
Adult Onset Leigh Syndrome in the Intensive Care Setting: A Novel Presentation of a C12orf65 Related Mitochondrial Disease.


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Adult Onset Leigh Syndrome in the Intensive Care Setting: A Novel Presentation of a $C12orf65$ Related Mitochondrial Disease

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Abstract

Background: Mitochondrial disease can present at any age, with dysfunction in almost any tissue making diagnosis a challenge. It can result from inherited or sporadic mutations in either the mitochondrial or the nuclear genome, many of which affect intraorganellar gene expression. The estimated prevalence of 1/4300 indicates these to be amongst the commonest inherited neuromuscular disorders, emphasising the importance of recognition of the diagnostic clinical features.

Objective: Despite major advances in our understanding of the molecular basis of mitochondrial diseases, accurate and early diagnoses are critically dependent on the fastidious clinical and biochemical characterisation of patients. Here we describe a patient harbouring a previously reported homozygous mutation in $C12orf65$, a mitochondrial protein of unknown function, which does not adhere to the proposed distinct genotype-phenotype relationship.

Methods: We performed clinical, biochemical and molecular analysis including whole exome sequencing on patient samples and cell lines.

Results: We report an extremely rare case of an adult presenting with Leigh-like disease, in intensive care, in the 5th decade of life, harbouring a recessively inherited mutation previously reported in children. A global reduction in intra-mitochondrial protein synthesis was observed despite normal or elevated levels of mt-RNA, leading to an isolated complex IV deficiency.

$^{1}$Denotes equal contribution.

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Conclusions: All the reported $C12orf65$ mutations have shown an autosomal recessive pattern of inheritance. Mitochondrial disease causing mutations inherited in this manner are usually of early onset and associated with a severe, often fatal clinical phenotype. Presentations in adulthood are usually less severe. This patient’s late adulthood presentation is in sharp contrast emphasising the clinical variability that is characteristic of mitochondrial disease and illustrates why making a definitive diagnosis remains a formidable challenge.

Keywords: Mitochondria, respiratory insufficiency, schizophrenia, human $C12orf65$ protein, protein biosynthesis, peptide termination factors

ABBREVIATIONS

Mt Mitochondrial
COX Cytochrome c oxidase, CIIV
H&E Hematoxylin and eosin
MRI Magnetic resonance imaging
BN-PAGE Blue native polyacrylamide gel electrophoresis
RC Respiratory chain
OXPHOS Oxidative phosphorylation
LONP Mitochondrial Lon Protease Homolog
CLPX Caseinolytic Mitochondrial Matrix Peptidase Chaperone Subunit.

INTRODUCTION

The mitochondrial genome (mtDNA) encodes 13 open reading frames in addition to the tRNAs and rRNAs required for intramitochondrial protein synthesis [1]. The remaining components that comprise or facilitate assemble the mitoribosome are encoded by the nucleus. Mutations in any of these genes that affect expression of mtDNA can potentially cause mitochondrial (mt) disease [2–5], a heterogeneous group of disorders characterized by compromised oxidative phosphorylation. The application of whole exome sequencing to patients with well-characterised mitochondrial disease has revealed new nuclear disease genes to be correlated with pathways where their involvement had not previously been known, or had not been predicted but not confirmed [6]. The $C12orf65$ gene falls into the latter category; it is a member of the mitochondrial release factor [7] family, some but not all members of which have been shown to cleave the terminal tRNA from the nascent peptide in the mitoribosome, allowing the newly made protein to escape the translation machinery. Despite its predicted role in mt-translation, this was not confirmed until the first report of a patient presenting with a pathogenic mutation in $C12orf65$ [OMIM 613541] leading to decreased mt-protein synthesis [8]. Subsequently a number of patients with $C12orf65$ mutations have been reported presenting with similar symptoms of mitochondrial disease [8–14] (Table 1). The predicted consequence of the mutations is that truncated versions of $C12orf65$ protein would be synthesised, where the length of the protein depended on the precise position of the stop codon. A clinical review of patients with $C12orf65$ mutations has proposed that distinct genotype-phenotype relationships can be distinguished in this patient group, despite the broad spectrum of symptoms [9]. These authors suggest that although the degree of protein truncation dictates the severity, an ‘obligatory clinical triad’ of optic atrophy, peripheral neuropathy and spastic paraparesis is characteristic for all patients.

Table 1

<table>
<thead>
<tr>
<th>Clinical Symptoms</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Optic atrophy†</td>
<td>[8, 9, 11–14, 32]</td>
</tr>
<tr>
<td>Pyramidal signs</td>
<td>[9, 10, 12, 13, 32]</td>
</tr>
<tr>
<td>Intellectual disability</td>
<td>[8–10, 12–14]</td>
</tr>
<tr>
<td>Neuroptathy (including distal weakness, foot deformity)†</td>
<td>[8, 9, 11–14]</td>
</tr>
<tr>
<td>Ophthalmoparesis</td>
<td>[8, 10, 13, 32]</td>
</tr>
<tr>
<td>Facial muscle weakness</td>
<td>[8, 13, 32]</td>
</tr>
<tr>
<td>Cerebellar involvement (intentional tremor, dysmetria, ataxia)</td>
<td>[8, 13, 32]</td>
</tr>
<tr>
<td>MRI changes consistent with Leigh syndrome</td>
<td>[8, 10, 13, 32]</td>
</tr>
<tr>
<td>Dysmorphic faces (hypertelorism, broad nasal bridge)</td>
<td>[10, 13]</td>
</tr>
<tr>
<td>Bulbar involvement</td>
<td>[8, 32]</td>
</tr>
<tr>
<td>Respiratory compromise/failure</td>
<td>[8, 32]</td>
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<tr>
<td>Muscular colloid bodies</td>
<td>[12]</td>
</tr>
<tr>
<td>Retinitis pigmentosa</td>
<td>[12]</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>[32]</td>
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<tr>
<td>Atherosclerosis</td>
<td>[10]</td>
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<tr>
<td>Scoliosis</td>
<td>[12]</td>
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<tr>
<td>Paralytic ileus</td>
<td>[8]</td>
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<tr>
<td>Urinary stress incontinence</td>
<td>[8]</td>
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<tr>
<td>Ovarian failure</td>
<td>[14]</td>
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*Previously reported patients (n = 27), †Clinical triad symptom.
Here, we describe a late presentation of LS and respiratory failure in a patient with long standing schizoaffective disorder, harbouring a previously reported C12orf65 mutation where the severity of the clinical presentation does not correlate with the length of the truncated C12orf65 protein. This mutation predicts one of the shortest aberrant C12orf65 proteins, and although the symptoms of the minimal obligatory triad were present, the patient only presented to the intensive care unit as an adult in the 5th decade of life. We have characterised respiratory capacity in skeletal muscle and fibroblasts, assessed de novo mitochondrial translation in patient fibroblasts and interrogated the steady state levels of respiratory chain proteins, confirming an isolated mitochondrial complex IV deficiency.

All the reported C12orf65 mutations have shown an autosomal recessive pattern of inheritance. Mitochondrial disease causing mutations inherited in this manner are usually of early onset and associated with a severe, often fatal clinical phenotype. The case presented here is in contrast to this pattern emphasising the importance of recognising the breadth of diagnostic clinical features, especially in light of the estimated prevalence of 1:4300, which indicates that these are amongst the most common inherited neuromuscular disorders [5].

MATERIALS AND METHODS

Case report

The male patient was a term birth, to healthy, non-consanguineous parents, and reached developmental milestones appropriately. At 2 years he underwent a surgical procedure for hypospadias and experienced perioperative respiratory arrest. He was felt to have suffered mild hypoxic brain damage, as evidenced by mild dysarthria, mild fixed limb spasticity and cognitive developmental delay but progressed through normal schooling and remained independent. No bulbar dysfunction or regression was ever documented. He underwent a tendon lengthening procedure at age 14 years with no untoward sequelae. He was diagnosed with schizoaffective disorder in his second decade of life requiring multiple hospital admissions but remained relatively asymptomatic in his later years.

