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The mitochondrial DNA variant m.9032T>C in *MT-ATP6* encoding p.(Leu169Pro) causes a complex mitochondrial neurological syndrome.

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Abstract

Diagnosing complex V deficiencies caused by new variants in mitochondrial DNA is challenging due to the rarity, phenotypic diversity, and limited functional assessments. We describe a child with the m.9032T>C variant in *MT-ATP6* encoding p.(Leu169Pro), with primary presentation of microcephaly, ataxia, hearing loss, and lactic acidosis. Functional studies reveal abnormal fragment F₁ of complex V on blue native gel electrophoresis. Respirometry showed excessively

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Ethics approval: Studies at the University of Colorado were done following IRB-approved protocols 16–0146 and 18–0128. Written informed consent was obtained prior to initiation of research as outlined in the IRB-approved study.

IACUC: No animals were used in this study.

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tight coupling through complex V depressing oxygen consumption upon ADP stimulation and an excessive increase following uncoupling, in the presence of upregulation of mitochondrial biogenesis. These data add evidence about pathogenicity and functional impact of this variant.

Keywords

complex V; phenotype; MT-ATP6; blue native PAGE; respirometry

1. Introduction

Mitochondrial diseases are a heterogeneous group of diseases caused by pathogenic variants in either the nuclear or mitochondrial genome. Diagnosing mitochondrial disease due to changes in mitochondrial DNA (mtDNA) has been difficult due to the large diversity in clinical and biochemical phenotypes^{1,2}. Diagnosing disease due to mitochondrial complex V variants has been particularly challenging given the limited availability of tools to confirm their functional significance. Complex V (ATP synthase EC 7.1.2.2) is composed of 15 structural and 2 assembly subunits, of which two, ATP6 and ATP8, are mtDNA-encoded^{3,4}. The mitochondrial DNA encoded *MT-ATP6* gene product, ATPase subunit a, is composed of six transmembrane domains. It contributes to the proton pore of the complex and is essential to couple proton translocation to the rotation of the complex V enzymatic subunit involved in ATP synthesis⁵. Recent reviews of all available cases of complex V deficiency due to pathogenic variants in *MT-ATP6* (OMIM 500015) highlighted the great diversity of clinical and biochemical features that exists among genetic variants^{6,7}. Currently 33 *MT-ATP6* variants are either known or suspected to cause disease, but 82% of all published cases are due to just 4 of these variants⁷. Defining the phenotype and the functional impact of those less well-described variants is of importance to better understand the disease pathophysiology, and to facilitate diagnosis in those with less specific phenotypes. Assays to evaluate the functional impact of a complex V variant have shown a decrease in complex V holoenzyme assembly or integrity, abnormal mitochondrial membrane potential (either increased or decreased), reduced ATP synthetic rate, ATP hydrolysis rate, ATP steady state levels, or an impact on oxygen consumption levels in the coupled versus uncoupled state^{7,8}. The most reliable way today to prove the pathogenic nature of a variant is the identification in unrelated, yet similarly affected patients⁷.

In this report we describe a 7 year-old male with a complex phenotype due to m.9032T>C, p.(Leu169Pro) in *MT-ATP6*. This variant has been reported previously only in a single family of a proband and mother both with a neuropathy, ataxia, retinitis pigmentosa (NARP) phenotype⁵. We document additional functional and biochemical findings providing functional confirmation of the pathogenic nature of this variant.

2. Methods

2.1 Clinical studies

All procedures were done in accordance with the standards of the ethics committee in accordance with the Helsinki Declaration of 1975 and as revised in 2000. The patient is

enrolled in a Colorado Institutional Review Board approved study (COMIRB# 16-0146), and informed consent was obtained for this study. Fibroblasts were further studied in agreement with COMIRB# 18-0128. Clinical information was obtained via chart review of the patient's medical record, and neuroimaging was reviewed by a pediatric neuroradiologist (DMM).

