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Mutation in the mitochondrial tRNA^{Val} causes mitochondrial encephalopathy, lactic acidosis and stroke-like episodes

Catherine Glatz^a, Kristin D'Aco^a, Sabrina Smith^{b,c}, and Neal Sondheimer^{a,c,d}

^a Section of Biochemical Genetics, The Children's Hospital of Philadelphia, 3615 Civic Center Boulevard, Philadelphia, PA, USA 19104

^b Section of Neurology, The Children's Hospital of Philadelphia, 3615 Civic Center Boulevard, Philadelphia, PA, USA 19104

^c Department of Pediatrics, The University of Pennsylvania. 3451 Walnut Street, Philadelphia, PA, USA 19104

Abstract

An m.1630A>G mutation in the mitochondrial tRNA^{Val} (MTTV1) was identified in a patient with hearing impairment, short stature and new onset of stroke. This mutation has previously been identified in a patient with the mitochondrial neurogastrointestinal encephalopathy syndrome (MNGIE). The mother of the proband also had high levels of the m.1630A>G allele present in blood and other tissues, without symptoms. To confirm the pathogenicity of this mutation, we created cybrid cell lines with various mutation loads. The m.1630A>G mutation impairs oxygen consumption, affects the stability of the MTTV and reduces the levels of subunits of the electron transport chain.

Keywords

mitochondrial DNA; mitochondrial disease; heteroplasmy; MELAS; mitochondrial tRNA; penetrance

Introduction

Mitochondrial tRNA mutations often cause archetypal mitochondrial disorders. The mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS) syndrome is most often caused by the m.3243A>G mutation in the tRNA^{Leu(UUR)} (Goto, et al, 1990) but other tRNA mutations (Bataillard, et al, 2001), and even mutations in non-tRNA mitochondrial genes can lead to a similar clinical presentation (Ravn, et al, 2001). tRNA mutations lead to disease through several mechanisms. The 3243A>G mutation reduces the synthesis of mitochondrial DNA (mtDNA) encoded proteins. Specifying the mechanism by which this reduction occurs has been complex, because the mutation causes

¹Abbreviations: MELAS – mitochondrial encephalopathy, lactic-acidosis and stroke-like episodes; MNGIE – mitochondrial neurogastrointestinal encephalopathy; MTTV – mitochondrial tRNA^{Val}; mtDNA – mitochondrial DNA; DNP – dinitrophenol;

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^dcorresponding author: sondheimer@email.chop.edu, 267-426-5033 (office), 215-590-3850, glatzc@email.chop.edu, dacok@email.chop.edu, smithsa@email.chop.edu.

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several defects affecting both the tRNA and the primary RNA transcript. These include a reduction in aminoacylation and reduced association of ribosomes with mRNA in cybrid lines with increasing levels of mutation (Chomyn, et al, 2000), the persistence of an aberrant RNA species in m.3243A>G cells that may interfere with ribosomal function (King, et al, 1992), and interference with post-translational modifications of the tRNA (Helm, et al, 1999). For other mitochondrial tRNA mutations, distinct pathogenic mechanisms have been identified, including amino acid misincorporation due to a dominant-acting mutation in tRNA^{Trp} that interferes with codon recognition (Sacconi, et al, 2008).

Mutations in mitochondrial tRNAs must generally reach a certain level of prevalence in order for symptoms to emerge. These thresholds of heteroplasmy, the ratio between mutant and wild-type genomes, are distinct for each mutation. Indeed, some mutations do not necessarily lead to symptoms even if they are homoplasmic, the state where no wild-type DNA is present. This variable penetrance is most clearly seen with the mutations that cause Leber's hereditary optic neuropathy (LHON). Many obligate carriers of mitochondrial complex I mutations linked to LHON have no symptoms. Suggested reasons for incomplete penetrance include the presence of environmental triggers (Kirkman, et al, 2009), the effects of nuclear modifiers (Phasukkijwatana, et al, 2010) and the influence of mitochondrial haplogroup (Ghelli, et al, 2009).

