ECHS1 mutations in Leigh disease: a new inborn error of metabolism affecting valine metabolism

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Two siblings with fatal Leigh disease had increased excretion of S-(2-carboxypropyl)cysteine and several other metabolites that are features of 3-hydroxyisobutyryl-CoA hydrolase (HIBCH) deficiency, a rare defect in the valine catabolic pathway associated with Leigh-like disease. However, this diagnosis was excluded by HIBCH sequencing and normal enzyme activity. In contrast to HIBCH deficiency, the excretion of 3-hydroxyisobutyryl-carnitine was normal in the children, suggesting deficiency of short-chain enoyl-CoA hydratase (ECHS1 gene). This mitochondrial enzyme is active in several metabolic pathways involving fatty acids and amino acids, including valine, and is immediately upstream of HIBCH in the valine pathway. Both children were compound heterozygous for a c.473C>A (p.A158D) missense mutation and a c.414+3G>C splicing mutation in ECHS1.

ECHS1 activity was markedly decreased in cultured fibroblasts from both siblings, ECHS1 protein was undetectable by immunoblot analysis and transfection of patient cells with wild-type ECHS1 rescued ECHS1 activity. The highly reactive metabolites methacryloyl-CoA and acryloyl-CoA accumulate in deficiencies of both ECHS1 and HIBCH and are probably responsible for the brain pathology in both disorders. Deficiency of ECHS1 or HIBCH should be considered in children with Leigh disease. Urine metabolite testing can detect and distinguish between these two disorders.

Keywords: biochemistry; metabolic disease; neurodegeneration

Introduction

Leigh disease (MIM 256000) is a devastating childhood, progressive, neurodegenerative disease characterized by bilateral, symmetric brain lesions. Patients typically present in infancy with hypotonia, ataxia, psychomotor delay and regression. The estimated incidence is 1:40,000 and the disorder is often fatal (Finsterer, 2008). Atypical and later onset forms of the disease occur and are often described as Leigh-like disease. Non-specific biochemical findings include increased lactate in CSF and blood and increased Krebs cycle intermediates in urine, pointing to a deficit in energy metabolism. Deficiencies of the mitochondrial
respiratory chain complexes I, II and IV, caused by mutations in either nuclear or mitochondrial DNA encoding subunits or assembly factors for these complexes, are frequent causes of Leigh disease with complex I deficiency being the most common (Tucker et al., 2010) but many other causative genes have been identified, most of them individually rare (Finsterer, 2008). The relative uniformity of clinical features in Leigh disease, lack of specific biochemical markers and the many causative genes present a diagnostic challenge and non-targeted exome and genome sequencing approaches are being increasingly used. These methods have revealed additional genes in patients with Leigh disease with >35 genes thought to cause Leigh disease (Gerards et al., 2013).

Here we describe a new inborn error of metabolism affecting valine metabolism in two siblings with fatal Leigh disease. Reactive intermediates in the valine pathway produce a characteristic metabolite signature and are likely to be responsible for the pathology of this disorder.

## Materials and methods

### Patients

The female proband was born at term (41 weeks gestation) by normal vaginal delivery after an unremarkable pregnancy. Her parents were non-consanguineous and of Greek ancestry. Her birth weight was 3270 g (25th–50th centile), length was 53 cm (90th–97th centile) and head circumference was 33 cm (3rd–25th centile). Apgar scores were 7 and 8 at 1 and 5 min, respectively. At birth she had generalized hypotonia and poor suck. At 12 h of age, the baby developed apnoea post-feeding. Septic work-up was negative. The baby continued to have apnoeas with associated bradycardias and desaturations and was intubated and ventilated by Day 3 of life. CSF lactate was 4.9 mM (reference range <3.0), pyruvate was 0.32 mM (0.06–0.13) with a normal CSF lactate to pyruvate ratio. Serial serum lactate levels were also increased, ranging from 2.8 to 4.7 mM (0.5–2.0). The activity of the pyruvate dehydrogenase complex was also decreased in cultured skin fibroblasts [0.15 nmol/(min·mg protein); controls 0.23–0.53]. He died at home at the age of 4 months due to respiratory failure after care was withdrawn.

