lower SFXN4 expression and lower expression of complex I compared to control (Fig 1 & 2)

**Figure 1. SFXN4 protein expression in patient.** Testing performed in triplicate. Error bars indicate SD. ***p<0.001

**Figure 2. Respiratory chain protein expression in patient.** Testing performed in triplicate. Error bars indicate SD. **p<0.01

ETC enzyme analysis revealed partially decreased activity of complex I while other complexes showed increased activities, indicating mitochondrial proliferation (Table 1). mtDNA copy number analysis confirmed mitochondrial proliferation in fibroblasts. (Fig 3).

**Table 1. Mitochondrial respiratory chain enzyme analysis (ETC) — fibroblast**

<table>
<thead>
<tr>
<th>ETC activities</th>
<th>ETC complexes</th>
<th>Patient</th>
<th>Normalized to Control</th>
<th>Normalized to citrate synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH: ferricyanide dehydrogenase</td>
<td>II</td>
<td>11.96</td>
<td>299%</td>
<td>322%</td>
</tr>
<tr>
<td>NADH: cytochrome c reductase</td>
<td>II + III</td>
<td>12.04</td>
<td>172%</td>
<td>186%</td>
</tr>
<tr>
<td>Total</td>
<td>IV</td>
<td>35.7</td>
<td>209%</td>
<td>225%</td>
</tr>
<tr>
<td>Rotenone sensitive</td>
<td>IV</td>
<td>35.7</td>
<td>209%</td>
<td>225%</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>II</td>
<td>11.96</td>
<td>299%</td>
<td>322%</td>
</tr>
<tr>
<td>Succinate: cytochrome c reductase</td>
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<td>12.04</td>
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<tr>
<td>Cytochrome c oxidase</td>
<td>IV</td>
<td>35.7</td>
<td>209%</td>
<td>225%</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td></td>
<td>63</td>
<td>93%</td>
<td>100%</td>
</tr>
</tbody>
</table>

1 Units: nmol/min/mg protein  
2 Percentage of the control mean  
3 Values normalized to citrate synthase activity

**Figure 3. Mitochondrial DNA copy number in patient.** Error bars indicate SD. ***p<0.001
Assessment of the oxygen consumption rate in fibroblasts demonstrated significantly reduced mitochondrial spare respiratory capacity in the patient compared to control (Fig 4).

Figure 4. Abnormal respiratory chain function in patients’ fibroblasts. A) Representative result from triplicate experiments. B) Average results from triplicate experiments. Error bars indicate SD. *p<0.05

Lactic acid as well as mitochondrial superoxide levels were increased in patient compared to control (Fig 5 & 6).

Figure 5. Mitochondrial ROS level in fibroblasts. Testing performed in triplicate. Error bars indicate SD. *p<0.05

Figure 6. Lactic acid levels in culture media. Testing performed in triplicate. Error bars indicate SD. **p<0.01
Analysis of Fe-S cluster proteins showed decreased expression of FECH and NUBPL, but not of aconitase2 (Fig 7).

**Figure 7. Protein expression of Fe-S cluster proteins.** Testing performed in triplicate. Error bars indicate SD. *p<0.05, **p<0.01

Surprisingly, mitochondrial iron levels were not increased in patient’s fibroblasts at baseline or after treatment with 100µM iron for 24 hours (Fig 8).

**Figure 8. Iron level in mitochondria.** Testing performed in triplicate. Error bars indicate SD.

**Summary**

We report the 4th patient with a SFXN4 defect due to a novel c.649C>T (p.R217*) mutation. Clinical presentation was similar to previously described. We demonstrate a clear mitochondrial defect in our patient’s fibroblasts. In comparison with previous reports in knockout cell lines, we did not observe elevated mitochondrial iron in our patient’s fibroblasts, which may be due to a milder defect and/or compensation from other sideroflexin family members. Additionally, our results suggest that SFXN4 may not be needed for ACO2 synthesis but appears to play an important role in the synthesis of FECH and NUPBL. Additional studies will be needed to further elucidate these findings.

**References**