

## A novel m.11406 T > A mutation in mitochondrial *ND4* gene causes MELAS syndrome

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### ABSTRACT

Pathogenic point mutations of mitochondrial DNA (mtDNA) are associated with a large number of heterogeneous diseases involving multiple systems with which patients may present with a wide range of clinical phenotypes. In this study, we describe a novel heteroplasmic missense mutation, m.11406 T > A, of the *ND4* gene encoding the subunit 4 of mitochondrial complex I in a 32-year-old woman with recurrent epileptic seizure, headache and bilateral hearing loss. Skeletal muscle histochemistry demonstrated that approximately 20% of fibers were cytochrome *C* oxidase (COX) deficient with increased activity of succinate dehydrogenase (SDH). Further investigations in muscle specimens showed significantly reduced level of *ND4* protein. It is interesting that the subunits of complex I (*ND1* and *NDFUB8*) and complex IV (*CO1*) were also remarkably decreased. These findings indicate that *ND1*, *NDFUB8* and *CO1* are more susceptible than other subunits to mutations in the mitochondrial *ND4* gene.

### 1. Introduction

Mitochondrial diseases are defined as disorders with primary dysfunction of the mitochondrial respiratory chain (MRC). Mitochondrial diseases usually affect multiple systems of the organism with a heterogeneous set of symptoms and tend to affect organs with higher energy consumption, such as skeletal muscle, central nervous system, the liver, and the heart (Chakrabarty et al., 2018). The etiology of mitochondrial diseases involves mutations in either the maternally inherited mitochondrial DNA (mtDNA) or Mendelian-inherited nuclear DNA (nDNA) (Chen and Butow, 2005; Koopman et al., 2013). Currently, more than 300 pathogenic point mutations have been found in human mitochondrial diseases (Liu et al., 2018).

Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) is one of the most common mitochondrial disorders. The onset of symptoms is typically in childhood or early

adulthood. Clinical manifestations include encephalopathy, stroke-like episodes, hearing loss, lactic acidosis, recurrent headaches, myopathy and short stature (Sparaco et al., 2003). These comorbidities may lead to significant disability and shorten life expectancy. Although MELAS is mostly caused by point mutations in mitochondrial tRNA gene, some MELAS-related mutations are also found in the genes encoding polypeptides, especially the genes related to complex I (Ruiz-Pesini et al., 2007).

Complex I (NADH: ubiquinone oxidoreductase) is the first step and the most abundant enzyme in the electron transfer chain (ETC) (Distelmaier et al., 2009). It catalyzes the oxidation of NADH and transfers electrons from NADH to ubiquinone (coenzyme Q10). Respiratory complex I consist of 45 subunits, including 7 subunits (*ND1*–*ND6*, *ND4L*) encoded by mtDNA, and 38 by nDNA (accessory subunits and assembly factors) (Fiedorczuk and Sazanov, 2018; Haapanen and Sharma, 2018). To date, about 20 disease-related