The second child was male and born at term (39 + 6 weeks gestation) by normal vaginal delivery after an unremarkable pregnancy. His birth weight was 3300 g (50th–90th centile), length was 52 cm (90th centile) and head circumference was 34.5 cm (50th centile). His Apgar scores were 2 and 6 at 1 and 5 min, respectively. He required suction and bag mask oxygen. This baby was also profoundly hypotonic and had poor feeding and desaturations; however, he did not require respiratory support. At 3 months of age, he had severe global developmental delay and abnormal neurological examination with severe generalized hypotonia and vertical nystagmus. In addition he also had a ventricular septal defect and severe progressive hypertrophic obstructive cardiomyopathy. MRI and spectroscopy at 7 weeks of age showed reduced myelination of the globus pallidus and putamina bilaterally with elevated lactate. Repeat MRI and spectroscopy at 4 months showed persistent elevated lactate, new diffusion restriction in the globus pallidus, anterior thalami, midbrain and hippocampi and interval parenchymal loss. Activity of the pyruvate dehydrogenase complex was also decreased in cultured skin fibroblasts [0.04 nmol/(min·mg protein); controls 0.23–0.53]. He died at home at the age of 8 months as a result of cardiorespiratory failure. In both siblings, dried blood spot acyl carnitine profiles and liver function tests were unremarkable.

### Biochemical and molecular analyses

Semi-quantitative urine screening for S-(2-carboxypropyl)cysteine was performed by flow injection tandem mass spectrometry of butyl derivatives as previously described (Pitt et al., 2002) with the addition of a 320.2 > 119 m/z transition. Quantitation of urine cysteine, cysteamine and glycine conjugates and the separation of 3-hydroxybutyryl-carnitine and 3-hydroxyisobutyryl carnitine is described in the Supplementary material. Urine 2-methyl-2,3-dihydroxybutyric was measured by gas chromatography-mass spectrometry. The structures of the metabolites are shown in Supplementary Fig. 2.

Measurement of short chain enoyl CoA hydratase (ECHS1) enzyme activity, immunoblotting of ECHS1 protein, ECHS1 and HIBCH sequencing, ECHS1 cDNA analysis and quantitative PCR of ECHS1 mRNA are described in the Supplementary material.

### Functional expression of wild-type and mutant ECHS1 in patient fibroblasts

We amplified the coding region of wild-type ECHS1 by PCR from reverse-transcribed RNA isolated from control fibroblasts and cloned this into the HindIII and BamHI sites of the eukaryotic expression vector pcDNA3 (Invitrogen). The entire fragment was sequenced to exclude PCR-induced errors. The mutations identified in the patients were subsequently introduced using the QuikChange® site directed mutagenesis kit of Agilent Technologies and primers 5'-CTGGGAC CACCTACACCGTGGGCGGCTGAGC-3' and 5'-GCTCACA GCCCCCCGCAAACTGGTGGTGGTCCACG-3' for mutation p.A158D and 5'-CTATGCCCCAGAAAGGACCAGTTTGCAGAC-3' and 5'-GCTGTCGAACATGCTTCTTCTTACCACGCCATAG-3' for mutation p.126_138del (i.e. the consequence of c.414+3G>C). The correct introduction of the mutations was verified by sequencing.

For functional expression, we transfected fibroblasts from Sibling 1 with the different pcDNA3 vectors containing either the ECHS1 wild-type, the p.A158D or the p.126_138del mutants using the AMAXA NHDF Nucleofector® kit and the Nucleofector® technology (Lonza).
Table 1 Biochemical findings in ECHS1 deficiency

