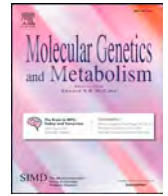




Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Mutations in the mitochondrial complex I assembly factor NDUFAF6 cause isolated bilateral striatal necrosis and progressive dystonia in childhood

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ARTICLE INFO

Keywords:

Leigh syndrome
NDUFAF6
Bilateral striatal necrosis
Complex I deficiency
Dystonia
Mitochondrial disease

ABSTRACT

Aim: To perform a deep phenotype characterisation in a pedigree of 3 siblings with Leigh syndrome and compound heterozygous *NDUFAF6* mutations.

Method: A multi-gene panel of childhood-onset basal ganglia neurodegeneration inherited conditions was analysed followed by functional studies in fibroblasts.

Results: Three siblings developed gait dystonia in infancy followed by rapid progression to generalised dystonia and psychomotor regression. Brain magnetic resonance showed symmetric and bilateral cytotoxic lesions in the putamen and proliferation of the lenticular-striate arteries, latter spreading to the caudate and progressing to cavitation and volume loss. We identified a frameshift novel change (c.554_558delTTCTT; p.Tyr187AsnfsTer65) and a pathogenic missense change (c.371T > C; p.Ile124Thr) in the *NDUFAF6* gene, which segregated with an autosomal recessive inheritance within the family. Patient mutations were associated with the absence of the *NDUFAF6* protein and reduced activity and assembly of mature complex I in fibroblasts. By functional complementation assay, the mutant phenotype was rescued by the canonical version of the *NDUFAF6*. A literature review of 14 *NDUFAF6* patients showed a consistent phenotype of an early childhood insidious onset neurological regression with prominent dystonia associated with basal ganglia degeneration and long survival.

Interpretation: *NDUFAF6*-related Leigh syndrome is a relevant cause of childhood onset dystonia and isolated bilateral striatal necrosis. By genetic complementation, we could demonstrate the pathogenicity of novel genetic variants in *NDUFAF6*.

1. Introduction

Leigh syndrome (LS) is a progressive neurodegenerative condition presenting in infancy, childhood or adolescence [1,2]. Neurologic features include psychomotor delay or regression, hypotonia, dystonia,

ataxia, spasticity, seizures and brainstem dysfunction. Early supplementation with thiamine and biotin can modify the clinical course and improve prognosis in LS patients due to biotinidase deficiency and genetic defects in thiamine transport and metabolism [3]. Thiamine responsiveness has also been described in patients with PDHA1

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<https://doi.org/10.1016/j.ymgme.2019.01.001>

Received 2 November 2018; Received in revised form 3 January 2019; Accepted 3 January 2019

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deficiency presenting at age > 12 months with relapsing ataxia and Leigh syndrome [3–5]. For the rest of aetiologies, the disorder is progressive, leading to severe disability and early death in most of the cases [1,6,7].

To date, 89 genetic defects have been reported to cause LS and Leigh-like syndromes [8–10]. Nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase or NADH dehydrogenase (complex I) deficiency accounts for approximately 35 to 50% of LS patients [11,12]. Complex I transfers electrons from NADH molecules to ubiquinone and pumps protons across the inner mitochondrial membrane [13–15]. Complex I contains 44 subunits [16–18]. The assembly of this complex requires at least 15 additional factors, being *NDUFAF6* one of them [19,20]. *NDUFAF6* participates in the first stages of the assembly of respiratory complex I and is associated with biogenesis of the mtDNA encoded subunit p.MT-ND1 [21,22].

Defects in *NDUFAF6*, initially considered a rare cause of LS [21,23,24] were recently identified in five Leigh syndrome Japanese patients with nuclear encoded respiratory chain complex defects [11,25]. However, little clinical information is available for the majority of cases, which hampers clinical recognition.

Our aim is to perform a deep phenotype characterisation in a pedigree of 3 siblings with compound heterozygous *NDUFAF6* mutations presenting with isolated dystonia and striatal necrosis, and to discuss clinical, biochemical and genetic data in the context of a literature review on *NDUFAF6* patients. Moreover, we confirmed that the mutant phenotype can be rescued by genetic complementation in patient fibroblasts, providing conclusive evidence for the pathogenicity of the mutations.

2. Material and Methods

2.1. Patients

The proband of this kindred was identified from a cohort of 34 children with movement disorders and basal ganglia necrosis on MRI, which were defined as hyper signal intensity on T2/FLAIR, restricted diffusion on DWI/ADC sequences (acute phase), or T1 hypo-intensity, volume loss or cavitation (chronic phase) in the putamen, caudate, pallidum or subthalamus.

This study was approved by the Ethics Committee of the Vall d'Hebrón Research Hospital in Barcelona, Spain. Blood samples, muscle biopsy and fibroblasts from patient 1 were collected with the approval of the Institutional Review Board at Hospital Virgen del Rocío in Sevilla, Spain. Informed consent from patients' guardians was collected.