In his fifth decade of life, six months prior to his acute admission, he was noted to have deteriorated physically reporting shortness of breath on exertion and worsening mobility requiring two sticks to mobilise with the need for a wheelchair for longer distances. His dysarthria had also progressively worsened.

At 45-years-old this man presented to his acute care hospital following a fall at home. On admission he was noted to be drowsy, with a reduced Glasgow coma scale of 13 out of 15, low grade pyrexia of 38 °C, tachypnoea (respiratory rate 21), tachycardia (100 bpm) and hypoxic (69% on room air). An arterial blood gas showed a pH of 7.20, pCO2 14.7kPa, pO2 9.3kPa and bicarbonate of 37.8 mmol/L consistent with type II respiratory failure. A chest X-ray showed patchy consolidation of the left base, white cell count was 12.2 and CRP less than 1. He was initially commenced on non-invasive ventilation but continued rapid deterioration warranted assisted ventilation. CT head was normal. On transfer to critical care he was treated for a presumed pneumonia, he responded well and was extubated after 48 hours, requiring intermittent non-invasive ventilation only for elevated pCO2. He then developed massive hematemesis and rapidly became haemodynamically unstable (systolic BP 90 mm Hg, pulse rate: 130 bpm) necessitating transfusion. He required urgent laparotomy and repair of a perforated duodenal ulcer but following the anaesthetic had a protracted hospital stay. He remained ventilator dependent via a tracheostomy related to gross disturbance of central respiratory drive compounded by diaphragmatic weakness and respiratory muscle weakness. Bulbar function remained profoundly poor, necessitating the placement of a percutaneous endoscopic gastrostomy feeding tube.

Histochemical and biochemical analyses

Standard histological (H&E) and histochemical (COX/SDH) analyses of skeletal muscle biopsies were performed on fresh frozen skeletal muscle sections (10μm) [15]. Mitochondrial respiratory chain complex activities were determined in skeletal muscle and fibroblast homogenates and expressed relative to the activity of the matrix marker enzyme, citrate synthase [16].

Molecular genetics

Total DNA was extracted from muscle, mtDNA rearrangements investigated by long-range PCR [17] and direct sequencing of the entire mtDNA genome was undertaken as previously described [18]. Alignment and variant calling were performed using SeqScape software v2.6 (Life Technologies, Warrington, UK)
using the revised Cambridge reference sequence for human mtDNA (GenBank accession: NC_012920.1) [19]. Whole exome sequencing and bioinformatics was performed as previously described [13]. Briefly, genomic DNA was fragmented and enriched by Illumina TruSeq™ 62Mb exome capture and sequenced (Illumina HiSeq 2000, 100 bp paired-end reads). Bioinformatics pipeline included alignment to the human reference genome (UCSC hg19) and variant detection (Varscan v2.2, Dindel v1.01) [20]. On-target variant filtering excluded minor allele frequencies (greater >0.01) in several databases and 343 unrelated in-house controls as previously shown. ANNOVAR was used to define homozygous/compound heterozygous variants and protein altering and/or putative ‘disease causing’ mutations [21]. Candidate genes were ranked by previous association with disease phenotypes [22]. The C12orf65 variant was confirmed by Sanger sequencing (primers; Forward 5′-TCATCTAACCCAGGTCCTCAG-3′; Reverse 5′-AGGCTGTGGAACCCTTGG-3′; GenBank accession: NM_152269.4) [18].

Cell culture

Cells were routinely cultured [23] in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1x NEAA, 1 mM pyruvate, 50μg/ml uridine, with either 4500 mg/L glucose or 10 mM galactose, at 37°C, in a humidified 5% CO₂ atmosphere. Transfection of HEK293 cells with siRNAs was performed with 30nM final concentration as previously described [7]. Control non-targetting (NT) and custom synthesised against C12orf65 (sense 5′-GGG AGA AGC UGA CGU UGU dTdT) siRNAs were from Eurogentec.