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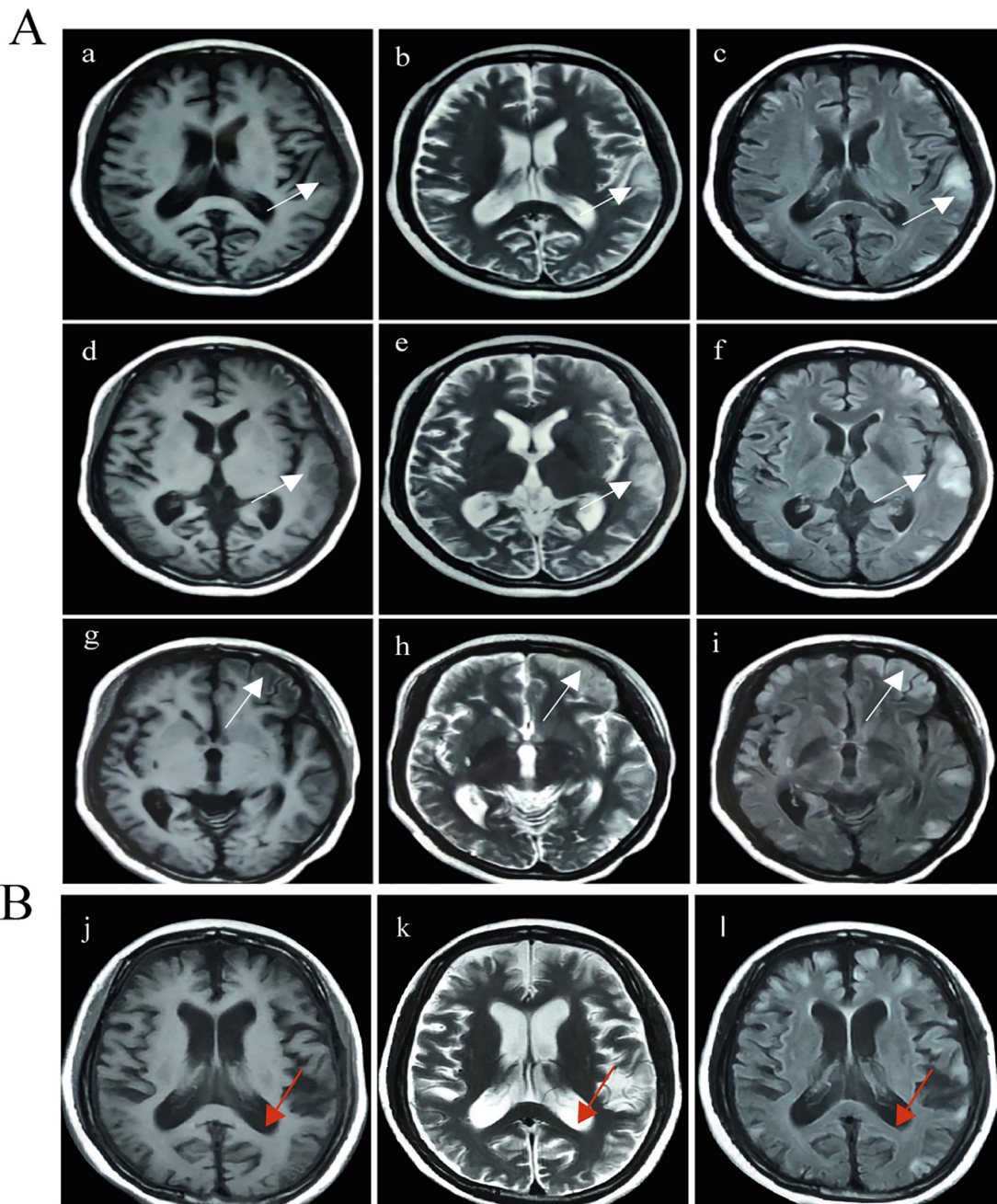
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**Fig. 1.** MRI findings of the patient at the age of 25 and 31. **A.** Brain MRI performed at 25 years of age demonstrating multiple cortical lesions (white arrows). (a, d, g: T1 weighted sequence; b, e, h: T2-weighted; c, f, i: FLAIR) **B.** Brain MRI at 31 years of age demonstrates progressive cortical atrophy and resulting ventriculomegaly (red arrows) (j: T1 weighted sequence; k: T2-weighted; l: FLAIR).

