



A novel mitochondrial m.4414T>C *MT-TM* gene variant causing progressive external ophthalmoplegia and myopathy

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Abstract

We report a novel mitochondrial m.4414T>C variant in the mt-tRNA^{Met} (*MT-TM*) gene in an adult patient with chronic progressive external ophthalmoplegia and myopathy whose muscle biopsy revealed focal cytochrome c oxidase (COX)-deficient and ragged red fibres. The m.4414T>C variant occurs at a strongly evolutionary conserved sequence position, disturbing a canonical base pair and disrupting the secondary and tertiary structure of the mt-tRNA^{Met}. Definitive evidence of pathogenicity is provided by clear segregation of m.4414T>C mutant levels with COX deficiency in single muscle fibres. Interestingly, the variant is present in skeletal muscle at relatively low levels (30%) and undetectable in accessible, non-muscle tissues from the patient and her asymptomatic brother, emphasizing the continuing requirement for a diagnostic muscle biopsy as the preferred tissue for mtDNA genetic investigations of mt-tRNA variants leading to mitochondrial myopathy. © 2019 The Author(s). Published by Elsevier B.V.

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1. Introduction

Mitochondrial disorders are clinically and genetically heterogeneous and are caused by mutations in genes of both mitochondrial DNA (mtDNA) and nuclear DNA. Of the mtDNA point mutations causing mitochondrial disease, ~50% involve mitochondrial (mt)-tRNA genes [1–3]. Pathogenic mt-tRNA mutations typically display heteroplasmy, the presence of both wild type and mutant mtDNA molecules in a cell, and are often associated with the presence of focal ragged red fibres (RRFs) and cytochrome *c* oxidase

(COX)-deficient fibres in skeletal muscle, leading to a combined respiratory chain deficiency reflecting a disorder of generalised mitochondrial translation. Pathogenicity scoring systems have been suggested for the accurate classification of novel mt-tRNA variants, taking into account a number of important factors including mtDNA heteroplasmy, tissue distribution and segregation with a clear biochemical defect [4,5].

Here, we report a new m.4414T>C pathogenic variant in the D-stem of the tRNA^{Met} (*MT-TM*) gene in a 66-year-old woman with mild mitochondrial disease manifesting as chronic progressive external ophthalmoplegia (CPEO) and myopathy associated with focal COX-deficient and ragged-red fibres in skeletal muscle.

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2. Patient and methods

2.1. Case report

The patient is the second child of non-consanguineous Dutch parents. She presented at the age of 66 years with a variable, bilateral, ptosis which had been present for approximately 3 years. Although she reported no evident diplopia, reading a book was somewhat difficult since the lines seemed to overlap each other. She had no difficulties with her speech or swallowing. In retrospect, she had exercise intolerance from childhood onwards, reflected by difficulties in bicycling long distances, and avoiding walking longer distances. Her medical history comprises a retroperitoneal liposarcoma of the right groin, successfully surgically removed, diabetes mellitus type 2, hypertension, ocular hypertension for which she had laser iridotomy, and a depression, for which she uses sertraline since 10 years.

The patient's brother is clinically unaffected. Her two daughters report no symptoms, but refused further examination. Both parents are deceased and were reported to be in good health.

On examination, at the age of 66, she had a bilateral ptosis, which worsened by provocation, with varying diplopia in all directions without clear ophthalmoplegia. At that time, there was no weakness in the extremities. Later on, she developed an ophthalmoparesis in all directions, bilateral proximal weakness of the arms (shoulder abduction/shoulder exorotation/elbow flexion MRC grade 4) and legs (hip flexors/knee flexors MRC grade 4) and some distal weakness of the feet (foot extensors MRC grade 4). Sensory investigations were normal.

Ancillary laboratory investigations showed normal thyroid stimulating hormone (TSH) levels, blood lactate concentration was elevated (3.1 mmol/l), as was fibroblast growth factor-21 (FGF21; 917 pg/ml, normal reference range 0–200 pg/ml). Myasthenia gravis was ruled out: antibodies against acetylcholine receptor and skeletal muscle were negative, while repetitive nerve stimulation and single fibre electromyography did not reveal any abnormalities. Subcutaneously administered neostigmine did not lead to improvement of the ptosis. Brain MRI to rule out brainstem pathology was normal. Cardiac evaluation did not reveal abnormalities.

2.2. Muscle histology and histochemistry

Standard histology (hematoxylin and eosin (H&E), modified Gomori trichrome staining) and oxidative enzyme histochemistry (cytochrome *c* oxidase (COX), succinate dehydrogenase (SDH) and sequential COX/SDH activities) were performed on 10- μ m transversely oriented frozen skeletal muscle sections, as described previously [6]. Quadruple immunofluorescence analysis to interrogate NDUFB8 (complex I) and COXI (complex IV) immunoreactivities was performed as previously reported [7].

2.3. Mitochondrial DNA (mtDNA) sequencing

Total DNA was extracted from a skeletal muscle, hair, urine and buccal samples by proteinase K digestion and a DNeasy Blood and Tissue Kit (Qiagen); DNA isolation from blood was performed using a DSP DNA Midi kit (Qiagen). Large-scale mtDNA rearrangements were analyzed by long-range PCR protocols [8]. Sequencing of the entire mitochondrial genome in muscle, including both detection and quantification of variants, was performed by next generation sequencing using a MiSeq platform (Illumina) exactly as reported [8]. Assessment of m.4414T>C heteroplasmy levels in the blood, hair, buccal and urine DNA was also performed by next generation sequencing.

2.4. Single muscle fibre segregation studies

A novel m.4414T>C variant identified following mtDNA sequencing was further assessed in individual (COX-deficient and COX-positive) skeletal muscle fibres isolated by laser-capture microdissection by quantitative pyrosequencing (Pyromark Q24 platform, Qiagen) using mutation-specific primers (details available on request). The allele quantification application of Pyromark's proprietary Q24 software was used to calculate heteroplasmy levels (level of test sensitivity >3% mutant mtDNA) [9].

3. Results

3.1. Histochemical and biochemical analyses

The histopathological assessment of the patient's skeletal muscle biopsy showed numerous COX-deficient fibres (20–25% of the total biopsy). Sequential COX-SDH histochemistry revealed that many of the COX-deficient fibres also showed subsarcolemmal mitochondrial aggregates, corresponding to ragged-red fibres (Fig. 1(A)), indicating an underlying mitochondrial aetiology. Quadruple immunofluorescence analysis in individual muscle fibres revealed the presence of fibres lacking NDUFB8 (approximately 20% of all fibres) and COXI (24% of all fibres) proteins, confirming a multiple respiratory chain abnormality (Fig. 1(B)). In addition, a clear correlation between the NDUFB8 and COXI expression levels in individual muscle fibres was found, which is indicative for a combined oxidative phosphorylation deficiency involving both complexes I and IV (Fig. 1(B)).

3.2. Molecular genetic analyses

The patient's muscle mtDNA was analysed for large-scale deletions, as these are frequently associated with a CPEO presentation [10], but long-range PCR assays were all normal. Sequencing the entire mitochondrial genome in muscle revealed a novel mt-tRNA^{Met} (*MT-TM*) gene variant: m.4414T>C. This variant is absent from all 47,412 mtDNA GenBank sequences listed on MitoMAP [11] and has not been

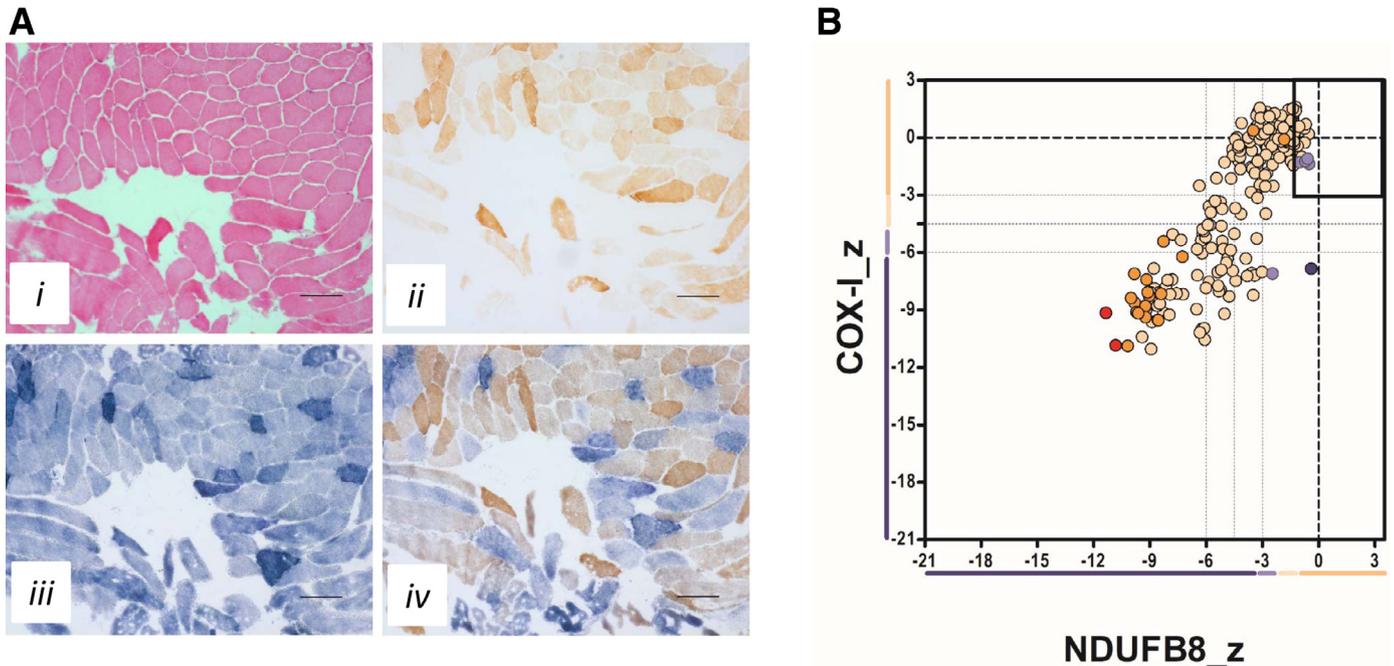


Fig. 1. Histological and histochemical analyses of the patient's skeletal muscle biopsy. (A) Hematoxylin and eosin (H&E) staining (i), cytochrome c oxidase (COX) histochemistry (ii), succinate dehydrogenase (SDH) histochemistry (iii) and sequential COX-SDH histochemistry (iv). Scale bar = 100 μm. (B) Quadruple immunofluorescence analysis of NDUFB8 (complex I) and COXI (complex IV). 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). Grey dashed lines represent SD limits for classification of the fibres. Lines next to x- and y-axis 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.

detected in >3450 in-house mtDNA sequences collated in Maastricht and Newcastle upon Tyne. The single nucleotide substitution was present at heteroplasmic levels (~30% mutant load) in the patient's skeletal muscle but was absent in the patient's blood, hair, urine and buccal samples, as well as in blood, urine and buccal samples of her clinically-unaffected brother, implying that it is a *de novo* sporadic mtDNA variant (Fig. 2(A)).

Given it was present at relatively low levels in skeletal muscle, single muscle fibre segregation studies were performed to determine whether the m.4414T>C variant segregates with the observed respiratory chain defect. Such an analysis revealed significantly higher levels of the m.4414T>C variant in COX-deficient fibres ($93.00 \pm 1.025\%$, $n=13$) compared to COX-positive fibres ($13.21 \pm 5.244\%$, $n=14$, $p<0.0001$ two-tailed Student's *t*-test), confirming that the m.4414T>C variant is responsible for the respiratory-deficient phenotype in the patient's muscle (Fig. 2(B)).

4. Discussion

We identified a novel pathogenic *de novo* sporadic mtDNA variant in the mitochondrial tRNA^{Met} gene (*MT-TM*), m.4414T>C, in an adult patient with CPEO and myopathy associated with focal COX-deficient and ragged-red fibres in skeletal muscle.

A previously validated scoring system was used to assign a pathogenicity classification to this variant [4]. The m.4414T>C variant is not reported before and is absent from available (online- and in-house-) databases. It is heteroplasmic in muscle, the clinically affected tissue, and absent from clinically-unaffected tissues and the unaffected brother. The m.4414T>C variant is situated within the D-stem of the mt-tRNA^{Met} molecule, affecting a highly evolutionarily conserved nucleotide and disturbing a strong evolutionarily conserved canonical Watson and Crick base pair [5] (Fig. 2(C) and (D)). In addition, the variant disrupts the tertiary structure of the tRNA molecule [12]. The presence of a combined oxidative phosphorylation deficiency of complex I and IV, as well as the focal COX-deficiency in the patient's muscle, are consistent with a pathogenic mt-tRNA variant. Finally, single fibre studies reveal a clear segregation of m.4414T>C mutation load with the focal respiratory chain defect. