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ECHS1 disease in two unrelated families of Samoan descent: Common variant - rare disorder

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Abstract

Mutations in the short-chain enoyl-CoA hydratase (SCEH) gene, *ECHS1*, cause a rare autosomal recessive disorder of valine catabolism. Patients usually present with developmental delay, regression, dystonia, feeding difficulties, and abnormal MRI with bilateral basal ganglia involvement. We present clinical, biochemical, molecular, and functional data for four affected patients from two unrelated families of Samoan descent with identical novel compound heterozygous mutations. Family 1 has three

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affected boys while Family 2 has an affected daughter, all with clinical and MRI findings of Leigh syndrome and intermittent episodes of acidosis and ketosis. WES identified a single heterozygous variant in *ECHS1* at position c.832G > A (p.Ala278Thr). However, western blot revealed significantly reduced ECHS1 protein for all affected family members. Decreased SCEH activity in fibroblasts and a mild increase in marker metabolites in urine further supported *ECHS1* as the underlying gene defect. Additional investigations at the DNA (aCGH, WGS) and RNA (qPCR, RT-PCR, RNA-Seq, RNA-Array) level identified a silent, common variant at position c.489G > A (p.Pro163=) as the second mutation. This substitution, present at high frequency in the Samoan population, is associated with decreased levels of normally spliced mRNA. To our understanding, this is the first report of a novel, hypomorphic allele c.489G > A (p.Pro163=), associated with SCEH deficiency.

KEYWORDS

ECHS1, Leigh syndrome, mitochondrial disease, Samoan population, silent variant

1 | INTRODUCTION

ECHS1 codes for the short-chain enoyl CoA hydratase (SCEH, also called crotonase), which plays a role both in branched chain amino acid catabolism and in fatty acid β-oxidation. The enzyme is composed of 290 amino acids and localizes to the mitochondrial matrix where its only nonredundant function is in valine degradation (Ferdinandusse et al., 2015; Yamada et al., 2015). Insufficient conversion of methacrylyl-CoA into 3-OH-isobutyryl-CoA and of acryloyl-CoA into 3-OH-propionyl-CoA, leads to accumulation of these highly reactive and toxic precursors. This then results in the inhibition of mitochondrial electron transport chain (ETC) as well as pyruvate dehydrogenase complex (PDHC) activities, leading to decreased ATP generation (Peters et al., 2014). Thus, deficiencies in SCEH can mimic metabolic defects in energy production with a clinical picture resembling PDHC deficiency or mitochondrial disease (Bedoyan et al., 2017; Ferdinandusse et al., 2015; Peters et al., 2014). Inheritance of *ECHS1* deficiency is autosomal recessive and onset typically occurs during infancy or childhood (Al Mutairi, Shamseldin, Alfadhel, Rodenburg, & Alkuraya, 2017). Most patients present with developmental delay, regression, dystonia, feeding difficulties, cardiomyopathy, and brain MRI changes consistent with Leigh syndrome. Since Sharpe et al., reviewed the mutational, clinical, and biochemical spectrum of 42 patients from 33 families with *ECHS1* disease, an additional nine new cases have been reported, none of which are due to synonymous mutations (Aretini et al., 2018; Carlston, Ferdinandusse, Hobert, Mao, & Longo, 2019; Pajares et al., 2020; Ronchi et al., 2020; Sharpe & McKenzie, 2018; Uchino et al., 2019; Wu et al., 2020; Yang & Yu, 2020). Here we report clinical, biochemical, molecular, and functional data on four patients from two unrelated families, presenting with two novel mutations in *ECHS1*. The missense mutation c.832G > A (p.Ala278Thr) decreases ECHS1 protein stability while the silent c.489G > A (p.Pro163=) mutation is associated with decreased levels of normally spliced mRNA.

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

All patients were followed at the Division of Metabolic Disorders, CHOC Children's hospital. Written consent was obtained from all study participants and/or their legal guardians via CHOC approved IRB# 130990.

2.2 | Biochemical testing

In Family 1, acryloyl and methacrylyl metabolites were measured in urine blotters via LC/MS–MS at the Victorian Clinical Genetics Services, Murdoch Childrens Research Institute, Australia, as published (Peters et al., 2015). In Family 2, measurements were performed in urine at Laboratory Genetic Metabolic Diseases, Amsterdam UMC, Academic Medical Center, and The Netherlands with similar methodology.

2.3 | Whole exome sequencing

Whole exome sequencing was performed on a clinical basis by Baylor Genetics (Family 1, Patient 2), GeneDx (Family 1, Patients 1, 3 and parents), and Ambry Genetics (Family 2, Trio) and on a research basis by Omega Bioservices for the unaffected brother of Family 1. All data was obtained under IRB# 130990 and reanalyzed at CHOC using

CLC-Bio Genomics Workbench and variants were filtered and annotated using Annovar as published (Wang, Li, & Hakonarson, 2010).

2.4 | Whole genome sequencing

Whole genome sequencing was performed at Rady Children's Institute for Genomic Medicine on DNA extracted from whole blood for Family 1, (Patient 1, 3, the unaffected brother and both parents) via published method (Kingsmore et al., 2019). Alignments against Hg19 were exported as BAM files and haplotypes were established using Integrative Genomics Viewer (IGV) tools as published (Thorvaldsdóttir, Robinson, & Mesirov, 2013).

2.5 | Cell culture

Primary human skin fibroblasts were derived from punch biopsies, 3 mm in size. The tissue was grown on fibronectin (10 μg/ml of PBS) (LifeTechnologies) coated plates in alpha MEM with 1× nonessential amino acids (NEAA) (LifeTechnologies), Primocin (Invivogen) and 15% FBS (Hyclone-Fetal Clone 3). The growing cells were maintained in DMEM-5 mM Glucose, 1× NEAA, 1× Pyruvate, 1× Antibiotic/Antimycotic (all LifeTechnologies) and 10% FBS (Hyclone-Fetal Clone 3) at 37°C in 5% CO_2. Cell pellets were made with 4–5 × 10^6 cells and stored at −80°C until extractions.

2.6 | Nonsense mediated decay experiment

Cells were cultured as described above. Emetine (Sigma) was diluted to 100 mg/ml in H_2O and used as a 1,000× stock at a final concentration of 100 μg/ml in sterile filtered growth medium. Exposure time prior to harvest for RNA generation was 5 hr.

2.7 | Immunoblotting

Protein was extracted from fibroblast cell pellets by incubation with RIPA buffer (Pierce) and HALT protease/phosphatase inhibitor cocktail (Thermo-Fisher) for 30 min at 4°C and then centrifuged at 16,000 rpm for 20 min at 4°C. DCA Assay (Biorad) was performed in triplicate to determine concentration of lysates. Lysates were stored at −80°C in NuPage LDS buffer (LifeTechnologies). Sample Reducing Agent (LifeTechnologies) was added to the lysates and antioxidant was added to the running buffer per the manufacturer recommendations. SDS-PAGE was run on 1.5 mm NuPage 4–12% Bis-Tris Gels with 1× NuPage MES Running Buffer (LifeTechnologies) for 1 hr at 200 V. The PVFD membrane was incubated with methanol and transfer buffer. The protein was transferred to the PVFD membrane using Trans-Blot Turbo RTA Blotting System run at the Mixed MW protocol: 1.3 A, 25 V for 7 min (Biorad). The membrane was washed with fast wash buffer (FWB) for 10 min and the incubated in Fast Blocking Buffer (FBB) (Pierce) for 30 min at RT. The membrane was incubated with ECHS1 antibody 1:10,000 (Abcam, ab174312) and beta-Actin antibody 1:5,000 (Abcam, 119716) overnight at 4°C. The next day, the membrane was washed with FWB 3 × 5 min at RT and then incubated with the Goat Anti-Rabbit IgG H&L (HRP) secondary antibody 1:20,000 (Abcam, ab6721). The protein was detected using SuperSignal Chemi-luminescent substrate (LifeTechnologies) and imaged on CL-Xposure film (LifeTechnologies). The results were analyzed using ImageJ and Microsoft Excel.

2.8 | RNA extraction

RNA was extracted from fibroblast cell pellets using PureLink RNA Mini Kit (Ambion) including on column DNAse treatment per manufacturer recommendations. Quality control of RNA samples was performed using a Bioanalyzer system (Agilent).

2.9 | Reverse transcriptase PCR

Reverse transcriptase PCR (RT-PCR) was performed using 300 ng of RNA and the SuperScript III one step RT-PCR kit with platinum Taq. We used a forward primer overlapping the exon 2–3 junction (5′-GCAGCTGGAGCTGATATCAAG-3′) and a reverse primer in exon 6 (5′-TTTTCTGCACACTGGATGGC-3′) to assess splicing efficiency for exon 4. To assess splicing for intron 6, a forward primer in exon 6 (5′-GTAGTAGCGATGGCCAAAGAA-3′) and a reverse primer overlapping the exon 7–8 junction (5′-CATCAGTGGCAAAGGTTGAATAA-3′) was used. PCR was run with an annealing temperature of 55°C and PCR products were separated on a 2% Agarose gel via electrophoresis for 1 hr at 140 V. Band intensity was quantified using ImageJ.

2.10 | Quantification of *ECHS1* expression

ECHS1 RNA expression levels were measured using a CFX Connect Real-Time PCR Detection System (Biorad). Two microgram of RNA extracted from fibroblast pellets were reverse transcribed using Superscript VILO cDNA synthesis kit (Invitrogen) according to manufacturer's recommendation. Quantitative PCR (qPCR) was performed via TaqMan gene expression assay (Applied Biosystems) using TaqMan Fast Advanced Master Mix and *ECHS1* assay Hs00929130_m1 spanning the exon 5–6 boundary. *GAPDH* assay Hs02786624_g1 and beta actin (*ACTB*) assay Hs99999903_m1 were used as the reference genes. Samples were run in triplicates and expression including SEM was generated via the ΔΔCt method using Microsoft Excel.

2.11 | RNA-Seq

Stranded libraries were constructed using 1 μg of total RNA as the input for the Illumina TruSeq total RNA kit in conjunction with the gold ribo-zero rRNA depletion kit. Quality control was performed via Agilent

Bioanalyzer. Hundred cycle paired end sequencing was performed on a HiSeq 4000 by the UCI High Throughput Facility. Trimmed reads were aligned to GRCh38 using CLC Genomics Workbench. Alignments were exported as BAM files and Sashimi plots were established using IGV.

2.12 | Transcriptome array analysis

RNA was submitted to the UCI High Throughput Facility for ClariomD transcriptome array assay (Applied Biosystems). CEL files were analyzed with TAC-Transcriptome Analysis Console 4.0 (Applied Biosystems). Summarization Method: Gene + Exon – SST-RMA was chosen with a Pos versus Neg AUC Threshold: 0.7 and analysis of variance (ANOVA) Method was ebayes. A Probeset (Gene/Exon) was considered expressed if ≥50% samples had DABG values below a DABG threshold of <0.05. Filtering criteria included a gene-level fold change of < –1.5 and p levels of $p < .05$ with a gene list filter focused on Mitocarta 2.0 genes (Calvo, Clauser, & Mootha, 2016).

2.13 | SCEH enzyme activity

SCEH Activity was measured in fibroblasts for Family 1 and for Patient 4 from Family 2, as published (Ferdinandusse et al., 2015).

3 | RESULTS

3.1 | Family 1

3.1.1 | Clinical findings

A detailed clinical description of Family 1 that includes the response to a valine restricted diet has been recently reported (Abdenur et al., 2020). Briefly, Family 1 consists of 4 boys with non-consanguineous parents. The mother is of Samoan descent and the father is of European derived (Portuguese) ethnicity. Of the four children, three are affected (Patient 1–3) and one of the affected (Patient 2) has passed away (Figure 1). Starting in infancy, the patients presented with developmental delay, regression, hypotonia in the trunk with rigidity and dystonic posturing in extremities. They all developed nystagmus and demonstrated changes in brain MRI (bilateral, symmetrical basal ganglia hyperintensities in T2) consistent with Leigh syndrome. Intermittent episodes of metabolic acidosis and ketosis with normal acylcarnitines were documented during intercurrent illnesses. The oldest brother (Patient 1) had a less severe clinical course. By 13 years of age he had developed spasticity and mild joint contractures and was only able to walk with assistance. He had dysarthria, was able to comprehend, read, and write at a second-grade level, and was still able to eat by mouth. Patient 2 had a more severe phenotype. He died at age 9 years after an intercurrent illness, complicated by respiratory insufficiency. By that time, he had little interaction, no head control, was unable to sit unsupported and had developed dysphagia necessitating gastrostomy tube feedings. The youngest brother (Patient 3) had a severe presentation, similar to Patient 2 and was alive at 6 years. None of the patients were on a valine restricted diet during the observation period of this study.

3.1.2 | Molecular and biochemical findings

Patient 2 had chromosomal microarray, whole exome sequencing (WES) as well as mitochondrial DNA (mtDNA) sequencing in 2012 and reanalysis of WES in 2013, which were not diagnostic. The patient also had an unremarkable MitoMet®Plus aCGH analysis (all done by Baylor Genetics Laboratory) with targeted probes against mtDNA and an additional 1,600 nuclear genes involved in mitochondrial biogenesis. This excluded deletions of larger than 1,000 base

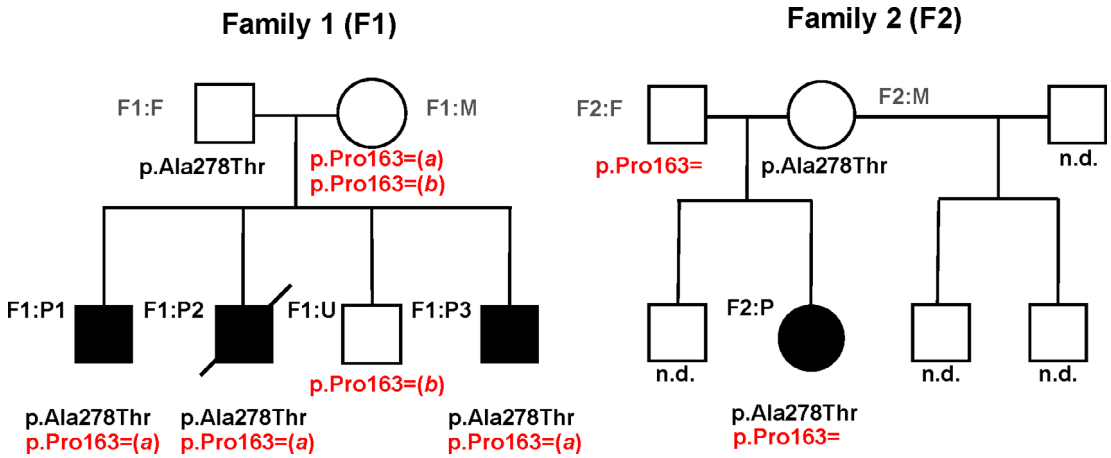

FIGURE 1 Pedigree of Family 1 (F1) and Family 2 (F2) with mutations in *ECHS1*. Haplotype is indicated below each tested individual. The silent exon 4 mutation is highlighted by red font. Black shading indicates affected status. P indicates patient, M indicates mother and F indicates father, U indicates unaffected, n.d. indicates studies were not done, (a) and (b) indicates phase of the maternal *ECHS1* allele in Family 1

pairs in the covered genes. Reanalysis of WES data in our laboratory revealed a novel, paternally inherited *ECHS1* variant in exon 8 at position c.832G > A (p.Ala278Thr) (rs933592081; NM_004092) in all affected children. It is present in the heterozygous state in the gnomAD database (accessed June 3, 2020) at a very low frequency (3/282,830 alleles), with no homozygotes reported (Karczewski et al., 2020). Its allele frequency is 6.5×10^{-5} in the South Asian population and 7.7×10^{-7} in the European non-Finnish population. The variant is predicted to be damaging in 10 out of 12 in silico pathogenicity prediction tools (SIFT, Polyphen2_HDIV, Polyphen2_HVAR, LRT, Mutation Taster, M_CAP, FATHMM_MKL, Mutation Assessor, Provean, Meta_SVM) (Wang et al., 2010). No other *ECHS1* exonic potential protein-altering (missense, nonsense, indel) or splice junction variants were observed in the family. Due to the compelling phenotype overlapping with reported *ECHS1* disease patients, SCEH activity was measured in fibroblasts of Patient 2 and shown to be significantly decreased at 14% of controls (14% of the mean SCEH activity in six control cell lines) (Figure 2a), consistent with the suspicion of *ECHS1* deficiency. Subsequently, SCEH activity was also measured in fibroblasts for all family members revealing even more reduced SCEH activity in the other 2 affected brothers (6% of controls in Patient 1 and 5% of controls in Patient 3), confirming the enzymatic diagnosis for this family. A reduction was also seen, but to a lesser extent, in samples from the parents as well as the unaffected brother (Figure 2a). Diagnostic markers for *ECHS1* disease in urine blotters revealed elevated S-(2-carboxypropyl)cysteamine, S-(2-carboxyethyl) cysteamine, N-acetyl-S-(2-carboxypropyl)cysteine and 2,3-dihydroxy-2-methylbutyric acid in the severely affected Patient 3 while the more mildly affected Patient 1 showed only mild elevations in N-acetyl-S-(2-carboxypropyl)cysteine (Table 1). A sample from the index patient was not available, while both parents and the unaffected brother had normal metabolite levels in urine blotters.