2.2. Genetic analysis

Total DNA was extracted from blood samples using the MagnaPure system (Roche Applied Science, IN, USA). A customised gene panel for movement disorders was designed using the Sure Design Tool (Agilent Technologies, Santa Clara, CA, USA). This panel included 241 genes linked to the following phenotypes: [1] LS due to mitochondrial defects; [2] Leigh-like syndrome due to inborn errors of intermediate metabolism; and [3] other phenocopies. Library construction was performed according to manufacturer's protocol using HaloPlex technology. Sequencing was carried out on MiSeq sequencer (Illumina, San Diego, CA, USA). Data processing, variant calling and variant annotation were done by DNAnexus platform and Variant Studio software. The average of mean-coverage in the sample gene panel was 95.4% for a read depth of 20×. Filtering was performed by minor allele frequency < 1% (extracted from ExAC general population database where 66,664 alleles were analysed from European non-Finnish population) and possible pathogenicity based on mutation effects (frameshift, insertions deletions, missense, stop gain and splice site regions). Variant validation and segregation studies were done by polymerase chain reaction (PCR) with Sanger sequencing using the Big Dye Terminator Cycle Sequencing

System (Applied Biosystems). Primers for validation of the identified change in *NDUFAF6* were forward primer 5'-CCATGTCCATGGTGATT TGT-3' and reverse primer 5'-GAAGGCTTTAGTGCTAAACTGG-3'. The *NDUFAF6* 'canonical' sequence (NCBI: NM_152416.3; NP_689629.2) was amplified from retro-transcribed total RNA extracted from control and patient 3 fibroblasts and cloned using TOPO-Cloning (Thermo Fisher Scientific) as in [26].

2.3. Mitochondrial parameters

Spectrophotometric assay for complexes of the respiratory chain in muscles was performed according to previously described methods [27]. Mitochondrial adenosine triphosphate (ATP) levels were measured 6 times as described previously [28] using the CellTiter-Glow Luminiscent Cell Viability Assay (Promega). In-gel activity staining of respiratory complexes was carried out as previously reported [29].

2.4. Complementation assay on patient fibroblasts

Control and patient-derived skin fibroblasts were grown at 37 °C in a 5% CO₂ atmosphere in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco-Life Technologies), supplemented with 10% foetal bovine serum (FBS from Gibco). For neomycin-resistant cells, a final concentration of 1 µg/mL in the medium was used.

The wild-type *NDUFAF6* cDNA (NM_152416.3) was cloned into the lentiviral expression vector pWPLD-ires-NeoR, a modified version of pWPLD (Tronolab, Addgene #12258). Lentiviral particles were generated, and transduction was performed as described (<https://www.addgene.org/tools/protocols/plko/#E>). Transduced cells were selected for neomycin resistance 24 h after the transduction.

NDUFAF6 mRNA levels were determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay in a LightCycler 2.0 system (Roche), using FastStart DNA MasterPLUS SYBR Green I (Roche).

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed as previously described [30].

For immunodetection, anti-*NDUFAF6* (Sigma, # HPA050545), anti-*NDUFS3* (Thermo Fisher Scientific, #), anti-*NDUFA9* (Thermo Fisher Scientific, # 459100), anti-*UQCRC2* (Abcam, # ab14745), anti-*p.MT-CO1* (Thermo Fisher Scientific, # 459600), anti-*SDHA* (Thermo Fisher Scientific, #459200) and anti-Actin (Sigma, #A 2066) antibodies were used. Chemiluminescence, image acquisition was performed using the amersham imager 600 system.

3. Results

3.1. Case report

The 3 siblings were conceived from healthy non-consanguineous Spanish parents. They were born at term after spontaneous delivery, birth weights were between 2.860 and 3.422 g. and perinatal history was unremarkable in all cases. Early psychomotor milestones were on average, and they walked unaided between 12 and 15 months. Initial concerns appeared between the age of 17 months and 2.5 years with toe walking and speech difficulties. Neurological deterioration was insidious, progressing to generalised dystonia and gait loss between the age of 3 and 6 years. Currently, patients are 11, 8 and 6 years old, they have prominent oromandibular and bulbar involvement causing speech difficulties (*i.e.*, dysarthria, stuttering) and dysphagia. None of them had ever experienced episodes of acute encephalopathy/deterioration with fever or intercurrent infections. Plasma amino acids, lactate and urine organic acids concentrations were normal in the first 2 patients, (P1 and P2) and were not performed in the youngest (P3). They received treatment with mitochondrial cofactors, including thiamine and biotin, as well as trihexyphenidyl and L-dopa for dystonia, without clinical improvement. The clinical phenotype and progression of the 3