BN-PAGE, SDS-PAGE and western blotting

For BN PAGE, mitochondrial lysates solubilised with dodecylmaltoside (3.2 g/g protein) from patient and control fibroblasts (25 μg per lane) were separated on 4.5–16% gels as described in [24]. For SDS-PAGE, mitochondrial lysates were prepared from patient and control fibroblasts and 12.5 μg per lane separated on 12% gels as described in [23].

Proteins were transferred to PVDF membranes (GE Healthcare) for immunoblotting. Primary antibodies used were; Complex I - NDUFB8, NDUFSA9; Complex II - SDHA (70 kDa flavoprotein subunit); Complex III - UQRC2 (Core 2); Complex IV - COX4, COX2; Porin; MRPL3, CLPX. Complex V (α-subunit) (all Abcam). ICT1, MRPS26, MRPS27, MRPL45 (PTG Labs); HSP90 (BD Biosciences); LonP (Sigma Life Science). Appropriate HRP-conjugated immunoglobulin secondary antibodies (Dako, Glostrup, Denmark) were used and detected with ECL-Prime (GE Healthcare) as per the manufacturer’s instructions.

Metabolic labelling of mitochondrially encoded proteins

This was essentially as described in Chomyn [25]. Patient and control fibroblasts were metabolically labelled [15S]-methionine/cysteine for 1 hour with emetine inhibition of cytosolic translation. Total cell lysates (50 μg) were separated by 15% SDS-PAGE. Signals from newly synthesised mt-proteins were detected from dried gels via PhosphoImaging and ImageQuant software (GE Healthcare). Gels were Coomassie blue stained to confirm equal loading.

Northern analysis

Total RNA from control and patient fibroblasts or HEK293 cells was extracted using Trizol (Life Technologies). Aliquots (4 μg) were electrophoresed through 1.2% denaturing agarose, and transferred to Genescreen Plus. Probes were generated using random hexamers on PCR generated templates corresponding to the internal regions of the genes of interest, as previously described [26].

RESULTS

Clinical findings

Neuropathological evaluation was consistent with an axonal neuropathy. On examination the patient had bilateral ptosis, horizontal gaze paresis and unilateral optic atrophy. He had mild facial muscle weakness, palatal myoclonus, a reduced gag reflex and dysarthria. There was severe distal muscle wasting of upper and lower limbs. Upper and lower limb reflexes were pathologically brisk, with the exception of ankle and plantar reflexes, which were absent. Initial neurology screen including serum protein electrophoresis, copper, caeruloplasmin, acylcarnitines, carnitine, phytic acid, very long chain fatty acids, α-fetoprotein and urinary organic acids were normal. Serum lactate was 3.7 mmol/L (normal range <2.0 mmol/L).

MRI of the head showed extensive swelling and abnormal high T2-weighted signal changes centrally within the medulla and dorsal pons. Additional small foci of restricted diffusion were seen within the...
Fig. 1. MRI revealed abnormalities consistent with mitochondrial disease. Cranial MRI was performed showing swelling and abnormal high T2-weighted signal changes centrally within the dorsal pons (A, red arrow) and medulla (B, C, red arrows). Other small foci of restricted diffusion were detected and also seen within the cerebellar hemispheres (A, yellow arrow).

Characterisation of respiratory capacity in skeletal muscle and fibroblasts

A diagnostic muscle biopsy was performed to formally investigate a likely mitochondrial disorder. Although the muscle appeared morphologically normal, histochemical analysis of oxidative enzymes including succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) revealed a generalised decrease in COX activity throughout the biopsy sample, confirmed by the sequential COX-SDH assay (Fig. 2A). Spectrophotometric assays of mitochondrial respiratory chain enzyme activities revealed a severe (approximately 20% of control values) and isolated COX (CIV) deficiency in the muscle of our patient (Fig. 2B). The identical biochemical defect was also observed, to a lesser degree, in the patient’s cultured fibroblasts (approximately 50% of control) (Fig. 2C). The activities of all other RC complexes were normal, confirming an isolated CIV deficiency.