Immunoblot analysis with antibodies against ECHS1 demonstrated reduced protein levels for all samples from affected family members, which were as low as 21% of control. The sample from the father, who is a carrier for c.832G > A (p.Ala278Thr) had protein levels reduced to 51% of control (Figure 2b) while the mother who was not reported to be a carrier of any nonsense variants in *ECHS1* had a ECHS1 protein level at 41% of control, indicating that she is likely a carrier of another, not previously reported variant in *ECHS1*. Samples from their unaffected son, who is not a carrier of the father's mutation, were reduced by 20%. Since we had previously excluded a maternal deletion or rearrangement in *ECHS1* via array CGH, these results indicated that the mother carried a variant either in a deep intronic or regulatory region of the gene. To identify this second variant, we started our investigations by amplifying the entire *ECHS1* cDNA from Patient 2 via RT-PCR using a forward primer in the 5′UTR and a reverse primer in the 3′UTR. Only one single band, representing the full-length *ECHS1* transcript (873 bp), was observed. We also performed RNA-Seq on all members of Family 1. Sashimi plots generated via Integrative Genomics Viewer (IGV) supported the RT-PCR findings and did not detect any novel splice junctions (Figure S1) (Thorvaldsdóttir

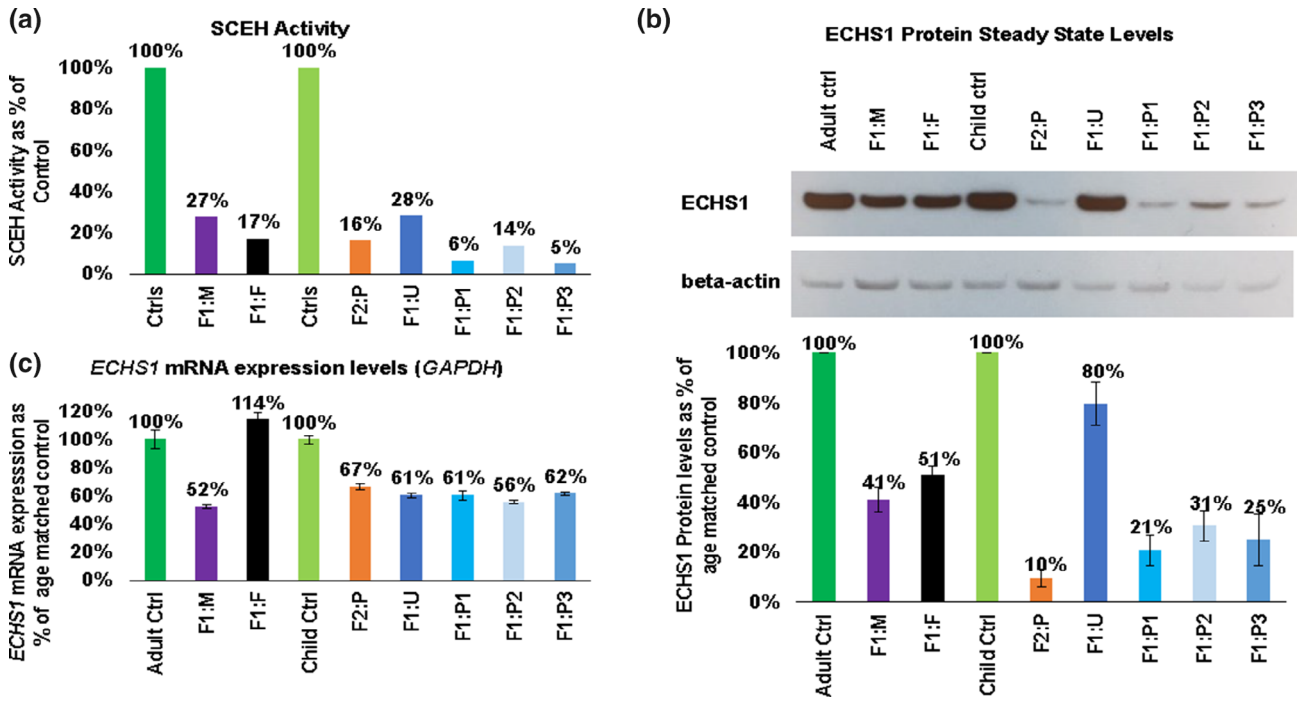

FIGURE 2 (a) SCEH enzyme studies on patient fibroblasts. SCEH activity is expressed as percentage of the mean activity in six control cell lines whereas results represent the mean of two independent experiments (b) SDS-PAGE and ImageJ quantification (bottom) of ECHS1 steady state protein levels of patient and control fibroblast lysates. Values are displayed as percentage of age-matched control and STD (n of Family 1 = 3 and *n* of Family 2 = 2). (c) Quantitative PCR of RNA extracted from patient and control fibroblast using TaqMan assay in triplicate. Values were calculated as ΔΔCt and SEM

TABLE 1 Metabolic markers characteristic of *ECHS1* disease in patient urine and/or urine blotters

Individual	S-2(2carboxypropyl) cysteamine	2,3-dihydroxy-2-methylbutyric	S-(2-carboxyethyl) cysteamine		N-acetyl-S-(2-carboxypropyl) cysteine		S-(2-carboxyethyl) cysteine-carnitine	S-(2-carboxypropyl) cysteine-carnitine
	Urine blotters	Urine blotters	Urine blotters	Urine	Urine blotters	Urine	Urine	Urine
F1:F	0.7	2.0	0.2	–	0.9	–	–	–
F1:M	0.9	4.0	0.4	–	0.5	–	–	–
F1:P1	1.0	16.0	0.5	–	**4.2**	–	–	–
F1:U	2.8	4.0	0.2	–	1.2	–	–	–
F1:P3	**11.0**	**46.0**	**1.6**	–	**14.0**	–	–	–
F2:P	–	–	–	**0.38**	–	**4.95**	**0.41**	**5.75**
Normal reference	**<10.0**	**<25.0**	**<1.2**	**<0.2**	**<4.2**	**<3.0**	**<0.24**	**<2.3**

Note: Measurements were performed in two different centers. Analytes from urine blotters were measured by UPLC-MSMS except for 2,3-dihydroxy-2methylbutyric acid, measured by GC/MS. Metabolites in urine were measured by LC/MS–MS. All units are in μmol/mmol creatinine. Values above the normal reference range are in bold.