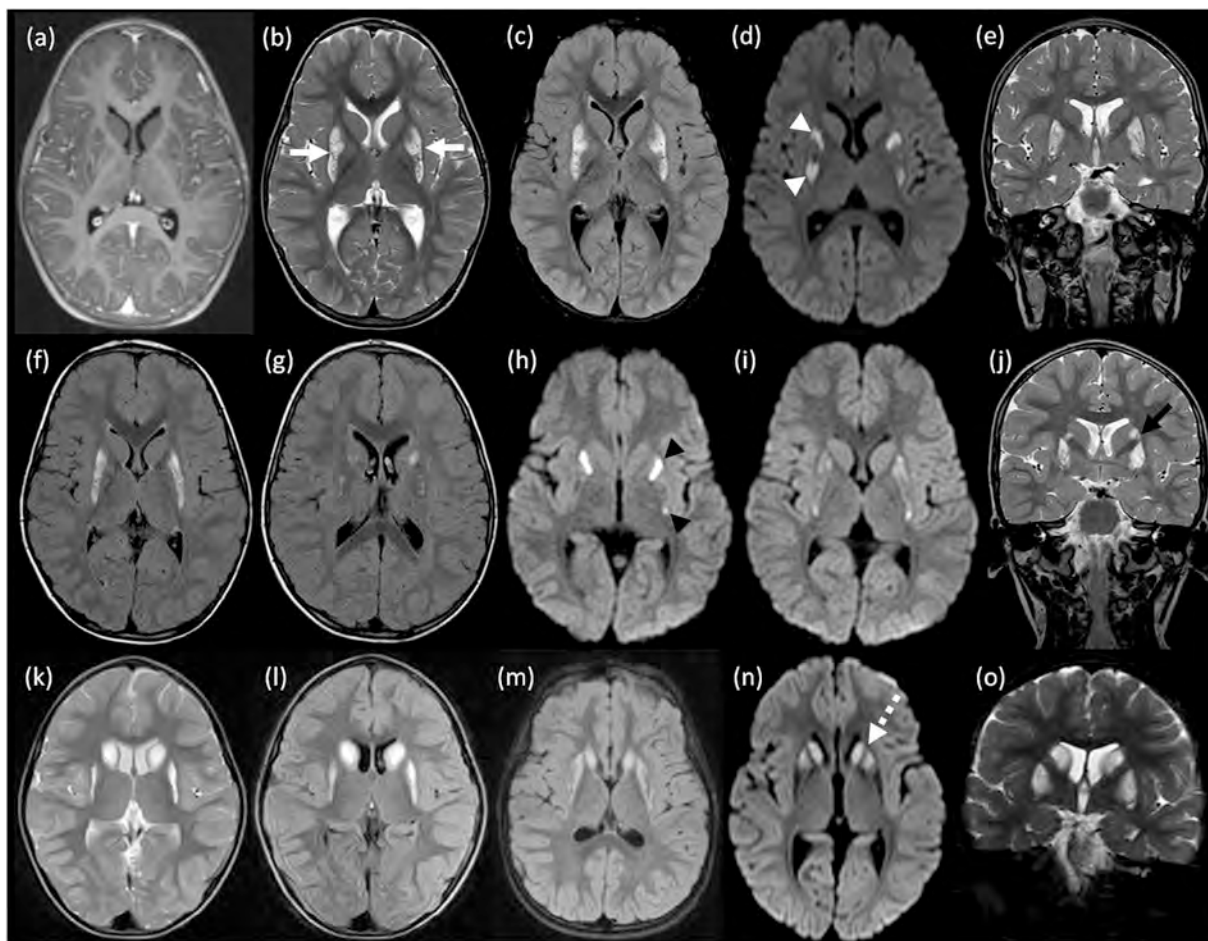


Fig. 1. Brain magnetic resonance imaging (MRI) in NDUFAF6 mutated patients. P3. MRI at 2 years and 7 months of age (a–e). Lenticulostriate arteries ectasia is seen on axial postcontrast T1WI (a). Axial T2WI (b) and FLAIR (c) images add putaminal hypersignal (white arrows), with sparing of its caudal edge and with apparently normal volume. High signal intensity in DWI (d), with low ADC signal (not shown), represents abnormal restricted diffusion within both putamen nuclei (white arrowheads), with a concentric distribution. Caudate nuclei are spared, better seen on coronal T2WI (e). Slight compensatory ventriculomegaly is also depicted. P2 MRI at 3 years and 4 months of age (f–j). Axial FLAIR images (f, g) show putaminal hypersignal with mild reduction in volume and central hypointensity reflecting cavitation. On DWI (h, i) putamen is observed with central patchy hypointensity but anterior and posterior hyperintensity (concentric distribution cytotoxic edema) (black arrowheads), with low ADC values (not shown). Partial involvement of the most outer part of the body of both caudates, more prominent in the left, and also affecting the grey matter caudate-putamen's bands (black arrows) better observed on coronal T2WI (j). P1 MRI at 5 years of age (k, l) and follow-up at 8 years of age (m–o). Axial T2WI (k) and axial FLAIR (l) images depict hyperintensity and moderate volumetric reduction of the whole putamen, and hypersignal and mild enlargement of the anterior part of the body of the caudate. Axial FLAIR (m) and coronal T2WI (o) show atrophy of the whole caudate and putamen nuclei, with mild compensatory frontal horns' dilation. Axial DWI (n) shows hypointensity within the posterior putamen (high ADC values, not shown), reflecting cavitation, and ventral putamen and caudate nucleus hypersignal (with high ADC values, not shown) due to T2 shine-through effect (white dashed arrows).

siblings is reported in Video 1.

Brain MRI of the three siblings is shown in Fig. 1. Early signs of compromise are shown in patient P3 fourteen months after the onset of dystonia, with acute lesions in the putamen in a concentric distribution, cytotoxic edema and apparently normal volume. Brain MRI in P2 at 10 weeks of age when the patient was asymptomatic was normal. A second MRI performed at 3 years and 4 months showed T2 hyperintensity in the putamen with partial involvement of the outermost part of the body of both caudates, and also affecting the grey matter bands' communicating with both nuclei. In DWI, the putamen is observed to have concentric distribution cytotoxic edema. Serial brain MRIs were performed in patient P1 at 5 and 8 years, showing lesions in the putamen extending to the whole body of caudate nucleus, hypertrophy and ectasia of lenticulostriate arteries, cystic patchy areas and reduced volume along the entire putamen nucleus axial view, and progression to prominent atrophy and volume loss of the whole caudate and the putamen. Brain magnetic resonance spectroscopy (MRS) revealed an inverted doublet in long TE spectroscopy, reflecting lactate and low levels

of N-acetylaspartate (NAA) in both the putamen and caudate.