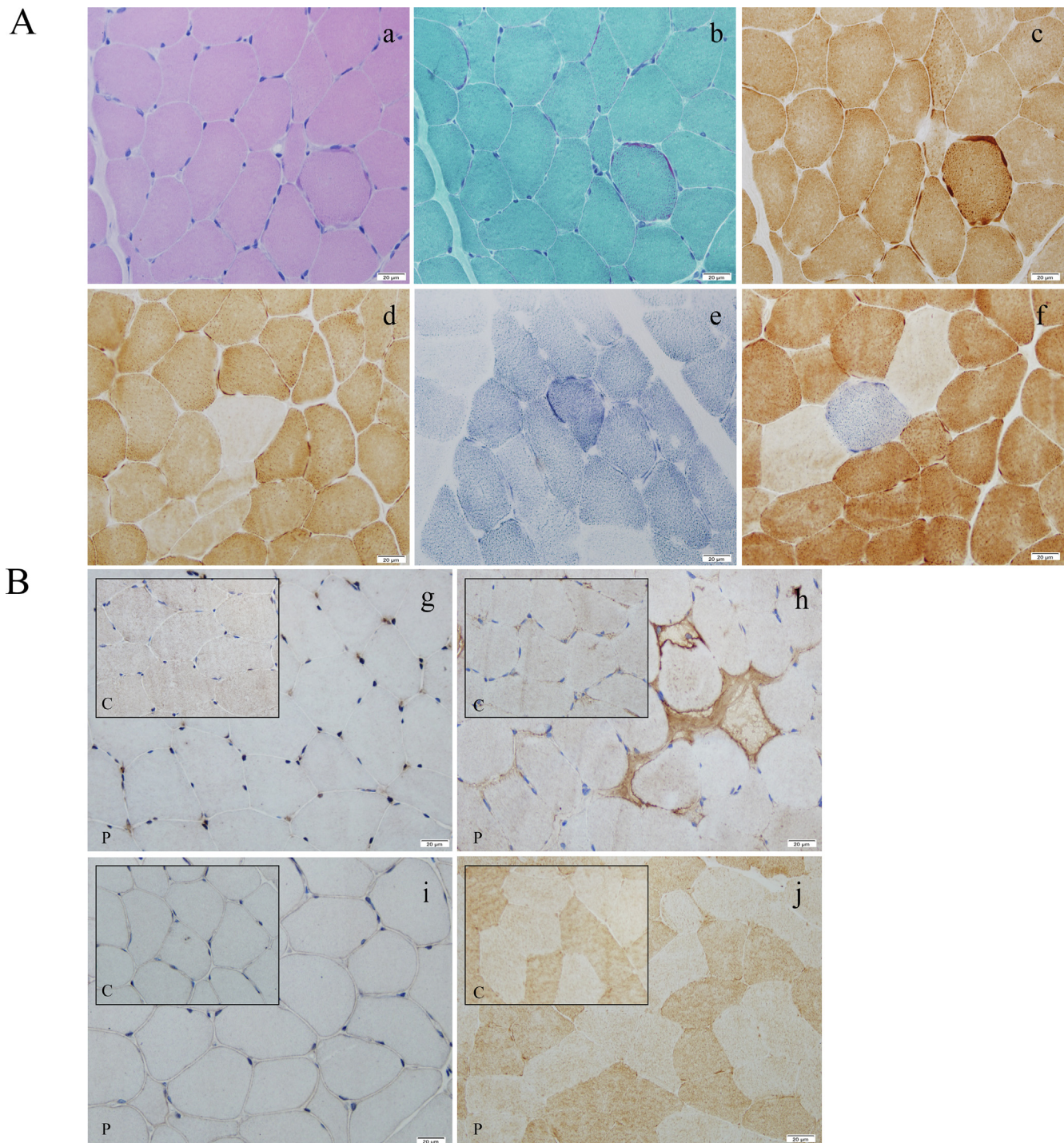
variants in complex I have been identified in various diseases, including Leigh syndrome (LS), Leber hereditary optic neuropathy (LHON), progressive external ophthalmoplegia (PEO), MELAS syndrome, exercise intolerance, diabetes mellitus, tumor, Parkinson's disease (PD), sporadic Creutzfeldt-Jakob disease (sCJD), and overlapping syndromes (Barshad et al., 2019; Fang et al., 2015; Kabekkodu et al., 2015; Lyu et al., 2019).

Here, we report a novel pathogenic mutation in the *ND4* gene (m.11406 T > A; p.Leu216His) in a 32-year-old female with MELAS syndrome and confirm the pathogenicity by combination of clinical analyses, muscle histochemistry analyses, and so on.

## 2. Materials and methods

### 2.1. Subject

This patient was a 32-year-old female, the second child of two non-consanguineous parents. Since she was 20 years old, the patient suffered from recurrent stroke-like episodes, seizures, headache, and bilateral hearing loss. At the age of 22, she was admitted into hospital for frequent seizures episodes. Mitochondrial hotspot sequencing did not reveal any positive information. And no any other systemic involvement, such as short stature, diabetes mellitus, or heart failure were found. Since then, her seizure was aggravated gradually. She has a healthy older brother. No family history for mitochondrial disorders or neurologic diseases was contributory. Neurological examination at the



**Fig. 2. Skeletal muscle histology and histochemistry findings of the patient. (\*400)** A. Histology findings of the patient. (a) HE stain showing muscle architecture is relatively normal. (b) Gomori trichrome stain showing dark staining in subsarcolemmal areas in several fibers. (c, d) COX stain showing approximately 20% of fibers were COX-negative, and some COX-positive fibers are darkly stained. (e, f) SDH enzyme histochemistry and COX/SDH double stain showing COX-negative fibers are increased activity of SDH. B. Histochemistry findings of the patient. (g) Immunohistochemical ND4 staining showed a remarkably decreased expression in almost all muscle fibers. (h) ND1 staining identified a small fraction of fibers with a subsarcolemmal accumulation of mitochondria. (i, j) The expression of ND5 and VDAC1 proteins was comparable in the patient and controls. Insets show staining of an age-matched control. Scale bar (a–f), 20  $\mu\text{m}$ . C, control; P, patient.

age of 32 revealed a decrement proximal muscle strength (grade 4). Muscle atrophy in all extremities was noted. Fasting blood lactic acid level was 2.8 mmol/L (0.5–2.2 mmol/L). Serum level of creatine kinase (CK) was within the normal range. Magnetic resonance imaging (MRI) of the head demonstrated abnormal high signal on T2 weighted image (T2WI) and fluid-attenuated inversion recovery (FLAIR) in the cortical and subcortical regions involving bilateral temporal, parietal, and

occipital lobes (Fig. 1). Results from electrocardiogram (ECG) and echocardiogram were normal.

## 2.2. Histologic analysis of muscle biopsy

A biopsy of the left quadriceps femoris was performed. Serial 10  $\mu\text{m}$ -thick cryosections were obtained and stained according to standard

**Table 1**  
mtDNA variants detected in this patient using whole mitochondrial genome sequencing on a skeletal muscle sample.

Position	Gene(location)	rCRS base	Mutation	Coverage	AA Change	mtDNA database
594	MT-TRNF		insC	5492	No	Polymorphic sites
750	MT-RNR1	A	G	7800	No	Polymorphic sites
1438	MT-RNR1	A	G	7600	No	Polymorphic sites
2706	MT-RNR2	A	G	7339	No	Polymorphic sites
3107	MT-RNR2		delA	7576	No	Polymorphic sites
8584	MT-ATP6	G	A	7332	p.Ala20Thr	Polymorphic sites
8701	MT-ATP6	A	G	6653	p.Thr59Ala	Polymorphic sites
8860	MT-ATP6	A	G	7169	p.Thr112Ala	Polymorphic sites
10,398	MT-ND3	A	G	6961	p.Thr114Ala	Polymorphic sites
<b>11,406</b>	<b>MT-ND4</b>	<b>T</b>	<b>A</b>	<b>7365</b>	<b>p.Leu216His</b>	<b>Not reported</b>
14,318	MT-ND6	T	C	6470	p.Asn119Ser	Polymorphic sites
14,766	MT-CYTB	C	T	6669	p.Thr7Ile	Polymorphic sites
15,080	MT-CYTB	A	G	7414	p.Thr112Ala	Polymorphic sites
15,326	MT-CYTB	A	G	6357	p.Thr194Ala	Polymorphic sites

rCRS: revised Cambridge Reference Sequence; AA: amino acid.

histologic protocols (Dubowitz et al., 2013; Old and Johnson, 1989). The stainings included hematoxylin and eosin (H&E), modified Gomori trichrome (MGT), NADH-tetrazolium reductase, cytochrome C oxidase (COX), succinate dehydrogenase (SDH), COX/SDH double histochemistry, oil red O (ORO), and adenosine triphosphatase (ATPase) at pH 4.3 and pH 10.4. The ND1, ND4, and ND5 subunits of complex I were detected by immunohistochemistry (ND1: ab181848, Abcam; ND4: A9941, Abclonal; ND5: ab92624, Abcam). Immunostaining for VDAC1 (ab15895, Abcam) was employed to detect mitochondria.

### 2.3. Mutational analysis of the mitochondrial genome

Total DNA from all available samples (skeletal muscle, blood, urine sediment, and buccal mucosa) was extracted by using the TIANamp Genomic DNA Kit (Tiangen, China). Direct sequencing of the entire mitochondrial genome (16,569 base pairs) was performed by next-generation sequencing using DNA from skeletal muscle homogenate utilizing previously described standard procedures (Rieder et al., 1998). Sanger sequencing of mtDNA was performed as previously described.

### 2.4. Western blotting

For western blotting analysis, proteins from the muscle tissue of the patient, as well as age- and sex-matched healthy control subjects were separated by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The gels were transferred onto polyvinylidene difluoride membranes (Immobilon-P PVDF-Membrane, Millipore, Burlington, MA, USA). After blocking with 5% dried skimmed milk and 0.1% Tween 20 in TBS, the membranes were incubated for 16 h at 4 °C with the following antibodies: total OXPHOS antibody cocktail (ab110413, Abcam), anti-MT-ND4 (A9941, Abclonal), anti-MT-ND1 (ab181848, Abcam), anti-MT-ND5 (ab92624, Abcam), anti-MT-ATP6 (ab192423, Abcam), anti-COX-IV (CST 4805 s, Cell Signaling Technology), anti-VDAC1 (ab15895, Abcam), anti-TOMM20 (ab56783, Abcam), and anti-GAPDH (10494-1-AP, Proteintech). The membranes were then incubated for 1 h at 25 °C with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Beyotime A0216, A0208). Protein signals were revealed with Immobilon Western Chemiluminescent HRP substrate (Millipore WBKLS0500). Digital images were acquired using the ECL system (Tanon 4600 SF) and quantified using the ImageJ software.

