Whole Exome Sequencing Identifies the Genetic Basis of Late-Onset Leigh Syndrome in a Patient with MRI but Little Biochemical Evidence of a Mitochondrial Disorder

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Abstract Leigh syndrome is a subacute necrotising encephalomyopathy proven by post-mortem analysis of brain

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tissue showing spongiform lesions with vacuolation of the neuropil followed by demyelination, gliosis and capillary proliferation caused by mutations in one of over 75 different genes, including nuclear- and mitochondrial-encoded genes, most of which are associated with mitochondrial respiratory chain function. In this study, we report a patient with suspected Leigh syndrome presenting with seizures, ptosis, scoliosis, dystonia, symmetrical putaminal abnormalities and a lactate peak on brain MRS, but showing normal MRC enzymology in muscle and liver, thereby complicating the diagnosis. Whole exome sequencing uncovered compound heterozygous mutations in NADH dehydrogenase (ubiquinone) flavoprotein 1 gene (*NDUFV1*), c.1162+4A>C (NM_007103.3), resulting in skipping of exon 8, and c.640G>A, causing the amino acid substitution p.Glu214Lys, both of which have previously been reported in a patient with complex I deficiency. Patient fibroblasts showed a significant reduction in NDUFV1 protein expression, decreased complex CI and complex IV assembly and consequential reductions in the enzymatic activities of both complexes by 38% and 67%, respectively. The pathogenic effect of these variations was further confirmed by immunoblot analysis of subunits for MRC enzyme complexes in patient muscle, liver and fibroblast where we observed 90%, 60% and 95% reduction in complex CI, respectively. Together these studies highlight the importance of a comprehensive, multipronged approach to the laboratory evaluation of patients with suspected Leigh syndrome.

Introduction

Leigh syndrome is largely an early-onset, progressive, neurodegenerative disorder caused by mutations in at least 75 nuclear- and mitochondrial-encoded genes involved in pyruvate metabolism or the mitochondrial respiratory chain complexes (MRC), with the latter mainly involving complex I (CI) and complex IV (CIV) (Lake et al. 2015). Clinical hallmark signs of Leigh syndrome are brainstem dysfunction, symmetrical spongiform lesions in the brain, dystonia, spasticity, movement disorders, visual disturbances, nystagmus, ophthalmoparesis, ptosis, cerebellar ataxia, seizures, scoliosis, bulbar dysfunction and peripheral neuropathy that lead to biochemical signs of mitochondrial dysfunction including lactate elevation in different body fluids, Krebs cycle intermediates in urine and dysfunction of the mitochondrial respiratory chain in different tissues (Lake et al. 2015). Mutations in the nuclear-encoded mitochondrial CI gene *NDUFV1* [MIM 161015] have previously been reported by Schuelke et al. (1999) in a patient with seizures, followed by a report by Benit et al. (2001) in a patient with the same mutations as reported here and a clinical phenotype of Leigh syndrome and CI deficiency (Benit et al. 2001; Schuelke et al. 1999). This gene encodes a 51 kDa subunit of the flavoprotein portion of CI, containing NADH, flavin mononucleotide (FMN) and Fe-S-binding sites (Mimaki et al. 2012). *NDUFV1* is highly evolutionarily conserved and plays a critical role in the catalytic activity of CI (Benit et al. 2001). Dysfunction of CI is the most common oxidative phosphorylation disorder in humans, likely due to the sheer number of subunits (each encoded by a different nuclear or mitochondrial gene) required for its structure, function and assembly, with defects in the CI assembly process commonly at fault (Mimaki et al. 2012).

In a bid to identify the genetic basis of late-onset childhood Leigh syndrome in the proband, we performed whole exome sequencing of DNA from the proband and four unaffected family members and identified compound heterozygous *NDUFV1* sequence variants (NM_007103.3) in the proband. Functional assays of patient fibroblasts revealed reduced levels of NDUFV1 protein, MRC assembly defects and dysfunction of CI and CIV, resulting in a probable reduction in ATP production. These variations have previously been associated with CI deficiency in a patient with moderately elevated plasma lactate levels and clinical features including cerebellar ataxia, persistent seizures, psychomotor regression, strabismus, ptosis and brain atrophy with multiple symmetric areas of hyperintensity in the brainstem (Benit et al. 2001).

Methods and Materials

Proband Summary

The female proband was the second child of unrelated parents of Caucasian Australian background. She had an older and younger brother, both of whom were well. The pregnancy was uncomplicated, and she was born at term by elective lower segment caesarean section due to cephalopelvic disproportion. The birth weight was 3.47 kg (50th percentile) and length was 50 cm (50th–75th percentiles). There were no concerns in the perinatal period, or with early growth and development, and hearing and vision were determined as normal. She was breastfed for the first 3–4 months.

She continued normally until 2 years of life, at which time she had febrile convulsions and subsequently developed a partial left ptosis. This ptosis resolved 2–3 months later. At the age of 7 years, her parents noted that she appeared to use her left arm and leg less than her right limbs. Her parents commented in retrospect that she had always appeared clumsier compared with her older brother. She subsequently developed progressive asymmetric bilateral dystonia of her hands and feet, worse on the right than the left, which was only partially responsive to Baclofen and Botox. Implementation of a ketogenic diet was of no obvious benefit.

Over the following 12 months, her dystonia progressively worsened, chewing became more difficult, she lost the ability to feed herself and her balance and speech deteriorated. She became wheelchair-bound by 10 years of age, developed progressive scoliosis and when last reviewed at the age of 12 years old had a resting tremor, dysarthria and dysphagia, requiring a gastrostomy. Her parents felt that a therapeutic cocktail of thiamine, coenzyme Q10, L-carnitine and biotin had been of some subjective benefit.

A brain MRI performed at the age of 7 years showed symmetrical putaminal lesions, and brain MRS showed a lactate peak (images not shown). A repeat MRI a year later showed progression of the changes in the putamen, as well as involvement of the left body of the caudate and the right quadrigeminal plate (images not shown). Blood and CSF lactate were normal on a number of occasions, as was a urine metabolic screen, serum CPK, copper and ceruloplasmin and liver function tests (results not shown). The clinical evolution together with the brain MRI changes was suggestive of a clinical diagnosis of late-onset Leigh syndrome.

Muscle and liver respiratory chain enzyme activities were found to be normal by spectrophotometric analysis

Table 1 Spectrophotometric MRC enzyme diagnostic data in skeletal muscle, liver and fibroblasts

	Enzyme activity			
	Patient	(Ref range)	% Activity	% CS ratio
<i>Muscle^a</i>				
CI (nmol/min/mg)	46	19–72	110	53
CII (nmol/min/mg)	121	26–63	269	126
CIII (min/mg)	72.2	12.8–50.9	248	112
CIV (min/mg)	11.88	3.3–9.1	180	86
CS (nmol/min/mg)	273	85–179	212	–
<i>Liver</i>				
CI (nmol/min/mg)	21	8–11	221	113
CII (nmol/min/mg)	166	54–73	272	138
CIII (min/mg)	15.1	5.2–10.3	199	100
CIV (min/mg)	0.78	0.5–0.9	110	56
CS (nmol/min/mg)	54	26–31	193	–
<i>Skin fibroblasts^b</i>				
	% Activity			
	Patient	Control		
CI in fibroblast	38*	100		
CIV in fibroblast	67*	100		

Bold characters indicate clinically significant abnormal values

^a Patient liver and muscle samples. Enzyme activity data are expressed as nmol/min per mg protein, except for CIII and CIV, which were expressed as /min/mg. Data are also expressed as % CS ratio, which represents % of the normal control mean value when expressed relative to citrate synthase. Complex I (CI), NADH-coenzyme Q1 oxidoreductase; complex II (CII), succinate-coenzyme Q1 oxidoreductase; complex III (CIII), decylbenzylquinol-cytochrome c oxidoreductase; complex IV, cytochrome c oxidase (CIV)

^b Dipstick MRC enzyme data from cultured fibroblasts. Enzyme activity data are expressed as % residual activity relative to protein (% protein)

* $p < 0.001$

(Table 1). She was negative for the common Leigh syndrome mutations m.8993T>C and m.8993T>G. Subsequent sequencing of the entire mitochondrial genome did not reveal any variants of interest, and fibroblast pyruvate dehydrogenase activity was found to be normal (results not shown).