Brain auditory and visual evoked potentials were normal for the first 2 patients and not performed in the third. Sensory and motor nerve conductions were characteristic for peripheral demyelinating involvement in P2. Muscle biopsy showed nonspecific myopathic changes in the absence of mitochondrial proliferation in P1 at 5 years, and no structural alterations in P2. Spectrophotometric measure of respiratory complex enzymatic activity in muscle demonstrated isolated MRC complex I deficiency in P1 (60% of activity) (8.88 nmol/CS Units, reference value 15–80 nmol/CS Units) and normal activity in P2.

3.2. Molecular analysis

We identified in the proband (P1, Fig. 2B) a frameshift novel change at genomic coordinate chr8:96057849_96057853 according to hg19 reference genome (c.554_558delTCTT; p.Tyr187AsnfsTer65) and a missense change previously reported in 2 unrelated patients (c.371 T > C; p.Ile124Thr) in the NDUFAF6 gene [25]. Both mutations

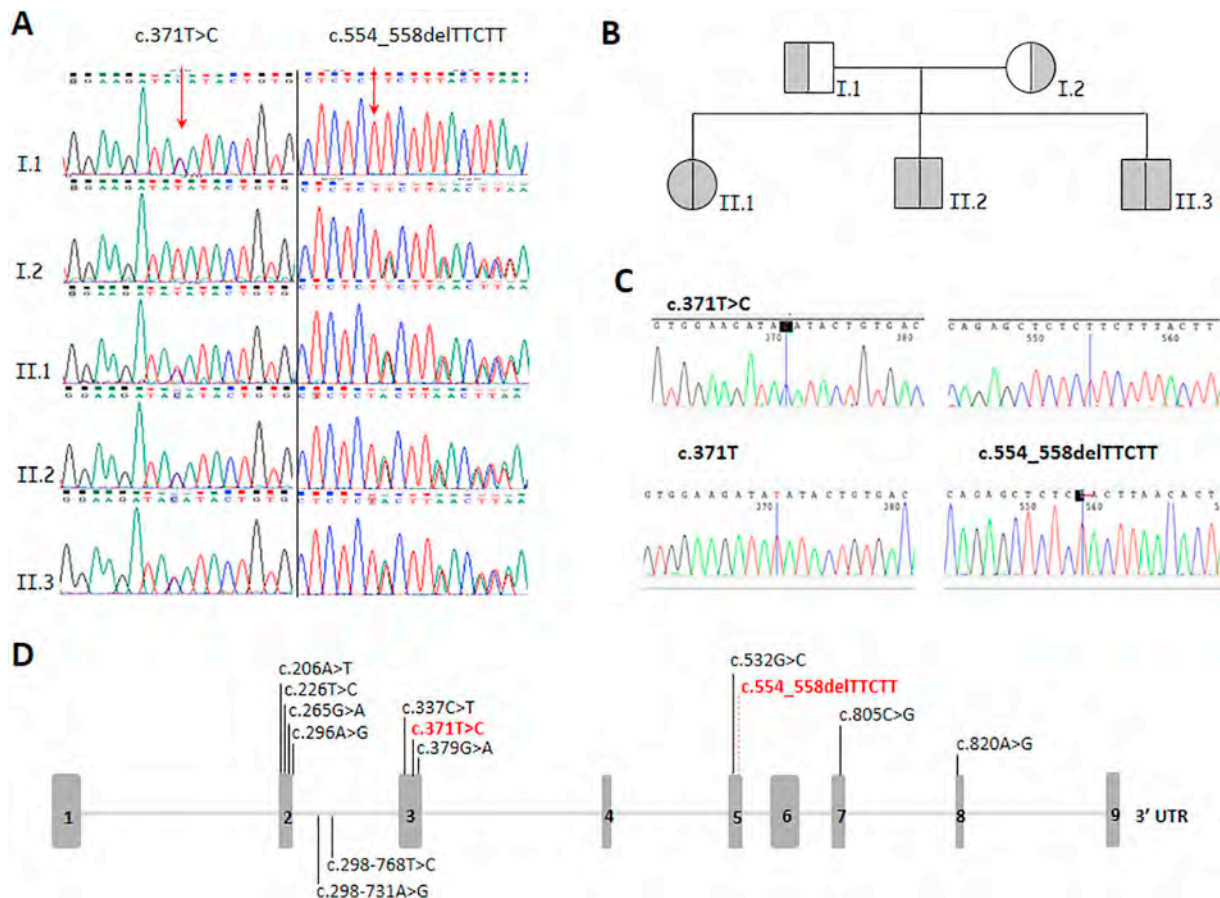


Fig. 2. Family pedigree and segregation analysis of the *NDUFAF6* mutations within the family. (A) Sanger confirmation of the missense (c.371 T > C) and frameshift (c.554_558delTTCTT) mutations in the *NDUFAF6* gene; (B) family pedigree; (C) amplification and cloning of patient *NDUFAF6* cDNA; (D) human *NDUFAF6* described mutations.

were in highly conserved regions according to UCSC Genome Browser and Clustal Omega software. The frameshift change was classified as pathogenic by Mutation Taster protein predictor, and it was not found in HGMD, dbSNP 1000 Genome project, ExAC database or CIBERER Spanish Variant Server. No other mutations were identified in other genes. SANGER analysis (Fig. 2A) showed that the other two affected siblings (P2, P3) carried both changes, the father carried the missense mutation and the frameshift variant came from the mother, consistent with an autosomal recessive mode of inheritance. Human *NDUFAF6* mutations described so far are also reported (Fig. 2D).