<table>
<thead>
<tr>
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<th>Sibling 1</th>
<th>Sibling 2</th>
<th>Controls</th>
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<tr>
<td><strong>Enzyme activities</strong></td>
<td></td>
<td></td>
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<tr>
<td>3-hydroxyisobutyryl-CoA hydrolase</td>
<td>Not done</td>
<td>10.3</td>
<td>7.9 ± 1.3 (11)</td>
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<tr>
<td>Short-chain enoyl-CoA hydrolase</td>
<td>&lt;9</td>
<td>&lt;9</td>
<td>379 ± 147 (12)</td>
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<td><strong>Urine metabolite levels</strong></td>
<td></td>
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<tr>
<td>Methacrylate metabolites (valine pathway)</td>
<td></td>
<td></td>
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<tr>
<td>S-(2-carboxypropyl)cysteine</td>
<td>33, 36</td>
<td>19–26 (3)</td>
<td>&lt;5.3 (19)</td>
</tr>
<tr>
<td>S-(2-carboxypropyl)cysteamine</td>
<td>27, 28</td>
<td>14–30 (3)</td>
<td>&lt;10 (19)</td>
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<td>Acrylate metabolites (valine pathway)</td>
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<tr>
<td>S-(2-carboxyethyl)cysteine</td>
<td>5.4, 3.6</td>
<td>1.6–2.0 (3)</td>
<td>&lt;2.2 (19)</td>
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<tr>
<td>S-(2-carboxyethyl)cysteamine</td>
<td>5.8, 6.2</td>
<td>5.0–6.8 (3)</td>
<td>&lt;1.2 (19)</td>
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<td>Tiglate metabolites (isoleucine pathway)</td>
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<tr>
<td>Tiglyl glycine</td>
<td>2.7, 2.6</td>
<td>0.9–4.5 (3)</td>
<td>&lt;7.8 (19)</td>
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<tr>
<td>Crotonate metabolites (fatty acid pathway)</td>
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<tr>
<td>Crotonyl glycine</td>
<td>1.6, 1.4</td>
<td>0.9–1.4 (3)</td>
<td>&lt;4.7 (19)</td>
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<td><strong>Other metabolites</strong></td>
<td></td>
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<tr>
<td>2-methyl-2,3-dihydroxybutyric</td>
<td>1560–2500 (4)</td>
<td>782, 972</td>
<td>&lt;25 (48)</td>
</tr>
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Enzyme activities in nmol/(min.mg protein), urine levels in μmol/mmol of creatinine.

a Mean ± SD.
b Number of measurements in brackets.

Cells were harvested 2 days after transfection and ECHS1 activity was measured in duplicate in cell homogenates.

**Molecular results**

No damaging mutations were identified in the exons and flanking regions of the HIBCH gene of the first sibling. Sequence analysis of the ECHS1 gene showed that both siblings were compound heterozygous for a c.473C>A (p.A158D) missense mutation in exon 4 and a c.414+3G>C mutation affecting the third base of intron 3 (Supplementary Fig. 3). Testing of the parents showed these mutations were maternally and paternally inherited, respectively and both mutations were absent from variant databases (dbSNP and NHLBI Exome Variant Server). The p.A158D mutation affects a highly conserved alanine residue at the entrance of the substrate binding pocket of the enzyme and is predicted to be damaging (PolyPhen-2).

ECHS1 cDNA was analysed to determine the effect of the c.414+3G>C splicing mutation (Fig. 2C). This resulted in the amplification of three products in both siblings, whereas only one band was present in control samples (Fig. 2C). The abnormal, smaller product was found to be missing 39 bp from the end of exon 3, whereas exon 4 was intact. This suggested the usage of a cryptic donor splice site in exon 3 as a result of the c.414+3G>C mutation (as predicted by Human Splicing Finder), resulting in the deletion of amino acids 126–138 from the ECHS1 protein. These amino acids make up part of the interface between the two trimeric units of the hexameric structure (Protein Data Bank ID: 2HW5).

The normal sized 193 bp ECHS1 cDNA band (Fig. 2C, lane 1) included both mutations and was sequenced to determine the extent of aberrant splicing caused by the c.414+3G>C mutation. This revealed that nucleotide position c.473 was predominantly the mutated c.473A allele with only a small amount of the wild-type c.473C allele (Supplementary Fig. 4), indicating that the c.414+3G>C mutation results in only a small amount of normally spliced product. Based on gel quantitation (SynGene Gene Tools)

**Results**

**Biochemical results**

Semiquantitative urine metabolite screening by tandem mass spectrometry showed increased levels of S-(2-carboxypropyl)cysteine, which was confirmed by quantitative analysis in both siblings. This analysis also revealed increases in several related cysteine and cysteamine conjugates. Urine organic acid screening by gas chromatography-mass spectrometry also showed increased levels of 2-methyl-2,3-dihydroxybutyrate. The urine metabolite levels are summarized in Table 1. The levels were normal in the parents (data not shown). These metabolites are features of 3-hydroxyisobutyryl-CoA hydrolase (HIBCH, Fig. 1) deficiency (MIM 250620), a rare defect of the valine catabolic pathway also associated with Leigh-like disease (Brown et al., 1982; Loupatty et al., 2007; Peters et al., 2013). However, sequencing of HIBCH did not reveal any pathogenic mutations and HIBCH enzyme activity (Loupatty et al., 2007) was normal in the second sibling (Table 1).

