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A mutation in *MT-TW* causes a tRNA processing defect and reduced mitochondrial function in a family with Leigh syndrome

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ABSTRACT

Leigh syndrome (LS) is a progressive mitochondrial neurodegenerative disorder, whose symptoms most commonly include psychomotor delay with regression, lactic acidosis and a failure to thrive. Here we describe three siblings with LS, but with additional manifestations including hypertrophic cardiomyopathy, hepatosplenomegaly, cholestatic hepatitis, and seizures. All three affected siblings were found to be homoplasmic for an m. 5559A>G mutation in the T stem of the mitochondrial DNA-encoded *MT-TW* by next generation sequencing. The m.5559A>G mutation causes a reduction in the steady state levels of $tRNA^{Trp}$ and this decrease likely affects the stability of other mitochondrial RNAs in the patient fibroblasts. We observe accumulation of an unprocessed transcript containing $tRNA^{Trp}$, decreased *de novo* protein synthesis and consequently lowered steady state levels of mitochondrial DNA-encoded proteins that compromise mitochondrial respiration. Our results show that the m.5559A>G mutation at homoplasmic levels causes LS in association with severe multi-organ disease (LS-plus) as a consequence of dysfunctional mitochondrial RNA metabolism.

1. Introduction

Mitochondrial diseases are the most common form of inherited metabolic disorders (Skladal et al., 2003), and most often affect multiple tissues and organs with high-energy demands such as the brain, skeletal muscle and the heart. These diseases are a result of mitochondrial dysfunction, which manifests with a wide range of clinical presentations including neurodegeneration, cardiomyopathies, visual impairment, muscle defects and exercise intolerance (Chinnery and Schon, 2003; Taylor and Turnbull, 2005; Vafai and Mootha, 2012). Mitochondrial diseases may be caused by mutations in mitochondrial DNA (mtDNA) or nuclear DNA encoding mitochondrial proteins, resulting in defective oxidative phosphorylation (OXPHOS) and energy metabolism. There are numerous downstream pathological effects as well, such as changes in cell signaling, increased oxidative damage and initiation of cell death (Montoya et al., 1981; Taylor and Turnbull, 2005; Vafai and Mootha, 2012). The mtDNA is a compact, circular, double-stranded genome encoding only 11 mRNAs that direct the synthesis of 13 electron transport chain protein subunits, 2 rRNAs and 22 tRNAs (Gardner et al., 2007; Montoya et al., 1981). Point mutations in the mtDNA can affect tRNA genes, mitochondrial polypeptide genes or rRNA genes causing diseases such as mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes, maternally inherited Leigh syndrome or aminoglycoside-induced non-syndromic deafness, respectively. In addition, mtDNA deletions or rearrangements can cause Kearns-Sayre syndrome, diabetes and deafness (Dimauro and Schon, 2003). MtDNA and consequently diseases caused by mutations in mtDNA are maternally inherited and like mutations in nuclear genes encoding mitochondrial proteins can cause a range of disorders with varying age of onset and severity (Vafai and Mootha, 2012).

Abbreviations: LS, Leigh Syndrome; MT-TW, mitochondrial tRNA tryptophan; OXPHOS, oxidative phosphorylation; rRNA, ribosomal ribonucleic acid; tRNA, transfer ribonucleic acid; NGS, next generation sequencing; IUGR, intrauterine growth retardation.

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Leigh syndrome is an early-onset progressive neurodegenerative disorder with a characteristic neuropathology consisting of focal, bilateral lesions in one or more areas of the central nervous system, including the brainstem, thalamus, basal ganglia, cerebellum, and spinal cord (Rahman et al., 1996). The most common underlying cause is a defect of oxidative phosphorylation (OXPHOS). The symptoms of LS vary but most commonly include severe developmental delay presenting in infancy with regression, central hypotonia and failure to thrive. Lactic acidosis, dysphagia, optic disk abnormalities, dystonia, ataxia and peripheral neuropathy are also common features. The condition is severe and usually results in death in early childhood (Baertling et al., 2014). Mutations in nuclear genes encoding mitochondrial proteins account for approximately 75% of LS and the remaining cases are a result of mutations in the mtDNA (Rahman et al., 1996). Mutations in *MT-ATP6* are the most frequent cause of mitochondrial DNA inherited LS (Thorburn and Rahman, 2014). However mutations in other mitochondrial mRNA coding genes (*MT-ND2*, *MT-ND3*, *MT-ND4*, *MT-ND5* and *MT-ND6* genes) and tRNA genes (*MT-TV*, *MT-TV1*, *MT-TW* and *MT-TK* genes) also have been reported (Thorburn and Rahman, 2014).

Mitochondrial ribosomes (mitoribosomes) decode the 11 mt-mRNAs using tRNA adaptor molecules. In animals the full complement of mt-tRNAs is encoded by the mtDNA providing the minimum set of tRNAs required for all sense codons, whereas the mitochondrial gene expression machinery is encoded on nuclear genes and imported into mitochondria post-translationally (reviewed in Suzuki et al., 2011; Rackham et al., 2012). The genetic code is established by aminoacyl-tRNA synthetases, which load specific amino acids onto the 3′ end of tRNAs. Modifications of tRNA residues, particularly at the first anticodon position (wobble position), are crucial for tRNA decoding (Suzuki et al., 2011). Mutations in mitochondrial tRNA genes cause a variety of mitochondrial diseases with over 260 different identified pathogenic mutations (Dimauro et al., 2013) affecting the stability of tRNAs, modification of tRNA bases, aminoacylation or processing of polycistronic transcripts (Suzuki et al., 2011) where the cleavage of tRNAs enables the release of mRNAs and rRNAs (Brzezniak et al., 2011; Lopez Sanchez et al., 2011).