Amplification and cloning of *NDUFAF6* cDNA from P3 fibroblasts yielded 2 sequences. One carried the missense change previously reported as a pathologic mutation (c.371 T > C; p.Ile124Thr). The other sequence carried the frameshift novel change (c.554_558delTTCTT; p.Tyr187AsnfsTer65) (Fig. 2C). These results confirmed the biallelic nature of the variants.

3.3. Literature review of patients with *NDUFAF6* deficiency

Our patients were compared with 11 previously reported cases with *NDUFAF6* defects and LS phenotype [23,25,45–47] (Table 1). Mean age at onset of these children was 24 (0–72) months. All patients were alive at the time of publication except for 1 case that died at 34 months [23]. One patient presented with an acute metabolic decompensation triggered by acute febrile illness, whereas onset of neurological features was insidious in the rest of the cases. All patients suffered from neurological regression, and the majority developed signs of basal ganglia dysfunction (dystonia, rigidity, tremor). Other neurological features included ataxia, seizures, myopathy and peripheral neuropathy. All

patients showed basal ganglia lesions on MRI, predominantly affecting the putamen and caudate. T2-hyperintensities in dentate nuclei and superior cerebellar peduncles were observed in three patients [45,46]. One patient showed a more diffuse brain involvement, with lesions affecting the parietal cerebral white matter and the dorsal pons [23]. Regarding biochemical findings, 4 out of 14 cases showed increased blood lactic acid levels. The activity of RCC was reduced in fibroblasts ($n = 9$, 40–86%) and muscle ($n = 6$, 28–80%).

3.4. Patient mutations are associated with the absence of the *NDUFAF6* protein and reduced activity and levels of mature complex I

The mitochondrial ATP levels measurements revealed that P3-derived primary fibroblasts displayed 40% reduction in mitochondrial ATP levels relative to control cells (Fig. 3A). *NDUFAF6* transcript levels in the patient were significantly reduced to 62% of the control (Fig. 3B). This reduction is probably due to nonsense mediated mRNA decay since the frameshift mutation in one allele of the patient introduces a premature stop codon into the *NDUFAF6* mRNA. Protein lysates extracted from a control, (C), an immortalised control (Ci) and P3 cells, were analysed by WB using a specific anti-*NDUFAF6* antibody. *NDUFAF6* cross-reacting material was detected as a 32 kDa band in control fibroblasts, but was almost undetectable in the patient (Fig. 3C), indicating that patient mutations are associated with a strong reduction of the *NDUFAF6* protein.

In order to determine if the absence of *NDUFAF6* caused a decreased in the activity and quantity of complex I, BN-PAGE analysis was performed. BN-PAGE followed by in-gel activity (IGA) staining of respiratory complexes of muscle samples from patients P1, and P2

Table 1 Clinical, biochemical and radiological features of patients with *NDUFA6* mutations reported in the literature.

Features	Pagliarini 2008		Bianciardi 2016		Kohda 2016		Fang2017		Catania 2018		This report (P12, 13 and 14 in the table refer to Patients 1, 2 and 3 in the text, respectively)			
	P 1	P 2	P 3	P 4	P 5	P 6	P 7	P 8	P 9	P 10	P 11	P 12	P 13	P 14
Phenotype	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS	LS
Consanguinity	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N
Age at onset (m)	10	7	42	72	17	0	2	48	21	12	60	30	30	17
Age of dead	34 months	alive	alive	NA	NA	NA	NA	NA	Alive	Alive	Alive	Alive	Alive	Alive
Current age (years)	-	1,8	8,5	NA	NA	NA	NA	NA	5	3	11	7	6,5	4,5
Clinical features	NA	NA	Insidious	NA	NA	NA	NA	NA	Acute/febrile illness	Insidious	Insidious	Insidious	Insidious	Insidious
Neurological regression	Y	Y	Y	Y	Y	Y	Y	NA	Y	Y	Y	Y	Y	Y
Extrapyramidal features	Decreased movement and strength and rigidity	Decreased movement and strength and rigidity	Dystonia	NA	NA	NA	NA	NA	Tremor, hypertonia	N	Oculomandibular dystonia, extrapyramidal hyper-tonia and a mild campto-cormic gait	Generalised dystonia	Generalised dystonia	Generalised dystonia
Cerebellar ataxia	Y	Y	N	NA	NA	NA	NA	NA	Y	Y	Y	N	N	N
Dysarthria	NA	NA	Y	NA	NA	NA	NA	NA	NA	NA	NA	Y	Y	Y
Seizures	Y	Y	N	NA	NA	NA	NA	NA	NA	NA	NA	N	N	N
Other symptoms and signs	N	N	Decreased fine manual motor abilities, scoliosis	N	Muscle atrophy	N	N	Exercise intolerance, weakness	Drizzling	N	Drizzling	N	Peripheral demyelinating neuropathy	N
Basal ganglia necrosis	Putamen	Caudate and putamen	Caudate and putamen	NA	Y	NA	Y	NA	Caudate and putamen	Y	Putamen	Caudate and putamen	Caudate and putamen	Putamen
Subcortical white matter	Parietal white matter	Normal	Normal	NA	NA	NA	NA	NA	putamen	NA	Normal	Normal	Normal	Normal
Spinal cord	Dorsal pons	Normal	Normal	NA	NA	NA	NA	NA	NA	NA	NA	Normal	Normal	Normal
Cerebellar involvement	NA	NA	Dentate nuclei	NA	NA	NA	NA	NA	NA	T2-hyperintensities in dentate nuclei and superior cerebellar peduncles.	NA	NA	N	N
MR spectroscopy lactate peak	NA	NA	Y	NA	NA	NA	NA	NA	NA	NA	NA	Y	NA	NA
Biochemical investigation	Elevated	Elevated	Normal	NA	NA	Elevated	NA	1.4-2.08 mmol/L	2.8 mmol/L	2.2 mmol/L	Normal	1.63 mmol/L	1.59 mmol/L	Not performed