## 3. Results

### 3.1. Morphological and immunohistochemical analysis

Serial sections of quadriceps muscle biopsy did not show any typical

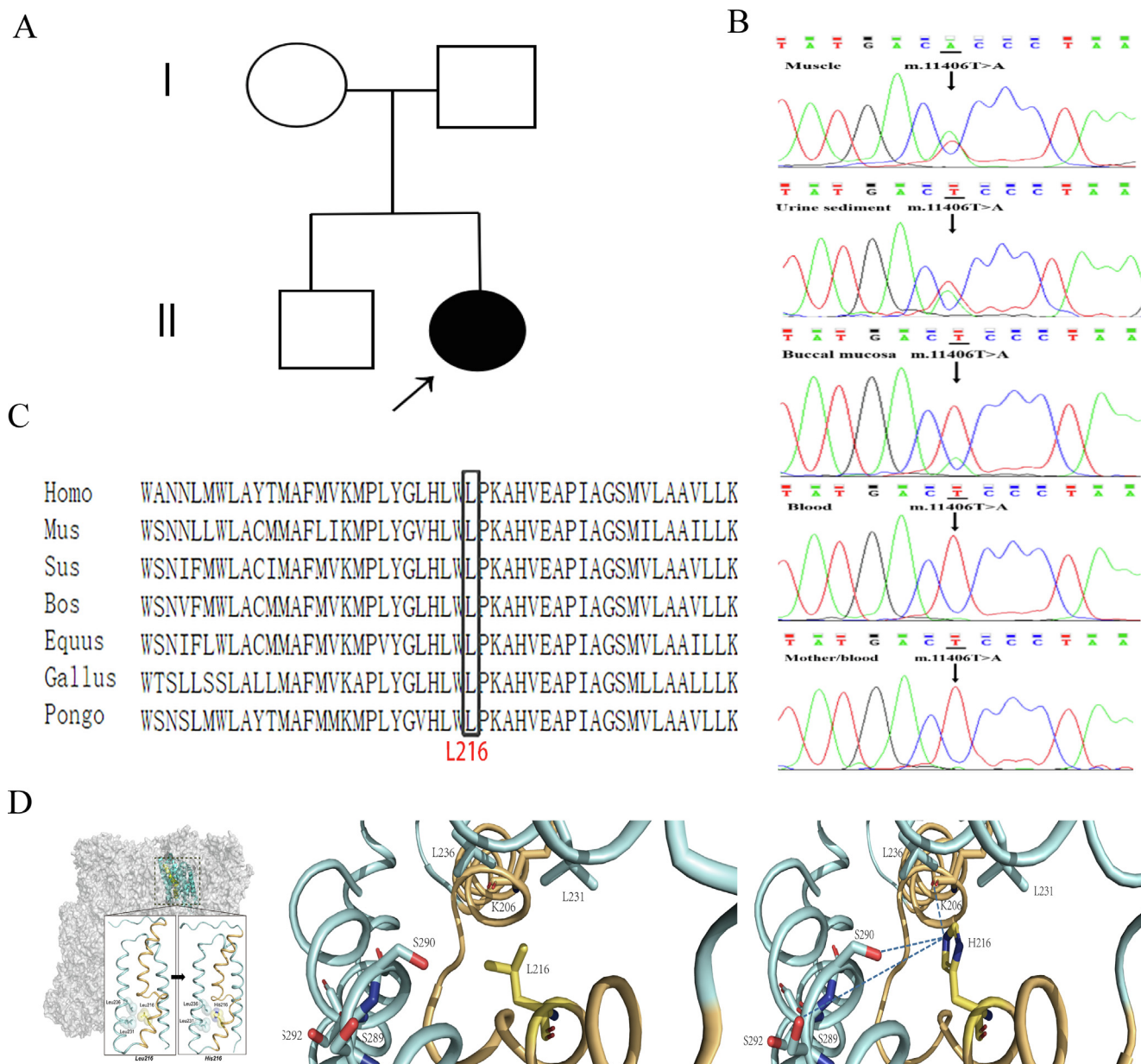
ragged red fibers (RRFs), although mildly increased subsarcolemmal staining was noted with MGT and COX stain (Fig. 2A a–c). Approximately 20% of fibers evaluated by both the SDH and COX/SDH stain (Fig. 2A d–f), were COX-negative, with most of them exhibited retained activity of SDH. Also, we gave the immunohistochemical stain to multiple subunits of complex I. Compared to healthy controls, muscle specimens from the patient had a remarkably decreased expression of ND4 (Fig. 2B g). ND1 staining identified a small fraction of fibers with a subsarcolemmal accumulation of mitochondria (Fig. 2B h). The expression of ND5 and VDAC1 proteins was comparable in the patient and controls (Fig. 2B i, j).