In this report, we present a case of a young woman diagnosed with MELAS and a m.1630A>G mutation in the mitochondrial tRNA for valine (MTTV). This mutation was reported previously in a patient with features suggestive of MNGIE who had COX-negative muscle fibers and a reduction in both complex I and complex IV activities by biochemical analysis (Horvath, et al, 2009). We have analyzed the mutant m.1630A>G allele in cybrid cell lines to confirm its pathogenicity and to further understand its effect on MTTV stability and function.

2. Patient and Methods

2.1. Case report

The proband, aged fifteen at the time of presentation, was the older of two children born to non-consanguineous parents. There was no family history of mitochondrial disease, seizures or developmental delay. She presented in status epilepticus. Head CT showed small foci of mineralization bilaterally in the basal ganglia and brain MRI showed acute right occipital lobe infarction. There were additional areas of signal abnormality in the right occipital and posterior temporal lobes that did not conform to a vascular territory and were not consistent with infarction (Figure 1). Lactate level at presentation was 6.4mM (normal<2mM) and magnetic resonance spectroscopy identified diffuse lactate peaks throughout her brain. Her past medical history is significant for bilateral sensorineural hearing loss requiring hearing aids, myopia, short stature requiring growth hormone supplementation and delayed onset of puberty. She was described as a "picky eater," but had no other specific gastrointestinal complaints. There was no prior history of stroke or seizures. She is in an age-appropriate educational setting but receives support in math. Because of suspicion for MELAS, mitochondrial sequencing for common tRNA mutations was performed with normal results. Subsequent whole mitochondrial genome sequencing identified a heteroplasmic m.1630A>G mutation.

2.2. Cell culture

Fibroblasts were obtained from a skin biopsy and cultured in DMEM (Gibco) supplemented with 10% Fetal Calf Serum, pyruvate (110µg/mL) and uridine (500µg/mL). Cybrids were

created from patient fibroblasts and 143B ρ^0 osteosarcoma cells (a gift from Michael King) using previously described protocols (King, et al, 1992).

2.3. Detection of Mitochondrial Heteroplasmy, Deletions and Depletion

Restriction fragment length polymorphism (RFLP) analysis was performed using DNA derived from the patient's blood, fibroblasts and urine as well as the mother's blood and urine using the last hot cycle method (Horvath, et al, 2009, Moraes, et al, 1992). Sequence at position m.1630 was detected by PCR-RFLP with primers amplifying nts. 1484–1787 of the mtDNA: CGCCCGTCACCCTCCTCA and CGGTACTATATCTATTGCGCCAGGTTTC and digestion with the restriction enzyme BseYI. Degree of heteroplasmy was quantitated using a phosphorimager (GE Life Sciences).

Heteroplasmy was confirmed using quantitative real-time PCR with TaqMan chemistry (Applied Biosystems). Probes were designed using PrimerExpress. Forward primer CATCAACAACCGCTATGTATTTTCG, reverse primer GTGGGTTGGGTTTTATGTACTACAG, Probe 1 CCATGAATATTGTACAGTAC, Probe 2 CCACCATGAATATTGTACGGT. qPCR was performed on an 7900HT (Applied Biosystems). A standard curve was created using cloned m.1630A>G and wild-type alleles cloned into pCR2.1 (Invitrogen).

Mitochondrial deletions were detected by long-range amplification of the entire mitochondrial genome as previously described (Coulter-Mackie, et al, 1998). Depletions were detected by qPCR of COXI DNA from genomic DNA normalized to COXIV1.

2.4. Transcription Profile

The levels of mitochondria-encoded and nuclear-encoded mitochondria resident mRNA were evaluated by RT-PCR. Fibroblast total RNA was converted to cDNA using the Archive kit (Applied Biosystems). TaqMan probes were designed for ATP5E, ATP6, ATP8, COXI, COXII, COXIII, COX17, CYTB, GAPDH, ND1, ND2, ND3, ND4, ND4L, ND5, ND6, NDUFS1, SDHB, and UQCRCQ. SYBR probes were designed for 12S, 16S, TrnP, TrnT. Results were analyzed by qPCR and were normalized to GAPDH expression.