Cell morphology (Fig. 2D) and growth rate of fibroblasts derived from the patient were comparable to control cells on glucose. However, these failed to proliferate on galactose (Fig. 2E, F), a substrate that forces cells to utilise oxidative phosphorylation rather than glycolysis for ATP production. This observation confirmed that the cells express a mitochondrial respiratory chain defect, consistent with the decrease in complex IV activity determined spectrophotometrically.

Identification of pathogenic C12orf65 mutation

Diagnostic molecular genetic studies excluded mtDNA depletion, mtDNA rearrangements and mtDNA point mutations as a cause of the respiratory chain defect in muscle. Whole exome capture and sequencing identified a homozygous exonic deletion (c.210delA, p.Gly72Alafs∗13) frameshift mutation (Fig. 3A), predicted to encode a truncated version of
the protein due to the introduction of a premature stop codon (Fig. 3B). This was identical to the mutation described in the first report of C12orf65 patients [8]. Subsequent C12orf65 reports also identified truncating mutations [8–13]. Although premature stop codons often trigger non-sense mediated decay (NMD) to degrade transcripts and so prevent synthesis of aberrant polypeptides, control levels of C12orf65 mRNA were reported (8, 12), and data not shown) and one study detected a truncated C12orf65 protein [11] suggesting that aberrant C12orf65 transcripts identified thus far escape normal NMD.

As with our patient’s mutation, a number of the reported mutations predict not only a truncated protein but also the loss of the highly conserved GGQ motif, a determining feature of release factors. This critical feature is essential for triggering peptidyl-tRNA hydrolysis, which cleaves the ester bond between the P-site tRNA and the nascent polypeptide chain [27, 28].

To identify if the synthesised polypeptides are stable, steady state levels of RC proteins were assessed by western blotting following SDS-PAGE. This confirmed decreased levels of complex IV (COX2 expression), consistent with our biochemi-
originally been reported in children with impairment of mitochondrial translation and severe decreases in activities of complexes I, IV and V [8]. In distinction to these expectations this patient presented in adulthood (aged 45 years) with milder symptoms and an isolated complex IV defect. Although the ‘obligatory clinical triad’ of symptoms was present there were other distinctive features in this patient’s presentation.

The patient suffered from shortness of breath and associated respiratory muscle weakness, consistent with a long term deficiency in respiratory chain (RC) enzyme activities. To date, only 3 published cases of C12orf65-related mitochondrial disease have reported respiratory failure; but age of onset varies from 4 to 14 years. This unusual cause of failure to wean from a respirator in a man in his fifth decade of life was, therefore, likely precipitated by a marked catabolic state, resulting from compromised respiratory chain activity. Spectrophotometric analysis of respiratory chain complexes indicated a severe and isolated complex IV deficiency, consistent with the growth defect seen in patient fibroblasts when challenged to respire on galactose. Intriguingly however, our molecular investigations of individual mt-RNA species, proteins and OXPHOS complex formation revealed a complex regulation of gene expression. Elevation of the mt-mRNAs encoding OXPHOS components did not correlate with elevated protein levels, nor did modest decreases in protein levels of specific complex I and V components compromise complex activities. Indeed, biochemically we were able to find only an isolated complex IV deficiency. This lack of complex I dysfunction also marked this phenotype as different from the biochemical defects reported in published cases.

