A novel pathogenic m.4412G > A MT-TM mitochondrial DNA variant associated with childhood-onset seizures, myopathy and bilateral basal ganglia changes

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ABSTRACT

Mitochondrial DNA variants in the MT-TM (mt-tRNA^Met) gene are rare, typically associated with myopathic phenotypes. We identified a novel MT-TM variant resulting in prolonged seizures with childhood-onset myopathy, retinopathy, short stature and elevated CSF lactate associated with bilateral basal ganglia changes on neuroimaging. Muscle biopsy confirmed multiple respiratory chain deficiencies and focal cytochrome c oxidase (COX) histochemical abnormalities. Next-generation sequencing of the mitochondrial genome revealed a novel m.4412G > A variant at high heteroplasmy levels in muscle that fulfills all accepted criteria for pathogenicity including segregation within single muscle fibres, thus broadening the genotypic and phenotypic landscape of mitochondrial tRNA-related disease.

1. Introduction

The circular mitochondrial DNA (mt-DNA) contains 13 genes that encode essential subunits of the oxidative phosphorylation complexes, 2 rRNA genes and 22 tRNA genes (Shon et al., 2012). The mitochondrial tRNA (mt-tRNA) genes, which contribute < 10% of the total coding sequence of the mitochondrial genome, are known as pathogenic hotspots because they are responsible for more than half of mtDNA-related diseases (Taylor and Turnbull, 2005). Although these mt-tRNA mutations are responsible for most of the mt-DNA-related diseases in adults phenotypes (Gorman et al., 2015) such as mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (Goto et al., 1990) and myoclonic epilepsy with ragged-red fibres (MERRF) (Shoffner et al., 1990), they are very uncommon in children (Darin et al., 2001; Scaglia et al., 2004). Furthermore, amongst the mutations in mt-tRNA genes, those in mt-tRNA^Met are rare with clinical cases associated with just a small number of reported variants (m.4403G > A, m.4409T > C, m.4440G > A, m.4437C > T and m.4450G > A (Vising et al., 1998; Lombes et al., 1998; Olsen et al., 2003; Peverelli et al., 2014; Scarpelli et al., 2018; Tang et al., 2013; Kuwajima et al., 2019; Born et al., 2015)). We report a novel m.4412G > A variant in the MT-TM gene with high levels of heteroplasmy in skeletal muscle in a young female with disease onset at age 10 years and our work to confirm its pathogenicity.

2. Materials and methods

2.1. Patient and clinical investigations

Our patient was identified following referral to the UK NHS Highly Specialised Service for Rare Mitochondrial Disease in Newcastle upon Tyne, UK. Informed consent from the patient's parents was obtained for the publication of relevant clinical information including photographs and all clinical investigations were carried out in accordance to the Declaration of Helsinki.
2.2. Histochemical and biochemical analyses

Standard histological (Hematoxylin & Eosin, modified Gomori trichrome staining) and histochemical (cytochrome c oxidase (COX), succinate dehydrogenase (SDH) and sequential COX-SDH reactions) analyses were performed on fresh frozen skeletal muscle sections. Mitochondrial respiratory chain complex activities were evaluated in a skeletal muscle homogenate and expressed relative to the activity of the matrix enzyme marker, citrate synthase, as described (Kirby et al., 2007). Additionally, the mitochondrial oxidative phosphorylation (OXPHOS) function was assessed using a quadruple immunohistochemical assay of Complex I (NDUF8), complex IV (COX-1) and porin (mitochondrial mass marker) immunoreactivity as previously reported (Rocha et al., 2015).

2.3. Molecular genetics

Total DNA was extracted from available tissues (skeletal muscle, circulating lymphocytes, urinary sediment and buccal epithelial cells) using standard methodologies. The entire mitochondrial genome was amplified in two overlapping fragments by long-range PCR of muscle DNA and analysed by Next Generation sequencing (NGS) using an Ion Torrent™ Personal Genome Machine (PGM) platform (Thermo Fisher Scientific). Sequences were aligned to the revised Cambridge sequence (GenBank Accession number NC_012920.1) for human mtDNA (Andrews et al., 1999). Almost all of the mitochondrial genome (99.99%) was covered at a read depth of 200× with a detection sensitivity of ≥5% heteroplasmy for single base substitutions. Data analysis was performed in Torrent Suite v5.0.4 using Variant Caller v5.0.4.0 and Coverage Analysis v5.0.4.0.

2.4. Assessment of mutation load by quantitative pyrosequencing

A novel m.4412G > A variant identified following mtDNA sequencing was further assessed by a quantitative pyrosequencing assay using mutation-specific primers (details available on request). mtDNA heteroplasmy levels were determined in DNA samples from the patient and her mother, as well as individual laser-captured COX-deficient and COX-positive muscle fibres. Quantification of mtDNA mutation loads was achieved using Pyromark Q24 software.