2.5. Cybrid Functional Studies

Cybrid lines were assayed for oxygen consumption using an XF24-3 (Seahorse Biosciences). Oligomycin (final concentration 1 μ M), dinitrophenol (90 μ M) and rotenone (1 μ M) were used to analyze response to inhibition and uncoupling. The activities of the electron transport complexes in isolated mitochondria were analyzed spectrophotometrically using a method previously described (Trounce, et al, 1996).

2.6. Northern Blot

RNA samples were produced from nearly confluent fibroblast cultures using Trizol (Invitrogen) and were resolved on a 15% acrylamide/TBE gel and transferred to a Hybond N + membrane (GE-Amersham). Mitochondrial tRNAs were identified by Northern blotting using 32 P-labeled probes for: Val (TGGTCAGAGCGGTCAAGTTAAGTT), Trp (TGGTGTCTTGGAAAAAGGTTTTC), Gln (TGGCTAGGACTATGAGAATCGAAC), and Glu (TGGTATTCTCGCACGGACTACAAC). The Val probe hybridizes to positions encoded by nts. 1650–1670 to avoid hybridization at the m.1630A>G mutation.

2.7. Western Blot

Whole cell extracts from cybrid cells were evaluated with the following antibodies: Complex I subunit NDUFA9 (MitoSciences MS111), Complex II subunit 70 kDa

(MitoSciences MS204), Complex III subunit core 2 (MitoSciences MS304), Anti-OxPhos Complex IV Subunit I (Invitrogen 459600), and ATP Synthase subunit alpha (MitoSciences MS507). Actin (Santa Cruz sc-1616) was used as a loading control. Western blots were imaged with a chemiluminescent detection system (VersaDoc).

3. Results

3.1. Patient Heteroplasmy

An analysis of heteroplasmy using the last hot cycle method confirmed the m.1630A>G mutation in the patient's blood (Figure 2A). In order to look at the tissue distribution of the mutation, we also evaluated DNA from fibroblasts and renal epithelial cells obtained from a urine sample. The mutational load was similar in the blood and renal epithelial samples, and was 60% in the fibroblast sample. To study the inheritance of this mutation we evaluated the patient's mother. We found high levels of mutated DNA in both blood and urine. Genotyping results were also confirmed by quantitative PCR (Table 1). Deletion and depletion of mtDNA were not identified (Figure 2B&C).

3.2 Transcription in Fibroblast Cell Lines

We evaluated the transcription of mitochondrial genes and nuclear genes used in mitochondrial electron transport in fibroblasts from the proband. When compared to a panel of controls, there were elevations in the transcription of several subunits of the electron transport chain (Figure 3). We have observed upregulated transcription of mitochondrial and nuclear subunits of electron transport in patients with other forms of mitochondrial disease (unpublished data), and this finding suggests an attempt to compensate for impaired mitochondrial function through regulation of gene expression.

3.3 Oxygen Consumption and Electron Transport in Cybrid Cells

The high levels of the m.1630A>G allele in the mother were not expected, given her lack of symptoms. However, in a previous report of a MNGIE patient with a m.1630A>G mutation, the unaffected mother also had a high mutation load (Horvath, et al, 2009). It is conceivable, therefore, that the mutation is actually a rare but benign variant. To exclude this possibility, we studied the mutation in cybrid lines created with the patient's fibroblast mtDNA. Several cybrids were obtained, including lines homoplasmic for the m.1630A>G mutation, lines which were homoplasmic wild-type, and several heteroplasmic lines. Sequencing of the homoplasmic wild-type genome confirmed that the genome was otherwise isogenic to the patient. The levels of mtDNA were not different in the cybrid line homoplasmic for the m. 1630A>G allele when compared to the wild-type cybrid (data not shown).

Oxygen consumption was evaluated in intact cells. Basal respiration was markedly reduced in the homoplasmic m.1630A>G cell line as well as in a heteroplasmic line with 67% mutation load (Figure 4). After the addition of oligomycin, an inhibitor of the F₀F₁-ATPase, the differences between strains decreased. Maximal respiration, provoked by the addition of dinitrophenol (DNP) was again significantly reduced in the homoplasmic mutant and heteroplasmic strains. These results suggest an impairment of electron transport in cybrid cells bearing the mutation.

To confirm this finding, the activities of mitochondrial electron transport complexes were investigated using mitochondria isolated from cybrids. There was a marked decrease in cytochrome-c oxidase activity with a smaller reduction in the activity of complex I, which was not statistically significant (Table 2).

3.4 tRNA and Protein Levels in Cybrids

Analysis of the secondary structure of MTTV predicts that the m.1630A>G mutation would interfere with tRNA folding by disrupting a conserved base-pairing in the anticodon stem (Figure 5) (Horvath, et al, 2009, Putz, et al, 2007). Therefore, we investigated the levels of MTTV by Northern blotting. We found a pronounced loss of MTTV in mutant cybrids when compared to control, but other tested mitochondrial tRNAs were either unchanged or even modestly elevated in the case of tRNA^{Thr} (Figure 6). This finding is consistent with instability of MTTV.

We next used Western blotting of mitochondrial extracts to study the effect of the m.1630A>G mutation on electron transport chain subunits (Figure 6). The mutant cybrid line had reduced levels of proteins in all complexes, excluding complex II, as compared to the wild-type control. The reduction was particularly severe for the mtDNA-encoded COXI subunit.

4. Discussion

The clinical evaluation of mitochondrial sequence is often complicated by the inability to confidently state that detected mutations cause disease. Uncertainty arises from the remarkable number of polymorphisms present within mitochondrial genomes and complications arising from tissue specificity or mitochondrial heteroplasmy. tRNA mutations present unusual difficulties in clinical diagnosis because of the inability to determine whether changes are deleterious. Previous studies have shown that *in silico* predictions of pathogenicity may fail to distinguish common tRNA variants from known disease-causing mutations (Florentz and Sissler, 2001).

Here, we have studied the m.1630A>G mutation, which was found in a patient with clinical features of MELAS. The same mutation was previously seen in a patient with MNGIE, but a comparison of the features of these patients shows similarities (Table 1). Both the mother of our patient and the mother of the patient studied by Horvath and colleagues had high levels of mutation without evidence of disease (Horvath, et al, 2009). Low penetrance, tissue-specific heteroplasmy or nuclear modifiers could explain this apparent paradox, and we sought to isolate the mutation to directly confirm its pathogenicity.

We have found that m.1630A>G is unlikely to be a common neutral polymorphism. The variant has not been observed in two major databases of mitochondrial variation (MitoMap and mtDB). The mutation would be predicted to destabilize the tRNA, since the base forms half of the final Watson-Crick pairing over the anticodon loop of the tRNA (Horvath, et al, 2009). Our studies confirm that the changes in MTTV levels are likely related to instability rather than a reduction of transcription. MTTV is produced from the heavy-strand promoter, a splice product from the maturation of 12S and 16S rRNA. We found that the synthesis of 12S and 16S rRNA is higher in our patient's fibroblast cell lines than in controls, making it unlikely that MTTV transcription is reduced by the mutation.

Cybrids homoplasmic for m.1630A>G have a reduction in the mitochondria-encoded electron transport subunit COXI, presumably from interference with translation, since the level of COXI transcription is higher in fibroblasts. Oxygen consumption in the cybrids is impaired. The milder decreases of UQCRCII, NDUFA9 and ATP5A1 – nuclear-encoded constituents of supercomplexes encoded in both genomes – may be due to the loss of mitochondrial-encoded subunits which destabilize the complex structures.

Although m.1630A>G is incompletely penetrant, it has consequences for mitochondrial function. Symptomatic patients with m.1630A>G should be considered at risk for both

gastrointestinal symptoms and stroke-like episodes, perhaps with dependence upon the tissue distribution of mutation. The investigation of this mutation using a cybrid model provides convincing evidence of its pathogenicity. Further studies will be required to determine how penetrance emerges for m.1630A>G and other mitochondrial mutations.