Of the mitochondrial disease patients for whom there is no current molecular diagnosis, approximately 30% exhibit defects in mitochondrial translation [6]. Incorporating whole exome sequencing into diagnostic algorithms is facilitating molecular genetic diagnoses but it is clear that identifying a pathogenic mutation in a gene encoding a relevant mitochondrial protein is only the first step towards understanding the molecular basis of mitochondrial disease. For example, mitochondrial aminoacyl tRNA synthetases (mt-aaRS) are responsible for adding the appropriate amino acid to the cognate mt-tRNA. The lack of a correctly charged mt-tRNA would be predicted to impair all intra-mitochondrial translation irrespective of which of mt-tRNA was in short supply. However, recessively-inherited defects in different aminoacyl mt-tRNA synthetases lead to very different clinical phenotypes exhibiting marked tissue specificity [29]. Similarly, different mt-tRNA mutations would be predicted to affect overall intra-mitochondrial translation but the degree to which each gene product is synthesised cannot be so easily correlated with either the particular mutant mt-tRNA or the relevant mt-aaRS. There are clearly other factors that play a part in modifying the severity of the defects and further research is required to dissect what these are and how they contribute to the clinical phenotype.

Defective C12orf65-induced RC deficiency may be responsible for a retrograde signal that causes the increase in mitochondrial RNA seen in all patient...
Fig. 4. Gene expression analyses of OXPHOS components and related proteins. A. Intramitochondrial protein synthesis was measured in cell lysates by incorporation of 35S-methionine and cysteine into fibroblasts derived from patient (P) or control (C). The relative positions of the mt-proteins are indicated. Protein loading was confirmed by Coomassie blue (CBB) staining of the gel. B. Northern blot analysis was performed on patient and control RNA (4 μg) to quantify the steady state levels of the RNA species indicated. The cytosolic 18S rRNA was used as a loading control. C. Similar analyses were performed on human kidney cells (HEK293) following 6 days siRNA mediated depletion of C12orf65 (lanes 1-3), or a non-targeting control siRNA (lanes 4-6) reflecting 3 experimental repeats. The cytosolic 18S rRNA was used as a loading control. Westerns of patient and control mitochondrial lysates (12.5 μg) were separated by 12% SDS-PAGE to detect levels of mitoribosomal proteins (D), respiratory chain proteins (E) or mitochondrial proteases (G) using porin as a loading control. F: Assembly and integrity of the OXPHOS complexes was determined by blue native 4.5–16% gradient PAGE of mitochondrial lysates (25 μg) from patient and control. After transfer, Western blot analysis was used to visualize all OXPHOS complexes with subunit specific antibodies as follows: complex I (NDUFA9), complex III (Core 2) and complex II (SDHA), complex IV (COX4) and complex V (α-subunit). samples where mt-RNA levels were analysed (ibid and [8]), and in cell lines following siRNA mediated depletion of C12orf65. This may potentially reflect a compensatory response directed at overcoming the OXPHOS defect, however the defective C12orf65 prevents any subsequent increase in protein synthesis.
The cellular ATP requirements in this patient were partially sustained by glycolysis, however under conditions of higher energy demands the consequences of the C12orf65 defect manifested as respiratory failure with defective OXPHOS.

Of the identified C12orf65 mutations this individual harbours a previously reported frameshift mutation that eliminates the GGQ domain and prematurely truncates the protein ten amino acids beyond this domain, generating one of the shortest reported C12orf65 proteins (Fig. 2B, [13]). In distinction to the published clinical reports of C12orf65 mutations, which are consistent with the shortest proteins correlating with the more severe symptoms (summarised in [13]), this individual only presented in his fifth decade of life with a milder and more restricted panoply of symptoms. Mitochondrial disease was until recently classified as a rare disorder [30, 31]. However, advances in our understanding of the genetics, biochemistry, and clinical presentations of these conditions have resulted in increasingly effective diagnoses. This improved recognition of mitochondrial disease is confirmed in a recent study indicating an estimated prevalence of 1:4300

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In summary, we demonstrate for the first time, an example where the severity of the clinical phenotype is independent of the position of the truncating mutation in the C12orf65 gene. Hence, cases such as the one presented here, reinforce the complexity of the clinical presentations of mitochondrial disease, where variable combinations of symptoms, with different degrees of severity, can manifest at any age.

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CONFLICT OF INTEREST STATEMENT FOR ALL AUTHORS

The authors have no conflict of interest to report.

COMPETING FINANCIAL INTEREST

The authors declare no competing financial interests.

REFERENCES