Mutations in *MT-TW* have been associated with mitochondrial myopathy, LS, neurogastrointestinal syndrome, myoclonic epilepsy, encephalomyopathy and a separate phenotype characterized by dementia, chorea, deafness and ataxia, as a result of combined complex I, III and IV deficiencies caused by a general mitochondrial translation defect and reduced OXPHOS complex assembly (Barić et al., 2013; Mkaouar-Rebai et al., 2009; Smits et al., 2010). There is only a single report of LS caused by an m.5559A>G mutation in *MT-TW* with a 43% load of the mutant mtDNA compared to wild type mtDNA, a state known as heteroplasmy. Although *in silico* prediction has suggested this mutation may cause secondary structure disruption of the tRNA (Mkaouar-Rebai et al., 2009), no further studies beyond the identification of the mutation have been carried out and functional studies on the effects of the m.5559A>G *MT-TW* mutation are lacking.

Here we have identified three siblings who are homoplasmic for the m.5559A>G mutation in *MT-TW* resulting in decreased $tRNA^{Trp}$ levels and generalized reduction of *de novo* mitochondrial protein synthesis. The siblings have LS associated with reduction in respiratory complexes and decreased OXPHOS. Interestingly we identify that the stability of mitochondrial RNAs is reduced and the mutation causes the accumulation of an unprocessed RNA intermediate containing $tRNA^{Trp}$.

2. Materials and methods

2.1. Approval

All sample collection and experimentation was performed with appropriate informed consent according to approvals given by the Human Research Ethics Office at the University of Western Australia.

2.2. Mitochondrial genome sequencing

MtDNA was extracted from patient blood using a Qiagen DNA extraction kit and amplified in two overlapping fragments using the following primer pairs (Tang et al., 2010): Fragment 1: forward 5′-AACCAAACCCCAAAGACACC-3′ and reverse 5′-GCCAATAATGACGTGA AGTCC-3′. Fragment 2: forward 5′-TCCCACTCCTAAACACATCC-3′ and reverse 5′-TTTATGGGGTGATGTGAGCC-3′ using an Expand LR kit (Roche) following manufacturers recommended protocols. The two fragments were combined in equimolar amounts for library preparation and sequencing on an IonTorrent sequencer (Life Technologies) at the Lotteries West State Biomedical Facility, Genomics node (LSBFG). Sequences were aligned to the mitochondrial genome (NC_012920) and variants called using the IonReporter software suite. Pyrosequencing was performed by the Australian Genome Research Facility (AGRF, Perth Node) according to the manufacturer's protocol and quantification of the heteroplasmy level was carried out using the PyroMark Q24 Method 012 that compared the peak heights at the wild-type and mutant nucleotides at position 5559.

2.3. Cell culture

Fibroblasts were obtained from a skin biopsy of patient (II-3) and a control subject and were cultured at 37 °C under humidified 95% air/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies) containing glucose (4.5 g/l^{-1}), 1 mM pyruvate, 50 μg/ml uridine, 2 mM glutamine, penicillin (100 U/ml^{-1}), streptomycin sulfate (100 $\mu g/ml^{-1}$) and 10% fetal bovine serum (FBS).

2.4. Mitochondrial isolation

Mitochondria were prepared from 10^7 cells grown overnight in 15 cm^2 dishes and isolated as described previously (Rackham et al., 2009), with some modifications. Mitochondria were lysed for 30 min in buffer containing 250 mM sucrose, 100 mM KCl, 20 mM magnesium acetate, 10 mM Tris–HCl pH 7.5, 0.5% Triton X-100 and EDTA-free Complete protease inhibitor cocktail (Roche).

2.5. Immunoblotting

Specific proteins were detected using mouse monoclonal antibodies against: β-actin, porin, NDUFA9 (a subunit of Complex I), Complex II subunit 70, Complex III subunit core 2 (UQCRC2), COX1, COXII, COXIV and ATP synthase subunit alpha (ATP5A) (Abcam, diluted 1:1000), in Odyssey Blocking Buffer (Li-Cor). IR Dye 800CW Goat Anti-Rabbit IgG or IRDye 680LT Goat Anti-Mouse IgG (Li-Cor) secondary antibodies were used and the immunoblots were visualized using an Odyssey Infrared Imaging System (Li-Cor).

2.6. Quantitative RT-PCR

The abundance of mitochondrial RNAs and unprocessed transcripts was measured on RNA isolated from cells using the miRNeasy RNA extraction kit (Qiagen). Levels of mitochondrial transcripts were measured from RNA isolated from cells or purified mitochondria. cDNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen) and used as a template in the subsequent PCR that was performed using a Corbett Rotorgene 6000 using SensiMix SYBR mix (Bioline) and normalized to 18S rRNA.

2.7. Northern blotting

RNA (5 μg) was resolved on 1.2% agarose formaldehyde gels, then transferred to 0.45 μm Hybond-N^+ nitrocellulose membrane (GE Lifesciences) and hybridized with biotinylated oligonucleotide probes specific to mitochondrial mRNAs, rRNAs and tRNAs. The hybridizations