2.2. Genetic and biochemical studies

Next generation sequencing of mtDNA was performed in a clinical laboratory as described¹⁰. Respiratory chain enzyme activities of complexes I, II, II+III, III, IV and citrate synthase were analyzed in post-600x g supernatants isolated from muscle and fibroblasts as described^{11,12}. Solubilized inner mitochondrial membrane fractions isolated by differential centrifugation from muscle and fibroblasts were separated by blue native polyacrylamide gel electrophoresis (BN-PAGE) and evaluated by in-gel activity staining assays for complexes I, II, IV, and V as described previously^{11,12}. The activity of the complex V oligomycin-sensitive ATPase activity (reverse reaction) was measured in duplicate by a modification of the previously published method as described^{13,14}. Fibroblasts were grown in α -MEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids and 1% antibiotic/antimycotic solution and harvested at 80% confluency. The cell pellet was resuspended in MegaFB buffer (250 mM sucrose, 2 mM HEPES, 0.1mM EGTA, pH 7.4), twice homogenized and centrifuged at 1000 g for 5 minutes, the supernatant was centrifuged at 14,400 g for 10 minutes to pellet the mitochondria before resuspending in 100 μ L MegaFB buffer. Protein was assayed using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). For the ATPase reaction, 50 μ L 10X assay buffer (400 mmol/L Tris-HCl, pH 8.0, 50 mmol/L $MgCl_2$, 100 mmol/L KCL, 40 U/mL lactate dehydrogenase, 40 U/mL pyruvate kinase, 2 mmol/L NADH, 20 mmol/L PEP, 5 mmol/L ATP, 1% BSA, 10 μ mol/L antimycin A, and 30 μ mol/L FCCP) and freshly prepared mitochondria equivalent to 5 μ g protein were combined, and then water pre-warmed at 30°C was added to complete the assay volume to 500 μ L. After mixing, it was sonified for 10 sec using a Branson Sonifier 450 at 10% output. The mixture was immediately placed in a Cary 300 UV/VIS spectrophotometer preset at 37°C and the oxidation of NADH was followed at 340 nm for 5 min, after which 1.2 μ L 2.5 mmol/L oligomycin was added to follow the reaction for an additional 5 minutes to determine the oligomycin-insensitive ATPase activity.

High resolution respirometry was performed on an Oroboros Oxygraph 2k system following a substrate inhibitor (SUIT) protocol as described¹⁵. Briefly, fibroblasts were grown to a confluency between 70 to 80% and harvested following treatment with trypsin. Cells were then resuspended in mitochondrial respiration buffer MIRO5 at a concentration of 5×10^5 cells/mL and 2 mL of the cell suspension were placed into the O2k chamber. Oxygen consumption (pmol O_2 /second/ 1×10^6 cells) was measured using a SUIT protocol performed in the following order: routine, malate (5 mM), pyruvate (2 mM), digitonin (2 μ g/mL), ADP (2.5 mM), glutamate (10 mM), succinate (10 mM), CCCP (uncoupler) (0.25 μ M, titrated until maximal rate), rotenone (0.05 μ M), antimycin A (0.125 μ M), tetramethyl-p-phenylenediamine (TMPD, 0.5 mM) + ascorbate (2 mM), and azide (150 mM) (Supplementary Figure 1). Calculated derived parameters include: acceptor control ratio (ADP/pyruvate), glutamate increase over pyruvate (glutamate-ADP)/glutamate), Q junction

(Succinate/glutamate), coupling ratio (succinate/CCCP) and uncoupling increase (CCCP-Succinate), complex I rate (CCCP-rotenone), complex IV rate ((TMPD+ascorbate)-azide)). Three replicates of the patient cells and 41 control fibroblast cell lines each done at least in duplicate were compared. Populations were described as mean and standard deviation, and comparisons between these two populations were done by Student t-test.

3. Case Report

The patient was born to non-consanguineous parents of mainly mixed European descent after a normal pregnancy by C-section for failure of progression of labor. After a normal neonatal period, he presented at age 2.5 months with myoclonic movements identified as right sided focal partial epilepsy and elevated lactic acid of up to 5.8 mM, but reportedly normal brain magnetic resonance imaging (MRI). He was initially treated with levetiracetam but developed choreatic movements that impaired fine motor skills and his ability to feed orally. The levetiracetam was cross titrated to oxcarbazepine and was discontinued by 6–7 months, and the abnormal movements resolved with the cessation of the levetiracetam. The patient did not have seizures after age 4 months, but did experience considerable developmental stagnation during the adjustment of anticonvulsant medications. He had a normal head circumference at birth, but his head growth plateaued at age 6 months and he developed progressive severe microcephaly. Anticonvulsant therapy was weaned off at age 9 months followed by remarkable developmental progression between ages 9 and 15 months including learning to sit independently at 11 months, crawl at 12 months, pull-to-stand and cruise by age 15 months, at which time he had a normal physical exam other than microcephaly and mild central hypotonia. He was able to ambulate independently by age 3 years.

During the next two years, he presented four times a year with febrile illness associated episodes of lactic acidosis (4 to 8 mM) often with associated Kussmaul breathing, with non-epileptic myoclonic or choreatic movements. These episodes improved with intravenous caloric support and were not associated with additional periods of developmental regression. He developed progressive mild to moderate high frequency hearing loss and required hearing aids from age 5 years on. Although he continued to make slow developmental progress, he started falling behind. He also exhibited an increasingly ataxic gait from age 3 years 7 months. Speech was limited to a few words with a few signs. At age 4 year 4 months he had a circling gait with some spasticity and benefited from ankle orthoses. He started treatment with ubiquinol 10 mg/kg/day at age 5 years without noticeable improvement. At age 6 years, leucovorin 1 mg/kg/day was added with resulting impression of increased alertness, focus and engagement. At age 6 years 11 month, he started having nocturnal epileptic events and on electroencephalogram (EEG) exhibited diffuse spikes of central vertex predominance, for which he was started on lamotrigine. He has dysmetria and ataxic gait on physical exam, but no spasticity.

At age 7 years, his weight was 17 kg, 0.5th percentile $Z=-2.53$ SD, his length was 112 cm 3rd percentile $Z=-1.86$ SD, and his head circumference was 45 cm, with the second percentile for age at 49 cm. Currently, at age 7 year 6 months, he takes all nutrition orally, but cannot feed himself. His developmental progress has been slow without evidence of

regression since seizure-onset. He takes a single antiepileptic agent, lamotrigine, and remains seizure free during the daytime but continues to have nocturnal events. His vision is normal. He has hearing aids for high frequency sensorineural hearing loss. He has thirty words, can dress himself, and count to ten.

Biochemically, in addition to intermittently elevated serum lactate during decompensations, outside an acute event he has serum lactate 2.24 mmol/L, pyruvate 0.20 mmol/L, and plasma alanine 477 μ mol/L (normal 143–439 μ mol/L), but normal 3-methylglutaconic acid 128 nM (normal 144 $\pm$ 58 nM), normal carnitine levels and acylcarnitine profile, and normal creatine kinase and plasma coenzyme Q levels. In cerebrospinal fluid he has elevated lactate 3.34 mmol/L (normal <2 mmol/L), alanine 62 μ mol/L (normal 13–48 μ mol/L), and increased protein 104 mg/dL (normal 12–60 mg/dL), but normal neurotransmitters, pterins, pyridoxal phosphate and 5-methyltetrahydrofolate levels 64 nmol/L (normal 40–187 nmol/L). Since age 3.5 years, he has elevated ACTH 195 pg/mL (normal 0–46 pg/mL) with normal serum cortisol 10.7 μ g/dL, with normal ACTH stimulation test response, and no evidence for other endocrinopathies. An echocardiogram showed normal biventricular function. Genetic testing included normal sequencing of *POLG1*, and normal microarray with only a loss in a region not associated with pathology arr 8q23.3(113347369-113396131)x1.

Brain MRI at age 10 months demonstrated mild cerebellar vermian hypoplasia and borderline low cerebellar volume. Repeat imaging at age 5 years 6 months confirmed both of those findings (Figure 1).

4. Results

4.1. Mitochondrial genetic studies

Next generation sequencing of mtDNA revealed a variant m.9032T>C, p.(Leu169Pro) at a heteroplasmy level of 94.1% in blood and 96.6% in skeletal muscle. Studies of blood samples of the asymptomatic mother and sister did not detect this variant. The mtDNA content in liver was 54% of the mean value of age and tissue matched controls. The variant m.9032C is not present in 30,181 controls in mitowheel (www.mitowheel.org) and in 50,175 controls in mitomap (www.mitomap.org). It is predicted to be pathogenic on Polyphen2 with a score of 0.999. The Leu169 is completely conserved through all species analyzed on Polyphen2.

4.2 Mitochondrial functional studies

On histology there was somewhat increased fiber size with mild type 1 fiber predominance and scattered hypertrophic type 2B fibers, but without further histologic abnormalities and with normal electron microscopy including mitochondrial ultrastructure. The histology of the liver was essentially normal and on electron microscopic examination some enlarged mitochondria bearing nonspecific paracrystalline inclusions were observed, but otherwise the mitochondria were of normal appearance, abundance and distribution.

In skeletal muscle, the respiratory chain enzyme activities showed high activities for all mitochondrial enzymes (Table 1). In fibroblasts, the activities of respiratory chain enzyme complexes I, II, III, and IV were normal. The activity of the oligomycin-sensitive ATPase

activity in fibroblasts in the direction of ATPase was slightly decreased but still within the normal range.

In contrast, blue native-PAGE analysis showed normal activities of complexes I, II, and IV, but for complex V revealed the presence of isolated F₁ subunits as additional bands of lower molecular weight, more pronounced in muscle than in fibroblasts (Figure 2). This finding is often seen in disorders of complex V^{8,16,17}.