(continued on next page)

Table 1 (continued)

Features	Pagliarini 2008		Bianciardi 2016		Kohda 2016		Fang2017		Catania 2018		This report (P12, 13 and 14 in the table refer to Patients 1, 2 and 3 in the text, respectively)			
	P 1	P 2	P 3	P 4	P 5	P 6	P 7	P 8	P 9	P 10	P 11	P 12	P 13	P 14
Lactate in CSF	NA	NA	Normal	NA	NA	NA	NA	NA	Normal	NA	Normal	Not performed	Not performed	Not performed
MRC CI (% of residual activity):	36% in muscle in fibroblasts in liver	14% in fibroblasts	72% in muscle 18% y 32% in fibroblasts*	(< 30% in a tissue, < 40% in a fibroblast cell line, or < 40% in 2 tissues)	(< 20% in a tissue, < 30% in a fibroblast cell line, or < 30% in 2 tissues)	(< 20% in a tissue, < 30% in a fibroblast cell line, or < 30% in 2 tissues)	(< 20% in a tissue, < 30% in a fibroblast cell line, or < 30% in 2 tissues)	NA	42% in muscle 38% in fibroblasts	NA	Normal	60% in muscle	Decreased enzymatic activity in muscle	Decreased enzymatic activity in muscle and in fibroblasts
Mutations	c.296A > G (hom)	c.296A > G (hom)	c.532G > C (hom)	c.371 T > C; p.I124T; c.805C > H269D	c.820A > G; p.R274G; c.820A > G; p.R274G	c.226 T > C; p.S76 P; c.805C > H269D	c.206A > T; p.D69V; c.371 T > C; p.I124T	c.337C > T* (paternal D) + -	c.532G > C; p.A178P; (c.420 + - 784C > T)	c.532G > C; p.- > C; p.- > A178P; (c.420 + - 784C > T)	c.532G > C; p.- > C; p.- > A178P; (c.420 + - 784C > T)	c.371 T > C; c.554_558delTTCTT	c.371 T > C; c.554_558delTTCTT	c.371 T > C; c.554_558delTTCTT

CSF: cerebrospinal fluid; MRC CI = mitochondrial respiratory chain complex I; MRI: magnetic resonance image; NA: not available; N: no; P: patient; Y: yes.

(Fig. 3D) and fibroblasts from P3 (Fig. 3E) showed decreased CI enzymatic activity relative to CII.

BN-PAGE followed by Western blot analysis using specific antibodies revealed that the amount of mature complex I in P3-mitoplasts was dramatically decreased compared to that of the control, consistent with the complex I activity defect detected in the 3 siblings. Mature complex II, complex IV and complex III dimer were present at only slightly decreased levels compared to the control (Fig. 3F).

3.5. Complementation assay on patient fibroblasts

In order to test functional complementation, wild-type *NDUFAF6* was transduced in patient and control fibroblasts. Robust over-expression of *NDUFAF6* was detected at both mRNA and protein levels (Fig. 3B and G). This was associated with the recovery of a normal amount of CI assembly in patient cells. In contrast, expression of wild-type *NDUFAF6* in control fibroblasts did not significantly modify the mature complex I amount (Fig. 3H).

Increased levels of mature complex I correlated with patient recovery of complex I in-gel activity (Fig. 3E), linking *NDUFAF6* gene with the biochemical defects.

4. Discussion

We report a pedigree of 3 siblings with progressive dystonia and striatal necrosis harbouring compound heterozygous mutations in the *NDUFAF6* gene. These children manifested with gait dystonia and speech difficulties in early childhood after a period of normal neurodevelopment. Gradual progression of symptoms led to generalised dystonia, gait loss, severe dysarthria and total dependence on activities of daily living in a period of 1.5 to 4 years. No systemic features of mitochondrial disorders were observed, and lactic acid, serum alanine levels and organic acids were normal in blood and urine.

The suspicion of mitochondrial disorders was based on neuroimaging studies, which detected cytotoxic oedema in the neostriatum on DWI and a lactate peak on MRS, and on inconsistent results in the activity of MRC complex I in muscle by spectrophotometric assay in two siblings. Massive parallel sequencing recognised the previously reported missense change c.371 T > C and the novel frameshift c.554_558delTTCTT variant in the *NDUFAF6* gene, encoding an assembly factor of NADH-ubiquinone oxidoreductase. Further investigations demonstrated reduced assembly of mature complex I and reduced complex I enzymatic activity by BN-PAGE and in-gel activity (IGA) staining.