Abbreviations: F, father; F1, family 1; F2, family 2; M, mother; P, patient; U, unaffecte.

et al., 2013). Sanger sequencing analysis of the RT-PCR product revealed 1 heterozygous variant at position c.489G > A (p.Pro163=) (rs140410716) with a peak height < 50% for the A base in exon 4 (Figure S2), for which we found the patients' mother to be homozygous. The reported overall population frequency (gnomAD v2.1.1) is 0.001 for the variant and further specifies an increased frequency in East Asians at 0.01 which, includes 2 homozygotes (accessed December 2, 2019) (Karczewski et al., 2020).

To assess whether *ECHS1* mRNA levels were significantly reduced in this family we performed transcriptome array analysis using the human ClariomD assay. We performed comparative analysis of one array each from the three patients of Family 1, their unaffected brother, their mother as well as the patient of Family 2 (n = 6) with arrays from five unrelated healthy controls including a total of four biological replicates for the controls (n = 9). We filtered for all significantly down regulated genes (p < .05, fold change <−1.5) of the 1,158 mitochondrial protein compendium Mitocarta 2.0 genes (Calvo et al., 2016). We then prioritized the resulting list of 22 genes by their p values and found *ECHS1* as the fourth most statistically significantly downregulated gene at p = .0008 and a fold change of −1.78 (Table S1). We also used RNA-Array results to choose glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the most appropriate housekeeping gene for our confirmatory results via qPCR due to its stable expression.

Quantitative PCR was performed using a TaqMan assay spanning *ECHS1* exon 5–6 junction and *GAPDH* as an internal reference gene. Results showed *ECHS1* mRNA to be decreased in all affected boys, their unaffected brother as well as their mother. The mother had the lowest relative *ECHS1* expression at 52% of control followed by the deceased patient at 56% of control. The other two patients and their unaffected brother all had levels approximating 60% of control, while the father's relative *ECHS1* mRNA level was slightly increased at 114% of control (Figure 2c) indicating no effect of c.832G > A (p.Ala278Thr) on *ECHS1* RNA stability for his sample gene (Figure S2). Trends were similar when using *ACTB* as the internal reference gene (Figure S3).

To assess whether there was a correlation between c.489G > A (p.Pro163=) and the observed decrease in *ECHS1* mRNA we interrogated the effect of the variant on splicing via Human Splice Finder (HSF) 3.1 (Desmet et al., 2009). Three HSF algorithms predict c.489G > A (p.Pro163=) to result in weakening of an exonic splicing enhancer, therefore suggesting the inefficient splicing of exon 4. We also interrogated c.489G > A (p.Pro163=) with SpliceAI, which predicted partial skipping of exon 4 with a splice donor loss score of Δ 0.16 and a splice acceptor loss of Δ 0.17 (Jaganathan et al., 2019). RT-PCR for control and maternal fibroblast RNA using a forward primer overlapping the exon 2–3 junction and a reverse primer in exon 6 of *ECHS1* however was essentially normal and a decrease in intensity of the PCR product was not observed. Skipping of exon 4 would have shortened the RT-PCR amplicon by 100 bp resulting in a frameshift after exon 3 with a stop codon in exon 6 (Figure 3a). Since we did not observe this shortened band, we reasoned that the frameshift could have led to nonsense mediated decay with insufficient template to produce the mutant amplicon. To investigate this possibility we cultured the cells from Patient 2, his mother and two age matched controls with Emetine, a known nonsense mediated decay inhibitor (Boichard et al., 2008). RT-PCR now revealed a shortened band in RNA from maternal fibroblasts, but findings were not significantly different in patient versus control (Figure 3b). This finding is consistent with the fact that the mother is homozygous for c.489G > A (p.Pro163=) and abnormal splicing would affect transcripts from both alleles. Our findings therefore indicate that c.489G > A (p.Pro163=) reduces normally spliced *ECHS1* mRNA resulting in nonsense mediated decay.

To further exclude whether there may have been additional variants from the mother which contributed to the reduction in mRNA we performed whole genome sequencing on all family members

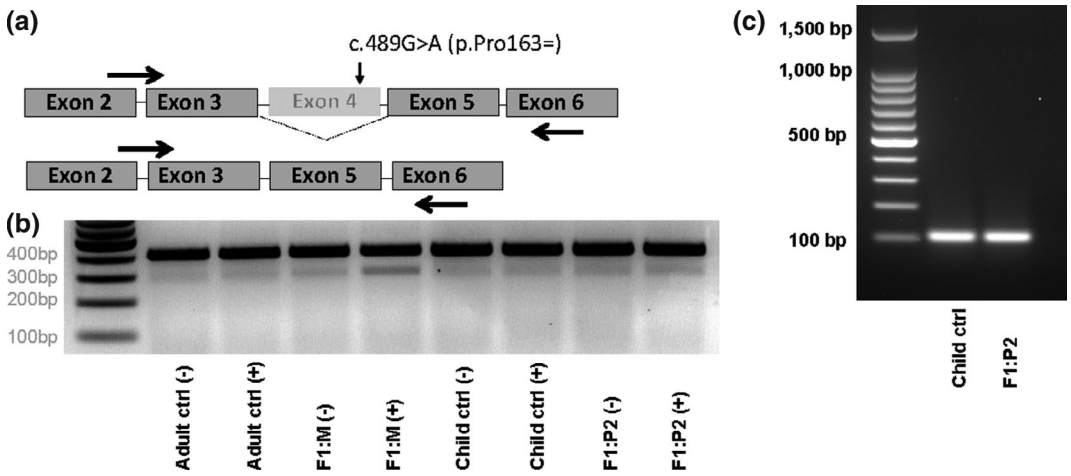

FIGURE 3 (a) Schematic of proposed impact of c.489G > A (p.Pro163=) on splicing. (b) Cells from adult and child control as well as the mother and Patient 2 of Family 1 were cultured with (+) and without (–) Emetine to halt nonsense mediated decay at 0.1 mg/ml for 5 hr prior to RNA extraction. RT-PCR was performed using a forward primer overlapping the exon 2/3 junction and a reverse primer in exon 6 (primers are denoted by arrows). PCR products were run on a 2% agarose gel. (c) RT-PCR to study the impact of the intron 6 variant on splicing using a forward primer in exon 6 and a reverse primer overlapping the exon 7–8 junction, a hypothetical band of 1,094 bp, representing abnormal splicing, was not observed

except for Patient 2. Data analysis of the WGS at Rady Children's Institute for Genomic Medicine did not produce any other potential gene candidate associated with Leigh syndrome for this family. WGS however revealed an additional rare maternally inherited *ECHS1* intron 6 variant at position c.740 – 277A > G (rs1008439511) (gnomAD allele frequency = 0.0001); 10 alleles in 6,822 Latinos, never in 1,516 South Asians, (accessed December 2, 2019), in Patients 1 and 3 and was not seen in the unaffected brother (Figure S4) (Karczewski et al., 2020). The intron 6 variant was later confirmed in Patient 2 of Family 1 and excluded in the patient of Family 2 via Sanger Sequencing. Since we had seen a reduction in SCEH enzyme activity, protein and mRNA levels in the unaffected brother, the intron 6 variant could at most have had a confounding effect on *ECHS1* mRNA levels. To assess the intron 6 variant's potential impact on splicing we interrogated HSF, which predicted the activation of an intronic cryptic donor site leading to the extension of exon 6. RT-PCR on fibroblast RNA from Patient 2 and control using forward primers in exon 6 and reverse primers overlapping the exon 7–8 junction however did not reveal any additional bands and a decrease in intensity of the PCR product was not observed in patient versus control (Figure 3c). We also interrogated via *ECHS1* WGS haplotype whether other noncoding variants segregated with the disease (Figure S4). Our results show that the only maternal heterozygous noncoding variant segregating with the disease is c.740 – 277A > G (rs1008439511) which is found neither in the unaffected brother who also has low *ECHS1* mRNA levels nor in the patient from Family 2.

To find additional evidence that the exon 4, c.489G > A (p.Pro163=) variant is indeed pathogenic we set out to find additional patients with this alteration.

3.2 | Family 2

3.2.1 | Clinical findings

The second family was ascertained by a database search for c.489G > A (p.Pro163=) (rs140410716) in patients with suspected mitochondrial disease and negative WES via Ambry Genetics. The database search resulted in a hit for an unrelated patient who curiously was also compound heterozygous for the same two variants as Family 1. In Family 2, c.489G > A (p.Pro163=) was inherited from the heterozygous father and the c.832G > A (p.Ala278Thr) mutation was inherited from the mother (Figure 1). The patient of Family 2 was found to not carry the intron 6 variant described for Family 1. We now had two completely unrelated families with the same primary variants of interest in *ECHS1*. The affected daughter of Family 2 was born without complications at 40 weeks of gestation after normal pregnancy via planned repeat cesarean section. Her birth weight was 3.17 kg. Parents are nonconsanguineous with the mother being of Chinese, Portuguese, Filipino, German, and American Indian descent and her father of Samoan descent. She has an 11 years old full brother as well as two older half-brothers. The patient developed normally until 8 months of age when she was admitted to the ED for fever and refusal to eat, leading to dehydration. She was treated with IV fluids and was discharged after 1 night. Few weeks thereafter, the patient was noted to have bilateral nystagmus and regression of motor skills. Ophthalmology examination revealed no retinal abnormalities and MRI of the brain, obtained at 10 months, showed hyperintense signal abnormalities on T2, T2-flair and diffusion weighted images in bilateral globus pallidus. A mitochondrial disorder was highly suspected. Lactic acid in blood was reportedly elevated, while amino acids, acylcarnitines,

carnitine, CK, urine organic acids, and Electron Transport Chain (ETC) activity in fibroblasts were normal. The patient had a "dual genome panel" testing via Baylor Genetics which did not reveal a potential gene candidate. This panel included full sequence of the mtDNA, as well as a genetic panel for 162 nuclear genes associated with mitochondrial disorders which did not include *ECHS1*.

The patient eventually regained the ability to crawl, was able to feed herself, and acquired some language. However, over time she continued to deteriorate, showing regression in cognitive, motor and language skills. At 2 years of age, a repeat brain MRI showed persistence of the basal ganglia abnormalities and interval development of bilateral periventricular and subcortical white matter hyperintensities. At 3 years, the patient had not experienced further regression but had plateaued in progress. She was nonverbal and non-ambulatory, had truncal hypotonia with hypertonia in extremities and dyskinetic and choreoathetoid movements. Levocarnitine and a "mitochondrial cocktail" were prescribed but they were stopped by the family when a new MRI showed further progression of the disease. At 4 years, WES was performed and was not diagnostic. The patient's condition remained overall unchanged, despite the families' attempts of unproven stem cells and hyperbaric chamber treatments.

Upon identification of the variants in *ECHS1* the patient was referred to CHOC Children's for further evaluation and management. She was 8 years old and her weight, height and HC were below the third percentile with a BMI at −1.21 *SD*. The patient was not dysmorphic, was aware of her surroundings but had limited interaction. She was able to track and follow with eyes but had intermittent nystagmus. Hearing appeared normal but she was only able to communicate with sounds and some word approximations. The patient was fed small pieces of food with care to avoid gagging or choking. A neurological exam revealed proximal hypotonia, no head control, and appendicular hypertonia with dystonic movements and brisk deep tendon reflexes.