3. Results

3.1. Clinical case report

A 10-year-old female Caucasian, born of non-consanguineous parents, had presented with generalised status epilepticus for more than 2 h, thus requiring intubation and admission to a paediatric intensive care unit. The patient was found to have bilateral horizontal nystagmus, ptosis and mild facial weakness. An MRI scan of the brain showed high T2 signal in the bilateral caudate nucleus, which was likely to have developed during the 1 year interval between the two scans and coincided with a deterioration in her ambulation (Fig. 1).

Fig. 1. Clinical features. (A) Clinical photography of the patient (aged 16 years) showing an underdeveloped lower jaw. (B) Retinal photography revealed pigmentary changes on her retina. (C) MRI T2 FLAIR coronal view performed at the age of 12 years showed normal T2 signal in the caudate nucleus. (D) Similar view performed at the age of 13 years showed high T2 signal in the caudate nucleus bilaterally; the red arrow indicates this abnormality on the left. This MRI signal change is likely to have developed during the 1 year interval between the two scans and coincides with a deterioration in her gait. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Her mother recalled that she had complained of fatigue and reduced exercise tolerance since early childhood. For long distances, she required the wheelchair to avoid excessive tiredness and myalgia. Since the onset of her first seizure, she had gradually struggled with her gait and felt significantly unsteady around the age 2 years and had tendencies to avoid physical exertion. Another medical observation was her short stature and poor weight gain since age 5 years. After an unsuccessful trial of nasogastric tube feeding, she eventually had percutaneous enteral gastrostomy (PEG) to improve her growth. Her birth history was unremarkable and her early neurodevelopment was entirely appropriate, but she had been struggling academically following the onset of her seizures. Both parents were healthy and her other two siblings were clinically unaﬀected.

On examination, both her height (1.44 m) and her head circumference were < 0.45th centile. She was 20 cm shorter than her predicted mid-parental height of 50th centile (1.64 m). Whilst, her weight had fallen from 75th centile at birth to the current 0.45th-2nd centile at age 16 years. From the neurological perspective, her gait was broad-based and her tone was low with proximal weakness. No other signiﬁcant bedside examination ﬁndings were observed apart from mild degree of underdeveloped lower jaw (Fig. 1A) and a PEG tube. Fundoscopic examination of her eyes showed evidence of retinitis pigmentosa (Fig. 1B).

3.2. Clinical investigations

Her lactate levels were high in both cerebrospinal ﬂuid, (6.9 mmol/L), and serum, (7.5 mmol/L) as well as mildly elevated creatine kinase at 316 units/L (normal 25–200 units/L). During her seizure episodes, her CSF lactate peaked at 17.2 mmol/L. All her other clinical laboratory results including inﬂammatory markers, kidney function, thyroid function, neuro-immunology screen, caeruloplasmin, vitamin levels, free fatty acids, acylcarnithine proﬁle, ammonia, amino acids, urinary organic acids, autoimmune screen and toxicology screen were normal. Her initial MRI brain scan at her ﬁrst seizure presentation age 10 years and her interim scan at age 12 years showed no signiﬁcant abnormalities (Fig. 1C) but her subsequent MRI brain imaging at age 13 years showed high T2 signal in the caudate heads bilaterally (more prominent on left) (Fig. 1D). In between these brain MRI scans, her gait and co-ordination deteriorated signiﬁcantly. Her cardiac review demonstrated no evidence of cardiomyopathy or conduction abnormalities. The electroencephalography (EEG) following her prolonged seizure age 10 years showed non-speciﬁc encephalopathic changes. Her subsequent inter-ictal EEG at age 11 years showed normal background activity with

Fig. 2. Histopathological and biochemical evaluation of skeletal muscle. (A) Histological and histochemical analyses of the patient’s skeletal muscle biopsy showing hematoxylin and eosin (H&E) staining (i), cytochrome c oxidase (COX) histochemistry (ii), succinate dehydrogenase (SDH) histochemistry (iii) and sequential COX-SDH histochemistry (iv), highlighting the marked COX defect; scale bar = 100 μm. (B) The assessment of individual respiratory chain enzyme activities identiﬁed a severe, multiple OXPHOS deﬁciency affecting complexes I and IV in patient muscle (blue bars) compared to controls (red bars); mean enzyme activities shown for muscle controls (n = 25) are set at 100%. (C) Quadruple immunofluorescence analysis of NDUFB8 (complex I) and COXI (complex IV) mitochondrial subunits, conﬁrming a multiple OXPHOS defect. Each dot represents the measurement from an individual muscle ﬁbre, colour co-ordinated according to its mitochondrial mass (low = blue, normal = beige, high = orange, very high = red). Gray dashed lines represent SD limits for classiﬁcation of the ﬁbres. Lines next to x- and y-axes represent the levels of NDUFB8 and COXI: beige = normal (> − 3), light beige = intermediate positive (−3 to −4.5), light purple = intermediate negative (−4.5 to −6), purple = deﬁcient (< −6). Bold dashed lines represent the mean expression level of normal ﬁbres. (For interpretation of the references to colour in this ﬁgure legend, the reader is referred to the web version of this article.)
a photoparoxysmal response at 22 Hz. There was frontal epileptiform activity during drowsiness, without clinical concomitant.

