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Restoration of complex V deficiency caused by a novel deletion in the human *TMEM70* gene normalizes mitochondrial morphology☆

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ABSTRACT

We report a fragmented mitochondrial network and swollen and irregularly shaped mitochondria with partial to complete loss of the cristae in fibroblasts of a patient with a novel *TMEM70* gene deletion, which could be completely restored by complementation of the *TMEM70* genetic defect. Comparative genomics analysis predicted the topology of TMEM70 in the inner mitochondrial membrane, which could be confirmed by immunogold labeling experiments, and showed that the *TMEM70* gene is not restricted to higher multicellular eukaryotes. This study demonstrates that the role of complex V in mitochondrial cristae morphology applies to human mitochondrial disease pathology.

1. Introduction

Complex V (or the mitochondrial (mt) ATP synthase, EC 3.6.3.14), uses the proton gradient to generate ATP during oxidative phosphorylation. Complex V is a multisubunit complex consisting of two functional domains, F_1 and F_o, connected by two stalks. The catalytic F_1 domain is composed of subunits α, β, γ, δ, ε and a loosely attached IF1 inhibitor protein. The membrane-embedded F_o domain consists of an additional ten subunits, a (or subunit 6), b, c, d, e, f, g, OSCP, A6L (or subunit 8), and F6 and functions as a proton channel (Collinson et al., 1996; Houstek et al., 2009). Subunits 6 and 8 of the F_0 domain are encoded by the mtDNA genes *MT-ATP6* and *MT-ATP8*, respectively. All other complex V subunits are encoded by the nuclear DNA. Mitochondria are enclosed by a double membrane. The inner membrane is composed of two subdomains: the inner boundary membrane and the cristae membrane. Cristae are invaginations of the inner membrane that are connected to the inner boundary membrane by narrow tubular structures, so-called crista junctions (Frey et al., 2002). It has been shown that complex V of mammalian mitochondria is arranged in long rows of dimeric supercomplexes (Strauss et al., 2008). Ribbons of complex V dimers are common to all eukaryotes (Strauss et al., 2008). The role of complex V dimers in the formation of tubular cristae has been hypothesized by Allen (1995). The link between the dimerization of mitochondrial complex V, through subunits *e*, *g*, and – as described just recently – subunits *i*, *k*, and the biogenesis of cristae has been provided by studying yeast cells (Paumard et al., 2002; Wagner et al., 2010). Complex V oligomers are found either on the crest of lamellae, or along the length of tubular cristae and introduce a positive curvature to the inner mitochondrial membrane (Strauss et al., 2008). In a recent yeast study, this effect has been shown to be attributed to the action of subunit *e* and subunit *g* (Rabl et al., 2009). Moreover, it has been suggested that the formation of cristae and crista junctions in

Abbreviations: mt, mitochondrial; HHH-syndrome, hyperammonemia–hyperornithinemia–homocitrullinuria-syndrome; MEGS, mitochondrial energy–generating system; CS, citrate synthase; PCR, polymerase chain reaction; RT-PCR, Reverse Transcriptase PCR; BN-PAGE, Blue Native Polyacrylamide Gel Electrophoresis; COX, cytochrome oxidase.

☆ The study has been carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees (Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen) and informed consent.

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mitochondria depends on antagonism between Fcj1 (formation of crista junction protein 1) and subunits *e* and *g* (Rabl et al., 2009). In HeLa cells, it has been shown that IF1 overexpression increases mitochondrial cristae formation and dimerization of complex V (Campanella et al., 2008). Other components such as prohibitins or OPA1 or others yet to be identified also could contribute to crista junction and cristae tip formation (Rabl et al., 2009; Zick et al., 2009). Complex V mutations have been described in *MT-ATP6* (see, among many others, (Morava et al., 2006)), in *MT-ATP8* (Jonckheere et al., 2008), in the nuclear encoded F_1-specific assembly gene *ATP12* (De Meirleir et al., 2004), and in the nuclear encoded structural subunit ε (*ATP5E*) (Mayr et al., 2010). A common splice site mutation and an isolated frameshift mutation were described in the *TMEM70* gene particularly in a homogeneous ethnic group (Romanies), and it has been shown that *TMEM70* is required to maintain normal expression levels of complex V (Cizkova et al., 2008). The *TMEM70* gene, located on chromosome 8q21.11, consists of three exons and encodes a 260 amino acid (AA) protein, transmembrane protein 70. GFP-tagged TMEM70 has been shown to be localized in mitochondria (Calvo et al., 2006). The exact molecular function of the TMEM70 protein is not known at present (Houstek et al., 2009), although it has been suggested that TMEM70 is involved in complex V biogenesis (Cizkova et al., 2008). *TMEM70* homologues have been found in genomes of multi-cellular eukaryotes and plants, while it has been described to be absent from yeast and other fungi (Houstek et al., 2009).

2. Case history

The boy, born at 40 weeks, was the third child of healthy consanguineous Iraqi parents with unremarkable family history. Two older siblings are healthy. Prenatal ultrasound revealed fetal ascites and oligohydramnion. He was small for gestational age (birth weight 2090 g, length 47 cm, head circumference 33 cm). Postnatal echocardiography was normal. He presented on day four of life with feeding difficulties and fever. Lactic acidosis was diagnosed (base excess −14.5 mmol/l) with a blood lactate of 13.9 mmol/l (normal<2). Also hyperammonemia (557 μg/dl, normal<45) occurred. Plasma amino acid analysis revealed elevation of ornithine (651 μmol/l, normal 0–235) and alanine (971 μmol/l, normal<450). In urine, excretion of homocitrulline was observed, together suggesting presence of hyperammonemia–hyperornithinemia–homocitrullinuria (HHH)-syndrome. After supplementation of sodium-benzoate and L-arginine and protein restriction, the clinical condition stabilized and hyperammonemia resolved. However, lactic acidosis and hyperalaninemia persisted, suggesting mitochondrial impairment. During follow-up lactate was between 3.5 and 7.8 mmol/l.

In the first months of life, hypertrophic cardiomyopathy developed. During follow-up motor and especially speech development was impaired. Cerebral magnetic resonance spectroscopy revealed an increased lactate peak within the basal ganglia and the white matter region as well as an increased choline peak.

All symptoms were initially interpreted as either a primary mitochondrial disease or HHH-syndrome with secondary mitochondrial impairment. After HHH-syndrome was excluded by molecular analysis, skin and muscle biopsies were performed in order to investigate a primary mitochondrial enzyme defect. Currently, at the age of 6 years, the cardiomyopathy has remained stable under treatment with calcium antagonists. The child's development is progressive but he retains a distinctive motor and mental retardation. He has an atactic gait and ptosis.

3. Materials and methods

3.1. Cell cultures

Fibroblasts were cultured in medium 199 (Gibco®, Invitrogen Corporation) supplemented with 10% fetal calf serum and penicillin/streptomycin (respectively 100 U/ml and 100 μg/ml). The amphotropic packaging cell line PA317 (# CRL-9078, LGC, Middlesex, UK) and Flp-In T-REx293 cells (Invitrogen) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/l glucose, 10% fetal calf serum and penicillin/streptomycin (respectively 100 U/ml and 100 μg/ml). Inducible cell lines were selected on 5 μg/ml blasticidin (Invitrogen) and 200 μg/ml hygromycin (Calbiochem), and expression of the transgene was induced by addition of 1 μg/ml doxycycline (Sigma Aldrich) for 24 h.

3.2. Biochemical assays

Measurement of the mitochondrial energy-generating system (MEGS) capacity was performed as described before (Janssen et al., 2006). The complex V (or mtATPase) activity was measured spectrophotometrically in mitochondria isolated from frozen pellets of the patient and control fibroblasts as described (Morava et al., 2006). The activities of the mitochondrial respiratory chain enzymes and citrate synthase (CS) were measured spectrophotometrically in muscle tissue and fibroblasts of the patient according to described protocols (Janssen et al., 2003; Srere, 1969).

3.3. Molecular genetic analysis

Total DNA from blood and cultured fibroblasts was extracted using the salting out procedure for human DNA extraction (Miller et al., 1988). The coding region in genomic DNA of the *TMEM70* gene was polymerase chain reaction (PCR) amplified using three primer pairs (Table 1a). PCR conditions were 95 °C for 5 min (denaturation), 92 °C for 45 s, 60 °C for 45 s and 72 °C for 45 s for a total of 35 cycles. Major deletions in genomic DNA were screened for by long template PCR (Expand Long Template PCR system, Roche, Germany) using new primers (Table 1a). PCR conditions were 95 °C for 3 min (denaturation), 93 °C for 10 s, 60 °C for 30 s, 68 °C for 7 min, for a total of 9 cycles followed by 93 °C for 10 s, 60 °C for 30 s, 68 °C for 7 min, plus 20 s each cycle for a total of 24 cycles. Sequence analysis of PCR amplified products was performed on an ABI3730 automatic capillary sequencer using BigDye® terminator chemistry (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). RNA from the patient cultured fibroblasts was extracted as described (Miller et al., 1988). Reverse Transcriptase PCR (RT-PCR) was performed to produce cDNA followed by PCR amplification (Table 1b). Sequence analysis was subsequently performed as described above.

Table 1a
Primers for PCR amplification of the genomic DNA.

Exon(s)	Forward primer	Reverse primer	Comment
1	5′-GGCATGCGCCACTTGTGCG-3′	5′-GCTGGGGCTCCCCAGGCC-3′	Conventional PCR
2	5′-AGGTTAGTTGACCATAATGATCCCTG-3′	5′-ACGTCTTGTAATTAAGGGATGCCA-3′	Conventional PCR
3a[a]	5′-GCAGATTTCCTGCCTGGAGAGG-3′	5′-TCTGGAATCTTCACATCATTCTGGTG-3′	Conventional PCR
3b[a]	5′-TGATTGGCCTTACATTTCTGCCA-3′	5′-AACAATCAGTCACTAACGGAATGCAA-3′	Conventional PCR
2	5′-GGATTGGCAAACCTGGTTAAAA-3′	5′-GGCTGAGGCAGGAAAATCCTT-3′	Long-template PCR

[a] Exon 3 of *TMEM70* is PCR amplified in two overlapping fragments (a and b).