Whole Exome Sequencing

Genomic DNA was isolated from whole blood collected from the patient, her parents and two unaffected siblings using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

WES and bioinformatics analyses were performed on all samples as previously described (Menezes et al. 2015).

Variants identified by WES analysis were confirmed by Sanger sequencing of DNA from all subjects. Sanger sequencing was performed at the Australian Genome Research Facility (AGRF), Westmead Millennium Institute (Westmead, Australia), using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

In Silico Analysis

In silico analyses of variants identified by WES were performed using Sift (<http://www.sift.jcvi.org>), PolyPhen2 (<http://www.genetics.bwh.harvard.edu/pph2/>), GVGd (<http://agvgd.iarc.fr/>), MutationTaster (www.mutationtaster.org/), PhyloP (<http://cvg.vital-it.ch/mga/hg19/phyloP/phyloP.html>), Human Splicing Finder (<http://www.umd.be/HSF/>) and PhastCons (<http://compugen.bscb.cornell.edu/phast/phastCons-HOWTO.html>).

Exon Skipping

Total RNA extracted from fibroblasts was converted to cDNA using a Superscript III first-strand cDNA synthesis kit (Invitrogen, Mulgrave, VIC, Australia), following the manufacturer's instructions. Each reaction contained 400 ng of total RNA and 250 ng of random decamers, 200 U of Superscript III and 40 U of RNaseOUT™ (Life Technologies, Carlsbad, CA, USA). Full-length cDNA was generated by incubation at 50°C for 60 min. cDNA was amplified by PCR to determine whether the intronic variant caused exon skipping. PCR products were gel purified (PCR Purification Kit; Qiagen) and sent to AGRF for Sanger sequencing.

Immunoblotting

Fibroblast cells were lysed and protein prepared as previously described (Riley et al. 2013). 20 µg of each protein sample was resolved on a 4–12% Bis–Tris gel (Life Technologies) and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were probed with 1:1,000 anti-NDUFV1 (ab174472, Abcam, Cambridge, United Kingdom) or 1:500 anti-OXPHOS cocktail (consisting of five antibodies, recognising labile subunits from each of the five MRC complexes; ab110411, Abcam). Protein loading was normalised to porin (1:5,000 anti-porin for 2 h at room temperature; ab14734, Abcam). All blots were then probed with either 1:2,500 anti-rabbit or anti-mouse IgG horseradish peroxidase (HRP; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 2 h at room

temperature. Membranes were then incubated with enhanced chemiluminescence (ECL) reagents and exposed to Hyperfilm ECL (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Films were scanned using a SRX-101A (Konica Minolta Holdings, Inc., Tokyo, Japan) scanner and analysed with ImageJ version 1.49 (<http://rsb.info.nih.gov/ij/>).

Protein was extracted from patient and control skeletal muscle and liver biopsy samples as previously described (PMID: 12847163). Relative concentration of protein was estimated by running samples on a 4–12% Bis–Tris gel (Life Technologies) and staining with Coomassie blue staining solution, after which samples were subjected to immunoblotting as above except blots were probed with 1:2,000 anti-mouse IgG horseradish peroxidase for 1.5 h and films were scanned using a CP1000 Processor (Agfa-Gevaert, Mortsels, Belgium).

Spectrophotometric MRC Enzyme Activity

MRC enzyme activities in liver and skeletal muscle biopsies from the proband were determined as previously described (Frazier and Thorburn 2012).

Complex I and IV Dipstick Enzyme Activity Assays in Fibroblasts

CI and CIV enzyme assays were performed on freshly isolated protein lysates from patient fibroblasts using dipstick enzyme assays, following the manufacturer's instruction (Abcam, Cambridge, United Kingdom).