On high resolution respirometry (Figure 3, Supplementary Table 1), the rate of oxygen consumption after the addition of malate and pyruvate and permeabilization of the plasma membrane with digitonin was normal, but was not increased much by the addition of ADP, resulting in a significantly low acceptor control ratio. The maximum ADP stimulated coupled rate using the substrates pyruvate, glutamate, and succinate (S) was normal, but after uncoupling the rate increased strongly, resulting in a significant increase with uncoupling, and representing a significantly below normal coupling control ratio (S/CCCP). After uncoupling, the rotenone-sensitive complex I was above normal, and the azide-sensitive complex IV rate was high normal. These results are compatible with increased respiratory capacity but a more restricted rate when coupled with ADP through the dysfunction of complex V.

5. Discussion

We report on a second independent patient with mitochondrial disease due to the m.9032T>C variant in *MT-ATP6* encoding p.(Leu169Pro). The clinical characteristics of all reported patients are summarized in Table 2. In the previously reported family⁹, the proband showed 70% heteroplasmy in blood. His history was notable for mild motor delays, mild ataxia and hypoactive deep tendon reflexes on exam and cerebellar vermian atrophy. He also had pigmentary retinopathy and sensorineural hearing loss. He had recurrent metabolic acidosis albeit without hyperlactatemia. His mother had 42% heteroplasmy in blood. Her course was notable for progressive gait instability, proximal weakness, and retinitis pigmentosa and hearing loss. This presentation is compatible with the neuropathy, ataxia and retinitis pigmentosa (NARP) phenotype.

Our patient with greater than 90% heteroplasmy in blood and muscle demonstrates a more severe phenotype. In addition, this variant was not detected in the blood of his asymptomatic mother and sister. Our patient also has features of NARP with sensorineural hearing loss, developmental delays, ataxia and cerebellar atrophy, but lacks retinal involvement. Furthermore, he exhibits poor growth and pronounced microcephaly, epilepsy, and risk of adrenal insufficiency. He has central lactic acidosis and recurrent lactic acidosis with illness. Remarkably, our patient did not present with the metabolites associated with multicarboxylase dysfunction previously associated with *MT-ATP6* variant carriers¹⁸, nor with elevated 3-methylglutaconic acid which is sometimes observed in this condition^{9,19}.

In addition to the shared clinical features and lactic acidosis in the patients associated with the m.9032T>C variant, several lines of functional evidence provide proof of pathogenicity of this variant's involvement in complex V dysfunction. In the previously published study,

the authors reported that cybrid models of the m.9032T>C variant developed in 143B cells demonstrated decreased endogenous oxygen consumption and decreased uncoupled respiration compared to controls, with no difference in leaking respiration⁹. The steady state ATP levels were decreased in these cells, reactive oxygen species (ROS) were increased, but the inner mitochondrial membrane potential was not different from controls.

In our patient, we demonstrate lability of complex V with isolated F₁ bands on blue native PAGE with in-gel activity staining present in both muscle and fibroblasts^{8,16,17,20}. Functionally, we showed a tight coupling resulting in inefficient stimulation of oxygen consumption with ADP driven by pyruvate and glutamate, substrates of complex I. This was reflected in a low acceptor control ratio and a depressed coupling ratio resulting in an excessive increase of the rate after uncoupling. These findings are most consistent with a reduced proton flux through the complex V pore driven by ADP, and increased capacity of the respiratory complexes probably through compensatory upregulation as reflected by increased enzyme activities in muscle and increased uncoupled ratios and high-normal complex I and complex IV driven respiration.

6. Conclusions

We conclude that the pathogenic variant m.9032T>C causes a NARP phenotype at lower heteroplasmy level, and at high heteroplasmy level a more complex, slowly progressive ataxic phenotype with growth impairment and severe developmental delay. The varied phenotype and inability to directly assess the enzymatic function of complex V often makes *MT-APT6*-related complex V deficiency challenging to diagnose. We propose the use of blue native PAGE with in-gel activity staining be pursued in the initial round of functional investigation of variants in complex V subunits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Brain magnetic resonance imaging of patient with m.9032T>C, p.(Leu169Pro) in *MT-ATP6*.

(A) Sagittal T1-weighted MR image of the brain demonstrates cerebellar vermian hypoplasia (white arrow). (B-C) Axial T2-weighted MR images of the brain reveal mild diffuse low cerebellar volume (white arrowheads) with sparing of the supratentorial brain.