Further metabolite analysis showed that, in contrast to HIBCH deficiency, the siblings had normal urine excretion of 3-hydroxyisobutyryl-carnitine (Fig. 2A), indicating a possible deficiency of short-chain enoyl-CoA hydratase (ECHS1, also known as crotonase, ECHS1 gene product, Fig. 1). ECHS1 activity was therefore measured in cultured skin fibroblasts and was below the limit of detection of the enzyme assay in both siblings [<9 nmol/(min.mg protein), controls 379 ± 147] (Table 1). The normal 27 kD ECHS1 band was completely absent in both siblings by immunoblot analysis (Fig. 2B).
of the three bands produced from the exon 3-exon 4 cDNA PCR, we estimated that ~46% is wild-type, 34% has a deletion and 20% is a heteroduplex (both wild-type and deletion carrying sequences in equal amounts, based on sequencing) resulting in ~56% wild-type and 44% incorrectly spliced mRNA overall. Thus, aberrant splicing of ECHS1 caused by the c.414 + 3G>C mutation is estimated to occur at least 80% of the time. Quantitative PCR of ECHS1 mRNA also showed that the siblings had 60% ± 26% (average ± average deviation, nine determinations) the levels of ECHS1 mRNA relative to controls (n = 2).

ECHS1 enzyme activity in the fibroblasts of Sibling 1 was rescued after transfection with wild-type ECHS1 [ECHS1 activity of 254 nmol/(min.mg protein) compared to 13 nmol/(min.mg protein) in mock transfected cells]. Transfection of fibroblasts of Sibling 1 with the two identified mutant forms of ECHS1 (mutations p.A158D and p.126-138del) did not result in an increase of ECHS1 activity [15 and 13 nmol/(min.mg protein), respectively].

**Figure 1** Valine catabolic pathway showing the formation of metabolites in short-chain enoyl-CoA hydratase deficiency. Enzymes are numbered: 1 = 3-hydroxyisobutyryl CoA hydrolase; 2 = propionyl-CoA carboxylase; 3 = (R)-methylmalonyl-CoA mutase; 4 = short-chain enoyl-CoA hydratase (ECHS1, crotonase, ECHS1 gene); 5 = isobutyryl-CoA dehydrogenase.

**Discussion**

ECHS1 is a mitochondrial enzyme that converts unsaturated trans-2-enoyl-CoA species to the corresponding 3(S)-hydroxyacyl-CoA species through addition of a water molecule to the double bond (Fong et al., 1977). It shows rather wide substrate specificity, but has greatest activity towards crotonyl-CoA. It is a key component of the β-oxidation spiral of short- and medium-chain fatty acid oxidation. In addition, it is active in the isoleucine and valine catabolic pathways. In the latter, it converts methacryyl-CoA to (S)-3-hydroxyisobutyryl-CoA and acryloyl-CoA to 3-hydroxypropionyl-CoA (Shimomura et al., 1994; Wanders et al., 2012). The latter reaction is also relevant to a secondary pathway of propionyl-CoA metabolism that feeds into the valine pathway (Fig. 1). Methacryyl-CoA and acryloyl-CoA are highly reactive intermediates that can react spontaneously with a variety of molecules including those with sulphhydryl groups such as cysteine and cysteamine (Brown et al., 1982). Methacryyl-CoA and acryloyl-CoA are therefore toxic to cells and can potentially disrupt many biochemical reactions and protein structures.