Blue Native Polyacrylamide Gel Electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed on freshly isolated protein lysates from patient fibroblasts as described elsewhere (McKenzie et al. 2007). 20 µg of protein sample was resolved on 4–16% Bis–Tris Protein Gels (Life Technologies, Carlsbad, CA, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software, San Diego, USA). All variables underwent statistical comparisons by Mann–Whitney *U* non-parametric testing. Two-tailed Student's *t*-test of measures was carried out at least in triplicate. Statistical significance was set at $P < 0.05$.

Results

Because of the clinical suspicion of late-onset Leigh syndrome, we initially performed MRC complex spectrophotometric assays in liver and skeletal muscle (Table 1). Since these results were within the reference ranges and the patient had a phenotype strongly indicative of Leigh syndrome, we moved to whole exome sequencing and identified compound heterozygous mutations (c.1162+4A>C and c.640G>A) in the MRC CI flavoprotein subunit encoding gene *NDUFV1*. Both mutations were confirmed by Sanger sequencing, with each parent carrying one of the mutations (Fig. 1a and c). *In silico* review of the c.640G>A variant predicted it to affect protein function with the most damaging score and showed that the p. Glu214 position is completely conserved across many species (Fig. 1b). *In silico* analysis of the c.1162+4A>C variation predicted it to be disease causing, affecting the donor splice site. These variations had very low minor allele frequencies (<http://www.1000genomes.org> & ExAc <http://exac.broadinstitute.org>). Amplification of patient cDNA, generated by reverse transcription of patient fibroblast mRNA, showed two bands on agarose gel electrophoresis: an intense higher full-length band and a shorter transcript (faint lower band) (Fig. 1d). Sanger sequencing of the shorter transcript confirmed the skipping of exon 8 (Fig. 1e), and the presence of the missense c.640G>A mutation in the higher full-length band in the patient mRNA.

We examined NDUFV1 protein levels in patient fibroblasts and its effect on MRC complex levels. Immunoblot analysis revealed a 72% reduction in NDUFV1 levels in patient fibroblasts compared to a control (Fig. 2a). Immunoblot analysis of subunits for MRC enzyme complexes, dependent upon MRC complex formation for protein stability, revealed a severe reduction in CI (95%), CIII (45%) and CIV (60%) in patient fibroblasts (Fig. 2b). However, in patient skeletal muscle and liver, only CI was reduced by 90% and 60%, respectively, suggesting that CI was the most vulnerable complex in our patient (Fig. 2c and Supplementary Fig. 1). It is not clear to us what factors may be contributing to the tissue specificity in our patient carrying *NDUFV1* mutations.

Since the *NDUFV1* mutations were only expected to affect CI, we examined MRC assembly in patient fibroblasts by blue native polyacrylamide gel electrophoresis (BN-PAGE). A relative reduction in CI (60%), CIII (35%), CIV (51%) and supercomplex I/III (95%) was observed (Fig. 2d). However, we were unable to examine whether

Fig. 1 (a) Sanger sequencing profile of *NDUFV1* from the proband and parents showing compound heterozygous mutations in the proband, including c.640G>A (p.Glu214Lys; heterozygous in the mother) and c.1162+4A>C (heterozygous in the father). (b) Evolutionary sequence conservation of the altered amino acid residue (p. Glu214) is denoted by a red rectangle. (c) Amplification of cDNA

shows two bands in the proband: an intense higher molecular weight band and a faint lower molecular weight band. (d) Sanger sequencing of the faint band from amplified cDNA showing skipping of exon 8 in the patient. (e) Representative image of the RT-PCR and sequencing analysis of exon 8 skipping

Fig. 2 (a) Immunoblot showing reduced *NDUFV1* expression in cultured fibroblasts from the proband (P) compared to controls (C). (b) Immunoblot showing OXPHOS expression in muscle and liver from the proband (P) compared to controls (C). (c) Immunoblot showing OXPHOS expression in cultured fibroblasts from the proband (P) compared to controls (C). (d) BN-PAGE immunoblotting

of skin fibroblast mitochondria lysed in 1% digitonin, using antibodies against the *NDUFA9* subunit of complex I, the *SDHA* subunit of complex II, the *UQCRC1* subunit of complex III and the *COX1* subunit of complex IV, shows reduced complexes I, III, IV and supercomplex CI/CIII

these assembly defects were also present in patient skeletal muscle and liver because we did not have enough tissue material to carry out these studies.