### 3.2. Identification of a novel pathogenic ND4 mutation

Next-generation sequencing (NGS) of the whole mitochondrial genome identified a novel missense mutation m.11406 T > A in ND4 gene in muscles of the patient with the average sequencing depth  $7657 \pm 579X$  (Table 1). This mutation resulted in a substitution of leucine at position 216 to histidine (p.Leu216His) in ND4. The mutation was further confirmed by Sanger sequencing in other samples from the patient. The highest level of mutation load was found in the muscle homogenate (66.1%), with lower levels present in the urine sediment (42.3%), buccal mucosa (20.4%) and no mutation was detected in the blood sample. The mutation was not detectable in the DNA extracted from urine sediment and blood of the patient's mother, suggesting that this mutation appeared de novo (Fig. 3B). Multi-alignment analysis demonstrated that this amino acid in ND4 protein was highly conserved among different species (Fig. 3C). In addition, we used Cryo-EM structure of ND4 domain in human respiratory complex I to analyze the effects caused by this mutation. From the close-up view, Leu-216 is located at the axis of the helix-loop-helix, contributing to the stabilization of the packing of ND4 domain. The axis is propelled to a new direction because of the replacement of the hydrophobic interaction by ionic bonds. The substitution is predicted to destabilize the ND4 domain, which is necessary for complex I assembly and function (Fig. 3D).

### 3.3. Downregulation of mitochondrial proteins

To determine whether the m.11406 T > A mutation may impair mitochondrial function, the levels of 6 mtDNA-encoded respiratory complex subunits and 5 nDNA-encoded respiratory complex subunits were evaluated by western blotting. The average levels of the 6 subunits encoded by mtDNA (ND1, ND4, ND5, CYB, CO1, ATP6) in the patient were 60.1%, 8.0%, 91.4%, 102.2%, 80.5%, and 99.3% of the average values of controls, respectively (Fig. 4A and B). The average levels of nDNA-encoded subunits (NDFUB8, SDHB, UQCRC2, COX4, ATP5A), were 47.7%, 97.5%, 103.6%, 98.4% and 99.3% of those in controls



**Fig. 3. The analysis of a novel variant in *ND4* gene.** **A.** Pedigree of the family. The proband is indicated by an arrow. **B.** Sanger sequencing of mitochondrial *ND4* gene from patient and her mother. Electropherograms of nucleotide position 11400–11412 showing heteroplasmic T–A transition at position 11,406 in patient’s muscle homogenate, urine sediment and buccal mucosa. The variant was not detected in patient’s blood sample and her mother’s blood and urine sediment. **C.** Multi-alignment analysis. The comparison of different eukaryotic species, showing that the base pair involving the nucleotide at position 11,406 in *ND4* is highly conserved. **D.** Cryo-EM structure of human respiratory complex I to analyze the point mutation m.11406 T > A in *ND4* gene. Left panel: The 216 leucine converts to histidine, this changing of interaction leads to rotation of the axis. Middle panel: Leu-216 is located at the axis of the helix-loop-helix, contributing to the stabilization of the packing of the domain. Leu-216, which has hydrophobic side chain, forms the hydrophobic interaction with Leu-231 and Leu-236. Right panel: Substitution of the Leu-216 to His-216 dramatically transfer the hydrophobic interaction to ionic bond among Ser-289, Ser-290 and the backbone group of Lys-206.