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Figure 1.

Representative images taken immediately following the onset of seizures. Computed axial tomography scan of the brain (A) shows basal ganglia calcification (^^^). Diffusion-weighted brain MRI images (DWI) show acute infarction (>) in the right occipital lobe (B, C). Areas corresponding to regions of increased DWI signal were dark on apparent diffusion coefficient (ADC) images (not shown). FLAIR MRI image (D) shows acute infarction (***) and additional region of abnormal signal (>) in the right hemisphere that did not show restricted diffusion.

Figure 2.

Detection of m.1630A>G heteroplasmy using last-hot-cycle PCR followed by BseYI restriction digest using various sources of DNA from the proband and her mother. The upper band (single arrow) is the uncut wild-type allele and the lower bands (a fused doublet represented by a double arrow) are created by the m.1630A>G mutation. DNA from an unrelated control is provided as reference (A). There were no significant differences in mitochondrial DNA level in blood or fibroblast samples from the proband as determined by qPCR of COXI normalized to a nuclear marker (B). Mitochondrial deletions, as assayed by long-range PCR of the full genome, were not appreciated (C).

Figure 3. Relative quantitation of gene expression in proband fibroblasts using real-time PCR. RNA was extracted from nearly confluent fibroblasts plated at identical densities. Mitochondria encoded subunits, rRNA and tRNA are on the left side, nuclear encoded are on the right. Normalization is to GAPDH. Control is a mixed population of unaffected individuals. * $p < 0.05$ by t-test.

Figure 4.

Oxygen consumption studies of intact cells. Cybrid lines homoplasmic for m.1630A>G, heteroplasmic or homoplasmic wild-type were analyzed in intact cells, along with a the respiratory deficient parental strain 143Bp⁰. Oligomycin, dinitrophenol and rotenone were added at the times indicated. Samples were analyzed in quadruplicate and values are normalized to protein concentration. The m.1630A>G strain had 9% of control activity under basal and DNP-stimulated conditions. The heteroplasmic strain (67% m.1630A>G) had a 26% activity under basal conditions, and 18% of wild-type activity under DNP stimulation.

Figure 5.

RNA secondary structure plot for MTTV (Horvath, et al, 2009, Gruber, et al, 2008). Position 1630 corresponds to position 31 of the 2-dimensional conventional structure. This position forms half of a conserved base-pairing in the anticodon stem.

Figure 6.

Loss of MTTV and subunits of electron transport in m.1630A>G cybrids. (A) Total RNA was extracted, resolved on acrylamide gels and blotted with probes for the mitochondrial tRNAs for valine, threonine, glutamine and glutamate tRNAs. (B) Western blotting for subunits of the electron transport chain in whole cell extracts from cybrid cell lines.

Table 1

Comparison of symptoms in the patient from this report and the patient studied by Horvath et al (Horvath, et al, 2009). qPCR analysis of heteroplasmy is provided for our family. The percentage of m.1630A>G heteroplasmy is given.

	Our patient	Horvath (2009) patient
Age at diagnosis	15	16
Hearing loss	yes	yes
Motor delay	yes	yes
Developmental Delay	mild	yes
Strokes	yes	no, normal MRI at age 7
Small stature	yes	yes
Ileus	no	yes
Seizures	yes, at time of strokes	focal EEG abnormality
Delayed puberty	yes	unknown
Heteroplasmy	blood 75%; urine 95%; fibroblast 60%	blood 70%, muscle >90%
Mat. Heteroplasmy	blood 93%, urine 98%	blood 60%

Table 2

Activities of electron transport complexes in wild-type and m.1630A>G cybrid lines. Enzyme activities are shown as nmol/min/mg protein. Data is based on triplicate runs.

Cell Line	NADH-Ubiquinone Oxidoreductase (I)	Succinate-Ubiquinone Oxidoreductase (II)	Cytochrome-c Oxidase (IV)
WT (1630A)	263±70	25±12	114±11
1630A>G	152±39	29±5	35±10*

*
 $p < .05$ by paired t-test.