In summary, these results confirm the deleterious nature of the *NDUFV1* variants as predicted by *in silico* analyses, with strong evidence that changes at the NDUFV1 subunit level have affected CI assembly in our patient. Mitochondrial respiratory chain enzyme function was then re-evaluated by dipstick enzyme assays to determine the relative specific activity of immunocaptured CI and CIV in patient fibroblast lysates. The results showed reduced activity of both CI and CIV reductions at 67% and 38%, respectively, in patient fibroblasts, relative to controls (Table 1). The reduced CI and CIV activity by dipstick enzyme assay was consistent with reduced CI and CIV levels seen on immunoblotting in patient fibroblast (Fig. 2b–d).

Discussion

This study highlights several challenges faced in the diagnosis of a patient with a clinical picture of Leigh syndrome but normal MRC enzyme activity in clinically unaffected tissues. The findings reinforce the value of using massively parallel sequencing for the identification of the underlying genetic basis of this genetically heterogeneous disorder and demonstrate the importance of confirmatory structural and functional analyses to build an evidence base linking genotype and phenotype.

In most cases, diagnosis of MRC diseases relies on identifying abnormal activity in one or more MRC enzymes (Hui et al. 2006). However, there are limitations in using MRC enzyme activity as a lead diagnostic tool. These relate primarily to dilute aqueous assay systems being an imperfect reflection of the function of membrane-embedded enzymes and the difficulty distinguishing primary from secondary MRC defects, as discussed elsewhere (Thorburn et al. 2004). Hence, only 29 out of 67 suspected Australian cases of Leigh syndrome showed a significant decrease in activity of the MRC complexes, underscoring the notion that enzyme activity investigations should be complementary and additive to other structural and functional evidence in the diagnosis of MRC disease and should be interpreted with caution (Rahman et al. 1996).

However, implementation of combined MRC enzymology methods such as spectrophotometric assays and polarographic or dipstick enzyme assays is not currently suitable as a frontline diagnostic assay but can be useful for confirmation and evaluation of the effect of novel mutations and to study animal models (Calvo et al. 2010; Wenchich et al. 2003). These complementary methods differ in their methodologies, potentially allowing differential interroga-

tion of the pathways involved (Barrientos et al. 2009; Tang et al. 2013; Wenchich et al. 2003).

Benit et al. (2001) has reported a patient with the same compound heterozygous variations, an onset of seizures at age 1 year, mildly elevated plasma lactate, neurological regression, strabismus, ptosis and a progressive course leading to death by 3 years. The MRI showed the typical bilateral, symmetrical basal ganglia and brainstem lesions. Leigh syndrome was proven histopathologically post-mortem (Benit et al. 2001). They reported CI deficiency in muscle and liver. However, CI was normal in cultured skin fibroblasts (Benit et al. 2001). The patient we describe here had different clinical manifestations compared with the case reported by Benit et al. (2001), with a later onset, slower progression and asymmetrical ptosis and dystonia, but no seizures. The patient is still alive 10 years after diagnosis. It is important to note that although we showed a significant reduction in both complexes I and IV in cultured fibroblasts as well as a significant reduction in complex I in muscle and liver in the current study, the overall evaluation of mitochondrial CI and CIV enzyme activity in the liver, muscle and fibroblasts was not considered helpful for the diagnosis of Leigh disease.

In mammals, all 44 CI subunits must be assembled correctly in the right sequence to form a mature functional complex. Among these components, disease-causing mutations in 14 nuclear-encoded structural subunits of CI (*NDUFS1*, *NDUFS2*, *NDUFS3*, *NDUFS4*, *NDUFS7*, *NDUFS8*, *NDUFV2*, *NDUFS6*, *NDUFV1*, *NDUFA1*, *NDUFA2*, *NDUFA9*, *NDUFA10*, *NDUFA12*), four assembly factor defects (*NDUFAF2*, *NDUFAF5*, *NDUFAF6*, *FOXRED1*) and 6 mtDNA subunits have previously been linked with Leigh syndrome (Ogilvie et al. 2005; Mimaki et al. 2012; Lemire 2015; Lake et al. 2015). Mutations in four CI subunits (*NDUFV1*, *NDUFS1*, *NDUFS4* and *NDUFS6*), all located in the matrix arm (N-module) of CI, lead to CI subassembly complexes of about 830 kDa on BN-PAGE analysis that appear to have lost the matrix arm of CI (Mimaki et al. 2012; Ogilvie et al. 2005; Tuppen et al. 2010; Leong et al. 2012). In our experience, patients and mice with mutations in these genes often show an apparently more severe impact on the amount of fully assembled CI on BN-PAGE than on spectrophotometric CI activity or functional analyses such as ATP synthesis (Leong et al. 2012). This is likely due to the amount of fully assembled CI in the intact inner membrane being higher than implied by BN-PAGE and enzyme studies where the membrane has been disrupted by detergent, freeze-thawing or sonication (Leong et al. 2012), destabilising the assembled CI. All four of these CI subunit genes are expressed ubiquitously, but the impact on CI enzyme activity and function also varies between tissues (Bird et al. 2014). Furthermore, as was seen in our case, mutations in

CI genes can affect the stability of other mitochondrial complexes, which may explain the reduced CIII and CIV levels and low CIV activity in patient fibroblasts (Ugalde et al. 2004; Suthammarak et al. 2010). Some ambiguity in being able to detect isolated CI defects in all patients with mutations in genes like *NDUFV1* is not surprising given the potential variability introduced by the differing severity of individual mutations, genetic background effects and different methods for assessing the activity and assembly of a membrane-embedded complex of 44 proteins that interacts with other membrane complexes.

In summary, we have presented *in silico* studies and abnormalities of the *NDUFV1* mRNA with consequent reductions of CI protein level in skeletal muscle, liver and cultured fibroblasts. The finding of normal CIV and CIII protein levels in the patient skeletal muscle and liver, also observed in a previous study (Benit et al. 2001), emphasises the need to investigate primary tissues, as fibroblasts alone may lead to misleading results. We have also highlighted the assembly defects in OXPHOS complexes CI, CIII, CIV and supercomplex I/III. Considering the limitations in the biochemical diagnosis of a child with clinical manifestations of late-onset Leigh syndrome, implementation of a multifaceted diagnostic pipeline, including whole exome sequencing and complementary structural and functional studies, helped to confirm the pathogenicity of *NDUFV1* mutations in this patient.

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Compliance with Ethics Guidelines

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 included in the study.

Synopsis

A definitive genetic diagnosis was provided by whole exome sequencing and functional studies in a patient with late-onset childhood Leigh syndrome and normal muscle and liver mitochondrial RC enzyme activities.

Conflicts of Interest

Michael Nafisinia, Yiran Guo, Xiao Dang, Jiankang Li, Yulan Chen, Jianguo Zhang, Nicole J Lake, Wendy A Gold, Lisa G Riley, David R Thorburn, Brendan Keating, Xun Xu, Hakon Hakonarson and John Christodoulou declare that they have no conflict of interest.

Details of Contributions of Individual Authors

Michael Nafisinia: design and implementation of structural and functional studies, data analysis and manuscript preparation

Yiran Guo, Xiao Dang, Jiankang Li, Yulan Chen, Jianguo Zhang, Brendan Keating, Xun Xu, Hakon Hakonarson: performance of whole exome sequencing, implementation of bioinformatics, data analysis and manuscript preparation

Wendy Gold, Lisa Riley: contribution to design of structural and functional studies, data analysis and manuscript preparation

Nicole J. Lake: implementation of structural and functional studies

David Thorburn: oversight of spectrophotometric assays, data analysis and manuscript preparation

John Christodoulou: overall oversight of the research project, clinical interface, data analysis and manuscript preparation

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