In HIBCH deficiency, the accumulation of methacryyl-CoA results in the excretion of an unusual amino acid S-(2-carboxypropyl)cysteine (Truscott et al., 1981). We added S-(2-carboxypropyl)cysteine to a panel of metabolites measured as a high-throughput urine screen for inborn errors of metabolism in order to detect HIBCH deficiency. The increased urine S-(2-carboxypropyl)cysteine in our two siblings with Leigh disease prompted more detailed analyses, showing that the related metabolites S-(2-carboxypropyl)cysteamine, S-(2-carboxyethyl)cysteine and S-(2-carboxyethyl)cysteamine were increased (Table 1). Urine 2-methyl-2,3-dihydroxybutyrate was also increased. The metabolic origin of this last metabolite has not been clearly established but there is evidence that it is derived from acryloyl-CoA (Peters et al., 2013). The above metabolite abnormalities occur in HIBCH deficiency which is associated with Leigh disease, but this diagnosis was excluded by sequencing of the HIBCH gene and normal HIBCH enzyme activity (Table 1). In contrast to HIBCH deficiency, the two siblings had normal excretion of 3-hydroxyisobutyryl-carnitine, derived from 3-hydroxyisobutyryl-CoA (Fig. 2A) and this suggested a defect of ECHS1, the enzyme immediately upstream of HIBCH in the valine pathway (Fig. 1). This was confirmed by the findings of undetectable ECHS1 enzyme activity (Table 1), absence of ECHS1 protein in fibroblasts (Fig. 2B), mutations predicted to be damaging in the ECHS1 gene and rescue of ECHS1 activity when Sibling 1’s cells were transfected with wild-type ECHS1. The damaging nature of the two mutations was further confirmed by demonstrating lack of rescue of ECHS1 activity when Sibling 1’s cells were transfected with mutated ECHS1.

There are many biochemical and clinical similarities between ECHS1 and HIBCH deficiencies. The metabolite patterns in both
Figure 2  Characterization of ECHS1 deficiency. (A) UPLC-MSMS analysis of urine hydroxy-C4 carnitines as butyl derivatives (304 > 85 m/z transition). Two peaks are formed as a result of derivatization. The black arrows indicate 3-hydroxybutyryl carnitine while the grey arrows indicate 3-hydroxyisobutyryl carnitine. (B) Immunoblot with antibodies against ECHS1 (indicated by arrowhead) with the lower panel showing loading controls with antibodies against β-actin. Lanes 1, 4: control fibroblasts; Lane 2: sibling 1; Lane 3: sibling 2. (C) Gel electrophoresis of ECHS1 cDNA in fibroblast samples after reverse transcriptase-PCR and PCR of exons 3–4 showing the effect of the c.414 +3G>C mutation. The 193 bp fragment shows the presence of normal sized ECHS1 cDNA. Lane 1: sibling 1. The abnormal lower band is the product of abnormal splicing while the abnormal upper band is a heteroduplex; Lane 2: control.
disorders reflect the accumulation of methacetyl-CoA and acryloyl-CoA and it is likely that the brain pathology in the two disorders is due to damage caused by these reactive intermediates. Deficiencies of respiratory chain enzymes and the pyruvate dehydrogenase complex in HIBCH deficiency (Loupatty et al., 2007; Ferdinandusse et al., 2013; Peters et al., 2013) also suggest secondary protein damage. Indeed, our patients also had decreased activity of the pyruvate dehydrogenase complex in cultured skin fibroblasts, increased lactate levels and normal lactate/pyruvate ratios indicating that secondary enzymatic deficiencies also occur in ECHS1 deficiency. However, they suffered a more severe clinical course and died within the first year of life, in contrast to HIBCH deficiency with four of the five reported patients surviving beyond 1 year of age (Loupatty et al., 2007; Ferdinandusse et al., 2013; Peters et al., 2013).

The metabolite abnormalities in the two siblings appeared to be confined to the valine pathway. Increased excretion of crotonylglycine and tiglylglycerine are predicted consequences of ECHS1 deficiency in the fatty acid and isoleucine pathways, respectively. However, both these metabolites were normal (Table 1). This apparent discrepancy may be due to low flux through these pathways at the times patients were sampled but further research into the role of ECHS1 in these pathways is required.

Our findings demonstrate that ECHS1 mutations result in ECHS1 deficiency and are another cause of Leigh disease. There is considerable overlap in the clinical and biochemical features of ECHS1 and HIBCH deficiencies. However, unlike most other genetic disorders causing Leigh disease, there is a specific pattern of urine metabolites that should facilitate the recognition of both ECHS1 and HIBCH deficiencies in future.

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Web Resources

NHLBI Exome Variant Server: http://evs.gs.washington.edu/EVS/
PolyPhen-2: http://genetics.bwh.harvard.edu/pph2/index.shtml
Protein Data Bank: http://www.rcsb.org/pdb/home/home.do
Exon primer design: http://ihg.helmholtz-muenchen.de/cgi-bin/primer/ExonPrimerUCSC.pl?db=hg19&acc=uc0011mu3

Supplementary material

Supplementary material is available at Brain online.

References