The novel variant c.554_558delTTCTT is a frame-shift mutation that is expected to result in a truncated translation product. Our patients' mutations were associated with a strong reduction in the steady-state levels of *NDUFAF6* protein. *NDUFAF6* transcript levels in the patient were also reduced, suggesting that the absence of transcripts from this allele contributed to the reduction of *NDUFAF6* protein levels.

Genetic complementation is a powerful tool to obtain strong evidence of pathogenicity of mutations [31]. Remarkably, the mutant phenotype was rescued in P3 fibroblasts by the canonical version of the *NDUFAF6*, thus demonstrating the pathogenicity of the *NDUFAF6* genetic variants detected in the 3 siblings.

Regarding neuroradiological investigations, the 3 siblings showed symmetric and bilateral cytotoxic lesions in the putamen nuclei, which started in its upper, lateral and central part, spreading in a concentric and centrifugal manner to its inferior and medial region, through the grey matter bands to the anterior part of the caudate nucleus body and then to the head ventrally and to the tail dorsally. The first grey matter structures affected developed diffusion restriction. The lesions progressed to volume loss and cavitation in the older siblings, more prominent in the putamen than in the caudate, and to compensatory ventricular enlargement. T2WI and T1WI after contrast media administration showed enhancement of the lenticulo-striate arteries, probably

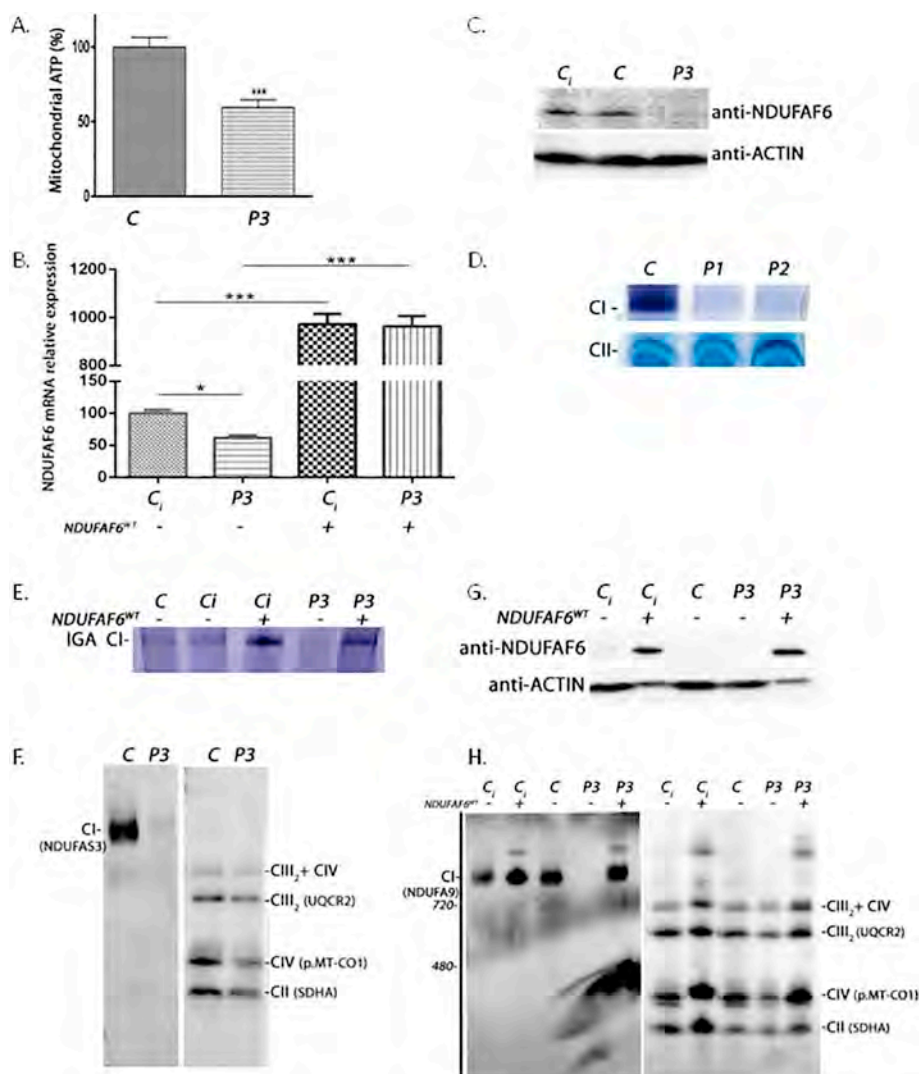


Fig. 3. Mitochondrial ATP synthesis, in-gel activity stain of respiratory complexes and Western blot (WB) immunodetection.

(A) Mitochondrial ATP levels in primary fibroblasts. Statistical significance (unpaired *t*-test) is shown, *** = $p < .001$ vs C. (B) qPCR quantitation of *NDUFAF6* mRNA expression in fibroblasts and *NDUFAF6*-transduced fibroblasts. Statistical significance (unpaired *t*-test) is shown, * = $p < .05$, *** = $p < .001$. (C) WB immunodetection of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in fibroblasts (100 μ g of total protein extract were loaded per lane). (D) In-gel activity staining of respiratory complexes in muscle samples of P1 and P2. (E) In-gel activity staining of complex I in fibroblasts and in *NDUFAF6*-transduced fibroblasts. (F) WB immunodetection of BNGE in fibroblasts and in *NDUFAF6*-transduced fibroblasts. (G) WB immunodetection of SDS-PAGE in fibroblasts (60 μ g of total cellular extract) and *NDUFAF6*-transduced fibroblasts (10 μ g of total cellular extract). (H) WB immunodetection of BNGE in fibroblasts and *NDUFAF6*-transduced fibroblasts. Ci: immortalised control fibroblasts; C: control primary fibroblasts; P3: patient 3 primary fibroblasts.

corresponding to vascular hypertrophy and proliferation in response to hypoperfusion and deoxygenation, a scenario previously reported in anatomopathological descriptions of LS [32–34]. Of note, none of the patients presented with pallidal compromise.