3.2.2 | Molecular and biochemical findings

Patient fibroblast culture was established to investigate the pathogenicity of the variants and for comparison to Family 1. Enzyme activity measurements revealed a residual SCEH activity at 16% of controls (n = 6) (Figure 2a). N-Acetyl-S-(2-carboxypropyl) cysteine, a metabolite characteristic for *ECHS1* disease was mildly elevated at 4.95 μmol/mmol creatinine (< 3.0) in patient's urine (Table 1). Additionally, consistent with findings in other *ECHS1* patients S-(2-carboxyethyl) cysteamine, S-(2-carboxyethyl) cysteine-carnitine and S-(2-carboxypropyl) cysteine-carnitine were also mildly elevated. Urine organic acids revealed only trace amount of 2,3-dihydroxy-2methylbutyric acid in a sample obtained out of crisis and 3-methylglutaconate was not observed. Western blot analysis for ECHS1 protein on patient fibroblasts revealed significantly decreased steady state levels at 10% compared with an age-matched control (Figure 2b). Quantitative PCR revealed *ECHS1* mRNA levels at around 67% of an age matched control (Figure 2c). All findings were consistent with a diagnosis of *ECHS1* disease, which further supports the pathogenicity of c.489G > A (p.Pro163=) (rs140410716). Since the allele had been inherited in both families by a parent of Samoan ancestry we contacted the Obesity, Lifestyle and Genetic Adaptations (OLaGA) Study Group, a genetic epidemiology research group with access to a large collection of DNA samples from participants living in the Independent State of Samoa and the U.S. Territory of American Samoa, a sub-sample of which had been whole genome sequenced as part of the NIH/NHLBI Trans-Omics for Precision Medicine (TOPMed) consortium (https://www.nhlbiwgs.org/studies/samoan-adiposity-study). With permission of the Samoan Ministry of Health, the database was interrogated and showed an allele frequency of 0.17 (439 alleles in 1284 Samoan participants) with 34 homozygotes for c.489G > A (p.Pro163=) (rs140410716). Based on the observed allele frequency, we would expect 38 homozygotes in this population, therefore the difference between the expected and observed genotype counts is not statistically significant (p = .786) and does not suggest reduced fitness for homozygotes. We also interrogated the database for the c.832G > A (p.Ala278Thr) mutation which was not observed in the Samoan sample collection.

4 | DISCUSSION

ECHS1 is a mitochondrial matrix protein, which catalyzes the hydration of medium- and short-chain enoyl-CoAs in fatty acid oxidation as well as branched chain amino acid catabolism (Wanders, Duran, & Loupatty, 2012). Although Janssen et al., elucidated the gene architecture of *ECHS1* in 1996 it was not until 2014 when the first patients with *ECHS1* defects were reported (Janssen, Davis, Le Beau, & Stoffel, 1997; Peters et al., 2014). Despite the fact that the enzyme has several substrates, *ECHS1* mutations have only been shown to cause a buildup of byproducts of acryloyl- and methacrylyl-CoA, intermediate metabolites in valine catabolism. These byproducts, which can be detected in urine, have been suggested to inhibit ETC and PDHC activity, which may explain the clinical presentation with developmental delay, regression, dystonia, feeding abnormalities and brain MRI abnormalities consistent with Leigh syndrome (Ferdinandusse et al., 2015; Haack et al., 2015). Patients with *ECHS1*-related disease therefore mimic classical mitochondrial disease with a variable phenotype ranging from mild paroxysmal exercise intolerance to neonatal lethal Leigh syndrome (Bedoyan et al., 2017; Olgiati et al., 2016).

For the families reported here, *ECHS1* disease was difficult to diagnose, despite their classic mitochondrial phenotype and the use of WES as well as WGS performed by multiple laboratories. One reason was that the c.832G > A (p.Ala278Thr) mutation had not been mentioned in the various clinical reports even though it is rare and predicted to be pathogenic. Most autosomal recessive disease variant filtering algorithms require two putative pathogenic alleles in the same gene to make the list. The reporting of one single heterozygous allele in an autosomal recessive disorder is generally limited to known/expected disease-causing variant(s) which *ECHS1* was not at the time. The reporting of single variants for recessive disorders, which fit the phenotype or suspected cellular functions, however, can be extremely

helpful to the clinician. These variants provide important clues to prompt the physician to order additional biochemical or enzymatic analyses which may otherwise not have been clearly indicated. Our delineation of *ECHS1*-related disease was made possible via segregation analysis with three affected and one unaffected sibling, pointing to a shared paternal *ECHS1* mutation. The clinical course in the family was also highly concordant with published *ECHS1* cases including regression, developmental delay and dystonia (Fitzsimons et al., 2018).

The clinical and molecular findings presented a strong indication for assessment of SCEH activity on fibroblasts for Family 1. SCEH enzyme activity was decreased in fibroblasts (5–16% of controls), confirming the diagnosis and showing that the c.489G > A (p.Pro163=) mutation affects enzyme activity (Ferdinandusse et al., 2015; Peters et al., 2014). Metabolite levels from urine blotters for the two living affected brothers in Family 1 were modestly increased and appeared to correlate with the disease severity in concordance with prior reports (Haack et al., 2015). Of note, biochemical markers were measured in samples obtained out of crisis which, in conjunction with the hypomorphic second mutation, may explain why levels were only mildly increased. Although the biochemical phenotype and residual enzyme activity could predict a "mild disorder," our patients, with exception of Patient 1 in Family 1, had a severe phenotype. This is consistent with a previous report of a patient with a severe phenotype and mild increase in metabolite levels (Huffnagel et al., 2018).