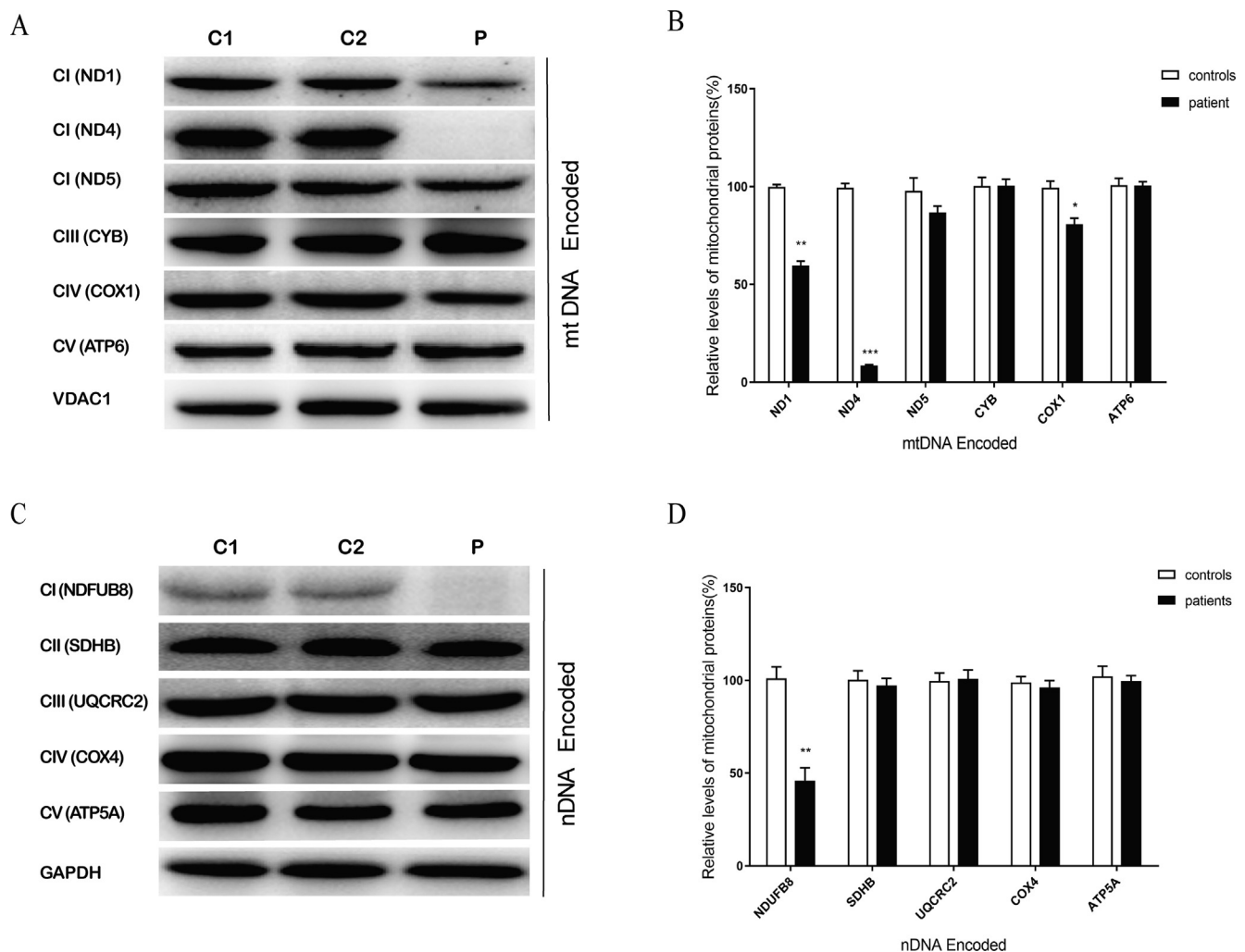
(Fig. 4C and D). Western blotting showed an almost undetectable amount of the *ND4* protein in muscle sample from the patient. The amount of other complex I proteins, probed by antibodies against ND1 and nuclear-encoded complex I subunit *NDUFB8* were also markedly decreased. Moreover, complex IV subunit *CO1* was also mildly reduced compared to controls.

#### 4. Discussion

In the present study, we found a novel missense mutation in the *ND4* gene, (m.11406 T > A; p.Leu216His) in a 32-year-old female presenting with MELAS syndrome. There are several pieces of evidence to

indicate the pathogenic nature of this mutation. Firstly, the variant is not recorded in any of the available databases including MITOMAP, mtDB and mtSNP. Secondly, this mutation is heteroplasmic and present at high levels in skeletal muscle of the patient (66.1%). Thirdly, the sequence homology analysis indicate that the nucleotide at position 11,406 is highly conserved among different species. Lastly, *ND4* protein level was dramatically decreased in patient’s muscle.

Complex I is the largest enzyme complex in MRC and the most common cause of oxidative phosphorylation diseases (Barshad et al., 2019; Hernansanz-Agustín et al., 2017; Sharma et al., 2009). The *ND4* gene encodes one of the major subunits of the MRC Complex I. *ND4* is probably a component of the hydrophobic protein fragment, and it is



**Fig. 4. Analysis of protein expression in the muscles of control and patient.** A. Western Blots were processed and probed with 6 mtDNA encoded subunits (ND1, ND4, ND5, CYB, COX1, ATP6). B. Quantification of total mitochondrial protein levels. The average levels mitochondrial proteins in controls and patient were showed. C. Western Blots were processed and probed with a total OXPHOS human antibody cocktail directed to subunits of complex I (NDFUB8), complex II (SDHB), complex III(UQCRC2), complex IV (cytochrome c oxidase subunit IV, COX4), and complex V (ATP5A). D. Quantification of 5 polypeptides were described. C, control; P, patient. Data are calculated based on three independent experiments. \* $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\* $P < 0.001$  compared with controls.

predicted to be essential for electron transfer through the membrane based on its molecular structure (Damm et al., 2012). Mutations in this gene cause mitochondrial ATP production failure that compromises cellular energy metabolism (Jiang et al., 2015; Xu et al., 2017).

According to MITOMAP, OMIM and ClinVar database, a total of 18 *ND4* variants have been reported, and most of them were frequently observed in patients with mitochondrial diseases (Table 2). m.11777C > A and m.11778G > A are the only two deemed pathogenic mutations, which are associated with LS, LHON and dystonia, respectively. (Charlmers and Harding, 1996; Komaki et al., 2003; Simon et al., 1999) In our study, patient with the novel m.11406 T > A mutation was presented with MELAS and significant deficiency in mitochondrial complex I.