By reviewing previous patients identified with homozygous or combined heterozygous mutations in *NDUFAF6* we could demonstrate a homogeneous phenotype consisting of an insidious onset movement disorders associated with neurological regression before the age of 6 years. All patients showed basal ganglia neurodegeneration on MRI. In two patients, a similar progression of basal ganglia abnormalities was reported with acute restricted diffusion lesions in the putamen spreading to the caudate and leading to atrophy and cavitation [45,46]. Three patients associated lesions in the cerebellar peduncles and dentate nuclei, and one single case showed involvement of the pons and parietal white matter. The vast majority of cases (11/12) showed decreased activity of RCC I in fibroblasts and/or muscle.

Brain lesions in our patients fulfilled the radiological criteria of bilateral striatal necrosis (BSN). This entity defines a group of disorders presenting with movement disorders and bilateral degeneration of the neostriata (caudate and putamen) [35–37]. BSN encompasses a subgroup of LS patients (MIM 256000), in whom the association with brainstem involvement and lactate peak in MRS is highly characteristic, as compared to other aetiologies [39]. Also, other brain areas may be affected in LS patients, the most common being the cerebellum, thalamus, spinal cord and white matter, stroke-like lesions and cortical and

cerebellar atrophy (BSN plus) [38–40].

Our results confirm that *NDUFAF6* may be included in a narrow subgroup of LS patients with brain lesions limited to the neostriata (isolated BSN), together with genetic thiamine metabolism defects [3], mitochondrial DNA genetic defects (ND1, ND6 and Complex V deficiency) [41,42] and *NDUFV1* mutations causing complex I deficiency [43,44]. These defects may show bilateral and symmetric involvement of the putamen and caudate, with swelling and cytotoxic oedema, and a lactate peak in the acute phase. Also, progression to atrophy and cavitation has been reported, similar to patients with *NDUFAF6*-related LS [35].

Etiologic diagnosis of early onset dystonia with BSN is a diagnostic challenge for paediatric neurologists due to the uniform and unspecific clinical and radiological features, the lack of biomarkers, and the increasing number of underlying genetic conditions described so far. Mitochondrial energy generation defects are the most frequent genetic conditions in children with dystonia and BSN [9]. Among them, genetic defects in thiamine transport and metabolism are of utmost importance due to the potential clinical benefit of biotin and thiamine supplementation [3,4]. Other genetic defects leading to neostriatal lesions were recently reviewed by Tonduti et al. [35] and included glutaric aciduria type 1 and other organic acidurias, Aicardi-Goutières syndrome caused by ADAR1 mutations, gangliosidosis type 2, sulfite oxidase and molybdenum cofactor deficiency, Wilson's disease, Alexander's disease, giant axonal neuropathy, H-ABC syndrome

(hypomyelination with atrophy of basal ganglia and cerebellum), Huntington's disease and some neurodegeneration with brain iron accumulation disorders.

5. Conclusion

NDUFAF6-related Leigh syndrome should be included in the differential diagnosis of LS patients presenting with insidious onset dystonia in early childhood, progressing from gait difficulties to generalised dystonia with predominant oromandibular involvement, dysphagia and dysarthria. Clinical deterioration is associated with symmetric and bilateral cytotoxic lesions in the putamen, with vascular hypertrophy and proliferation of the lenticular-striate arteries, spreading to the caudate, and progressing to cavitation and volume loss, fulfilling radiological criteria of isolated striatal necrosis in the majority of cases. Reduced assembly of mature complex I and reduced complex I enzymatic activity is characteristic, while systemic mitochondrial biomarkers may be normal. Even when next generation sequencing techniques permitted the identification of candidate variants, functional genetic complementation assays allowed us to obtain conclusive evidence for pathogenicity in this family.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jymgme.2019.01.001>.

Disclosures

Authors report no disclosures.

Contributions

HB and BPD participated in the study design, acquisition, analysis and interpretation of data and drafting of the manuscript. OAL and MC were responsible for the collection and analysis of clinical data. LMS and RA participated in the analysis and interpretation of biochemical and molecular genetic studies. ASM and EV participated in the analysis and interpretation of brain MRIs. PG, SE and NG-P participated in the molecular analysis and complementation test. ER-P, JM and MPB-B participated in experiment design, analysis and interpretation of data and drafting of the manuscript. BPD conceived the idea for the study, designed and supervised the study, interpreted data and drafted and revised manuscript content. All authors read and approved the final manuscript.

Funding

This work was funded by grants from the “Instituto de Salud Carlos III” (FIS-PI15/00287, PI18/01319, PI 17/00021, PI17/00166), Fundación Mútua Madrileña, the European Social Fund (ESF) and the Fundació La Marató TV3 (20143130 to BPD).

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