The results of the enzymatic and biochemical assays then justified additional testing for the identification of a second variant on the RNA level. Although we were able to readily demonstrate that the maternal allele resulted in decreased *ECHS1* expression, the delineation of the second variant proved to be more difficult than expected, despite the availability of WGS data for the entire family. Ultimately, we show that a very unlikely candidate, which violates all currently accepted filtering criteria, contributed to the disease. The variant is common, silent, and homozygous in an unaffected parent. Although synonymous variants are rarely identified as disease causing there is now increasing evidence that the impact of silent mutations is vastly underappreciated. For example a recent study, investigating the mutational spectrum for galactosemia in Sweden, revealed two pathogenic silent variants (p.Pro36 = and p.Pro109=) in *GALT*, resulting in abnormal splicing leading to a decreased residual GALT activity and classic galactosemia phenotype (Ohlsson, Hunt, Wedell, & von Döbeln, 2019). A machine learning algorithm using a deep neural network process to more accurately predict splice junctions estimates that ~10% of disease causing mutations are due to cryptic splice sites including synonymous variants (Jaganathan et al., 2019). Our suspicion that the maternal silent c.489G > A (p.Pro163=) variant was the culprit had been supported by in depth RNA studies but the fact that the mother was homozygous and symptom free was surprising. Investigations of the unaffected son were most important to address this issue and revealed carrier status for *ECHS1* disease in him on the RNA and enzymatic level. Since he neither shares a paternal nor a maternal *ECHS1* allele with his affected brothers, the homozygous maternal c.489G > A (p.Pro163=) mutation became a plausible candidate. Eventually the identification of a second family with the same compound heterozygous genotype and a confirmed SCEH defect provided additional evidence for the pathogenicity of c.489G > A (p.Pro163=). Since both carrier parents of c.489G > A (p.Pro163=) in Family 1 and 2 are of Samoan descent we postulated that the c.489G > A (p.Pro163=) is enriched in that ethnic group. Data from a cohort of Samoan ancestry showed the variant to be highly represented with a carrier frequency of approximately 28%. Although common variants are not generally known to cause rare severe disease this phenomenon is not without precedent. For example, in the Amish a carrier frequency of 12% for a missense mutation in the gene *LONP1* causing CODAS syndrome has been reported. In contrast to c.489G > A (p.Pro163=) in *ECHS1*, the *LONP1* mutation is pathogenic in the homozygous state with high perinatal mortality (Strauss et al., 2015). High carrier frequencies of 20% are also known for sickle cell disease and beta thalassemia attributed to increased fitness against Malaria infection (Ferreira et al., 2011; Lazarin et al., 2013). It might be speculated that c.489G > A (p.Pro163=) in *ECHS1* provides some sort of advantage with respect to fitness to have become that enriched in this population. Synonymous variants have also been reported in other inborn metabolic diseases (Ohlsson et al., 2019; Salomons et al., 2007). They produce different levels of both, normal and aberrant transcripts from the same allele. Thus, the resulting enzymatic activity will be higher or lower depending on levels of the normal or aberrant RNA transcribed. We did not detect aberrant *ECHS1* RNA species for the mother and so despite the fact that the mother has two alleles of the "hypomorphic" mutation she is not affected as protein levels are decreased but are representing functionally normal enzyme above a threshold levels which would cause pathogenicity.

Ultimately, the patient's phenotype will depend not only on the severity of the silent variant, but also on the presence of a pathogenic alteration on the second allele. In our patients, the mild silent variant in combination with the severe c.832G > A (p.Ala278Thr) mutation resulted in severe disease in three patients and a milder phenotype in the remainder. As severe disease was seen in two different families, and both phenotypes were present within the same family, it is unlikely that environmental factors were determinants of the variability in disease expression. It is possible that the severity of the metabolic crises and/or the age when they occurred may have played a role in the different degree of brain damage observed. Alternatively, it is possible that the four patients described in this study may have VOUS in modifier genes involving related metabolic pathways affecting expression of the disease. In fact, c.489G > A (p.Pro163=) (rs140410716) has been revealed as a disease modifier itself and has been associated with worse cardiac outcomes in a genome study of Han Chinese patients who had undergone STENT for acute coronary syndromes. Additionally, the study found lowered *ECHS1* expression to be associated with a more severe clinical phenotype (Liu et al., 2018). Another study interested in genomic modifiers of coronary disease found a clear correlation of drug refractory dilated cardiomyopathy and several polymorphisms in *ECHS1* (Campbell et al., 2018). Interestingly, cardiac involvement in *ECHS1* deficiency has been described in patients with severe disease presentation, the patients reported in this study, however, did not show any cardiac

involvement (Haack et al., 2015). ECHS1 has also been suggested as a nutrient sensor which bridges the gene's involvement in metabolism to obesity and heart disease. The investigations by Zhang et al. show that high nutrient states result in the acetylation of ECHS1 protein at p.Lys101 leading to impaired binding of its substrate enoyl-CoA followed by ubiquitination of the protein. Ubiquitinated ECHS1 protein however is resistant to mitochondrial import and is prematurely degraded. (Zhang et al., 2017). Further investigations in the Samoan population correlating increased incidence of common, multigenic disease with variants in *ECHS1* may shed light on the interplay of this gene with metabolism in the future.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Mariella T. Simon and Shaya S Eftekharian established the cell cultures, contributed to study design, data collection, analysis, and interpretation and wrote the article. Sacha Ferdinandusse and Frédéric M. Vaz contributed to data collection, analysis, and interpretation, critically revised the manuscript and approved the final version. Sha Tang performed data analysis and interpretation, critically revised the manuscript, and approved the final version. Take Naseri, Muagututi'a Sefuiva Reupena, Stephen T. McGarvey, Ryan L. Minster, Daniel E. Weeks together with the Samoan Obesity, Lifestyle, and Genetic Adaptations (OLaGA) Study Group performed acquisition, analysis, and interpretation of the Samoan allele frequency data as well as critically revised the manuscript and approved the final version. Daniel D. Nguyen assisted with data collection and analysis and approved the final version. Sansan Lee contributed to data acquisition and approved the final version. Katarzyna A. Ellsworth and David Dimmock contributed to data acquisition, analysis, and interpretation, critically revised the manuscript and approved the final version. James Pitt performed and interpreted metabolite analyses, critically revised the manuscript and approved the final version. Jose E. Abdenur was responsible for diagnosis and management of all patients, contributed to study design and writing, critically revised the manuscript and approved the final version.

DATA AVAILABILITY STATEMENT

c.832G>A (p.Ala278Thr) as well as c.489G>A (p.Pro163=) including phenotypic information has been submitted to the Leiden Open Variation Database (LOVD) https://www.lovd.nl/.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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