Figure 2: Blue native PAGE with in-gel activity staining.

Blue native PAGE with in-gel activity staining showed normal activities of respiratory chain enzyme complexes I, II, and IV, but for complex V it reveals the presence of subunits of complexes of lower molecular weight in muscle (A) and less prominent in fibroblasts (B).

Figure 3: High resolution respirometry.

High resolution respirometry shows similar rates with pyruvate, glutamate, and succinate, but less stimulation with ADP as reflected in a significantly decreased acceptor control ratio (pyruvate+ADP/pyruvate). After uncoupling, patient cells show a higher uncoupled rate, reflected in significantly higher uncoupling increase and depressed coupling index (Succinate/CCCP). The calculated complex I rate was significantly increased as well. Average and SD of 3 patient experiments and 41 controls is shown. * $p < 0.05$, ** $p < 0.01$

Table 1:

Results of biochemical testing in muscle and fibroblasts

Respiratory chain enzyme activities									
	Controls	Patient		Controls	Patient		Controls	Patient	
	Activity	Activity	SD	Ratio /CS	Ratio/CS	SD	Ratio/CO II	Ratio/CO II	SD
MUSCLE									
Complex I	23.6–74.8	82.5	+1.9	98–271	199	+1.0	285–767	625	+0.9
Complex II	49.0–133.4	132.2	+1.3	251–573	318	0.1	NA	NA	NA
Complex III	5.7–31.4	65.3	+2.3	19–172	157	1.8	45–369	494	1.7
Complex II-III	34.2–107.6	87.8	+1.1	173–472	211	-0.2	549–1226	664	-0.3
Complex IV	1.1–3.8	2.1	-0.4	4–23	5	-0.5	11–68	16	-0.5
Citrate synthase	159.3–353.3	415.3	+1.9	NA	NA	NA	NA	NA	NA
FIBROBLASTS									
Complex I	49.3–131.1	83.3	-0.1	145–396	213	-0.1	237–754	431	0.2
Complex II	130.9–364.4	281.5	-0.4	297–863	493	-0.5	NA	NA	NA
Complex III	8.0–29.2	12.0	1.1	19–56	56	1.1	36–114	114	1.4
Complex II-III	61.8–158.8	126.9	1.2	131–376	387	1.4	263–1100	786	0.9
Complex IV	2.2–7.1	2.0	-0.8	6–23	8	-0.8	12–35	17	-0.8
Citrate synthase	253.5–544.1	323.4	0.0	NA	NA	NA	NA	NA	NA
Complex V ATPase	155.4–260.4	179.0	-0.9	NA	NA	NA	NA	NA	NA

The activities in muscle and fibroblast tissue of each respiratory chain complex and of the combined complex II-III are shown expressed as $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, and as a ratio over the activity of citrate synthase (activity/CS) and as a ratio over the activity of complex II (activity/CO II). The values are also expressed as standard deviations (Z-score) of the log transformed values of controls, which are normally distributed. Abbreviations: CS = citrate synthase, CO II = complex II. The activity of complex V is reported in the direction of hydrolytic ATPase activity. Results are provided as mean and standard deviation (SD), and for controls the 5th and the 95th percentile are shown. P-values on the Student t-test below 0.05 are shown in bold.

Table 2:

Symptoms in patients with the m.9032C>T; p.(Leu169Pro) pathogenic variant

Feature	Patient 1	Patient 2	Patient 3
Publication	Ref [8], proband	Ref [8], mother	This report proband
Age of onset	12 months	Adult	3 months
Sex	Male	Female	Male
Heteroplasmy levels			
Blood	70%	42%	94%
Other tissue	hair 93%, urine 96%	hair 73%, urine 57%	Muscle 96%
Inheritance	Maternal	Unknown	<i>de novo</i>
Symptoms			
Ataxia	Present age 16 yr	Present, adult	Present, age 3
Sensorineural hearing loss	Present age 16 yr	Present, adult	Present, age 3 yr
Seizures			Present
Retinitis pigmentosa	Present, age 7 yr	Present, adult	Not present
Episodic lactic acidosis	Present, age 1 yr		Present, age 2 yr
Learning difficulties	Present		Present
Decreased DTR	Present age 16 yr		Not present
Muscle weakness		Proximal legs	
Electrophysiology studies	Sensory neuropathy	Normal	ND
Brain MRI	Vermis atrophy	Normal	Vermis atrophy

The presentation and heteroplasmy levels of the three known patients from two families with this condition are summarized. Abbreviations used: yr = year, mo = months, MRI = magnetic resonance imaging, DTR = deep tendon reflexes, ND = not done.