Interestingly, we find this novel mutation in *ND4* may affect the stability of ND1 and the nDNA-encoded subunit NDFUB8. Additionally, a slight decrease in the subunit of complex IV protein (CO1) was also found. Recent advances in the technology of separation of mitochondrial membrane proteins, as well as techniques for the reconstruction of complex structures based on electron microscopy images, promoted the notion that the five mitochondrial respiratory complexes are tightly connected to form a supercomplex (Fedor et al., 2017; Minocherhomji et al., 2012; Porras and Bai, 2015; Vartak et al., 2013). Complex I, responsible for the initial step in the electron transfer participates in the

assembly of each subunit, requiring the cooperation of numerous assembly factors (Gorman et al., 2015; Vartak et al., 2014). Mutations from any of the subunits of complex I result in the failure of the complete assembly of this subunit and may negatively affect the activity of the remaining subunits of complex I. Using the yeast two-hybrid system, Gershoni et al. (2010) predicted the interaction between NDUFC2 and ND4, as well as the interaction among NDUFA1, ND1, and ND4. Yan et al. (2017) concluded that ND1 and ND4 interact with each other and are involved in oxidative damage to the dopaminergic system of the substantia nigra. Sugiana et al. (2008) demonstrated that a mutation in *NDUFA5* gene affects the formation of 400 kDa assembly subcomplexes and in turn, inactivate the ND1, NDUFS2, -3, -7, -8, and NDUFA9 subunits.

In conclusion, the novel pathogenic m.11406 T > A mutation not only significantly affects the expression of the ND4, but also the that of ND1, NDFUB8 and complex IV subunit CO1. Our findings support the notion that subunits of respiratory complex I cooperate with each other during their assembly, although the mechanism of the interaction remains unclear and warrants further research.

**Table 2**  
Summary of the cases reported to harbor mutations in *ND4* gene.

Nucleotide Position	Nucleotide Change	AA Change	Disease association	Status	Reference
11,084	A-G	p.Thr109Ala	AD, PD, MELAS	Conflicting reports	(Pereira et al., 2011; Lertrit et al., 1992)
11,126	G-A	p.Glu123Lys	Papillary thyroid carcinoma	Reported	(Abu-Amero et al., 2005)
11,204	T-C	p.Phe149Leu	sCJD	Reported	(Zhang et al., 2015)
11,232	T-C	p.Leu158Pro	CPEO	Reported	(Mitchell et al., 2006)
11,240	C-T	p.Leu161Pro	LS	Reported	(O'Keefe et al., 2018)
11,253	T-C	p.Ile165Thr	LHON, PD	Reported	(Leo-Kottler et al., 2002)
11,330	G-C	p.Ala191Pro	LHON	Reported	(Zhou et al., 2006).
11,406	T-A	p.Leu216His	MELAS	<b>Present work</b>	<b>Present work</b>
11,696	G-A	p.Val313Ile	LHON, Leber's optic atrophy	Likely Pathogenic	(Pereira et al., 2011)
11,777	C-A	p.Arg340Ser	LS	Pathogenic	www.mitomap.org
11,778	G-A	p.Arg340His	LHON, Dystonia	Pathogenic	www.mitomap.org
11,832	G-A	p.Trp358Ter	Exercise intolerance	Reported	(Taivassalo et al., 2002)
11,874	C-A	p.Thr372Asn	LHON	Reported	(Abu-Amero and Bosley, 2006)
11,919	C-T	p.Ser387Phe	Thyroid Cancer	Reported	(Abu-Amero et al., 2005)
11,984	T-C	p.Tyr409His	LS	Reported	(Vanniarajan et al., 2006)
11,994	C-T	p.Thr412Ile	OAT	Conflicting reports	(Selvi Rani et al., 2006)
12,015	T-C	p.Leu419Pro	Atypical MELAS	Reported	(Endres et al., 2019)
12,018	C-G	p.Thr420Ser	LS	Reported	(Bacalhou et al., 2017)
12,026	A-G	p.Ile165Val	Diabetes mellitus	Reported	(Tawata et al., 1998)

AA: amino acid; PD: Parkinson's disease; AD: Alzheimer's disease; MELAS: mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; sCJD: sporadic Creutzfeldt-Jakob disease; CPEO: chronic progressive external ophthalmoplegia; LHON: Leber hereditary optic neuropathy; LS: Leigh syndrome; OAT: Oligoasthenoteratozoospermia.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mito.2020.06.011>.

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