3.3. Histochemical and biochemical analyses

Muscle biopsy analysis showed normal fibre size following H&E staining, with evidence of fat deposition between fibres and fascicles (Fig. 2A). In addition, we noted remarkable mitochondrial histochemical abnormalities characterized by a mosaic pattern of COX-deficiency affecting > 80% of all fibres on both the individual enzyme reaction and the sequential COX-SDH reaction and subsarcolemmal mitochondrial accumulation (ragged-blue fibres affecting ~5% of the total biopsy) on the individual SDH reaction, consistent with a mitochondrial aetiology (Fig. 2A). The assessment of mitochondrial respiratory chain enzyme activities in a frozen skeletal muscle homogenate revealed evidence of severe, multiple respiratory chain defects involving complexes I and IV with sparing of complex II activity, suggestive of a generalised defect of mitochondrial translation (Fig. 2B). In agreement with this, quadruple OXPHOS immunofluorescence confirmed the presence of many muscle fibres lacking both complex I (NDUFB8) and complex IV (COX-1) expression, confirming a multiple respiratory chain defect (Fig. 2C).

3.4. Molecular genetic analyses

A peripheral blood leucocyte sample was screened for common pathogenic mtDNA variants but no abnormalities were detected. Sequencing of the complete mitochondrial genome revealed a novel m.4412G > A MT-TM variant at high levels of heteroplasmy (92% mutation load based on NGS reads) in skeletal muscle. Quantitative pyrosequencing confirmed high levels of heteroplasmy in skeletal muscle (90% mutation load), with much lower levels of heteroplasmy in buccal and urine (6% and 7% respectively) DNA samples. DNA samples obtained from the patient’s mother showed no evidence of the novel m.4412G > A variant in buccal epithelia, urinary sediment or a blood sample implying the variant had likely arisen de novo during embryogenesis and not been maternally-inherited (Fig. 3A). Single muscle fibre analysis of individual COX-positive and COX-deficient fibres revealed a statistically-significant higher mutation load in COX-deficient fibres (92.89 ± 0.36% (n = 19 fibres)) than in COX-positive fibres (COX-positive fibres: 70.13 ± 7.12% (n = 16 fibres); p < .0001) confirming pathogenicity of the m.4412G > A variant (Fig. 3B).

4. Discussion

We described a young female who carried a novel variant, m.4412A > G in the MT-TM gene, resulting in the childhood onset of mitochondrial disease with multi-system involvement that included prolonged seizures, hypotonia, fatigue, proximal muscle weakness, retinopathy and short stature secondary to growth failure. Gross motor dysfunction was gradually progressive with significant deterioration of her gait and coordination, which appear to coincide with the development of bilateral signal change in the head of caudate on cranial MRI at age 13 years. Although she had some degree of learning difficulties following her first seizure, bilateral basal ganglia changes and elevated

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**Fig. 3.** Mitochondrial DNA studies revealing a pathogenic m.4412A > G variant. (A) Family pedigree identifying the level of the novel MT-TM variant in the proband (indicated by an arrow), and the absence of this variant in all tissues tested in her mother. (B) Single fibre PCR analysis clearly shows a marked segregation of the m.4412A > G mutation with a biochemical defect in individual COX-deficient muscle fibres which harbour higher levels of mutation than COX-positive fibres (see text for details); each symbol represents data for one fibre. (C) Phylogenetic conservation of this region of the MT-TM gene sequence indicates the mutation affects an evolutionary conserved residue and base pair within the DHU stem, as further illustrated on the schematic representation of the mt-RNAmet cloverleaf structure (D).
<table>
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<tr>
<th>MT-TM gene variant</th>
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<th>m.4409T &gt; C</th>
<th>m.4403T &gt; C</th>
<th>m.4440G &gt; A</th>
<th>m.4450G &gt; A</th>
<th>n.d.</th>
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<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Clinical presentation</td>
<td>Age at onset</td>
<td>Sex</td>
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<td>Epilepsy</td>
<td>Stroke-like episode</td>
<td>Other features</td>
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<tr>
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<tr>
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<td>n.d.</td>
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<tr>
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<td>n.d.</td>
<td>n.d.</td>
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In summary, we report a novel m.4412A > G variant in the MT-TM gene presenting with childhood-onset mitochondrial disease characterized by myopathy, prolonged seizures, retinopathy, short stature, lactic acidosis and bilateral basal ganglia changes. This mtDNA novel variant adds to the list of pathogenic MT-TM variants and illustrates the importance of a diagnostic muscle biopsy in demonstrating histocytological mitochondrial abnormalities and supporting single-fibre segregation studies.

5. Conclusion

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Conflict of interest statement

The authors have no conflicts of interest to declare.

References


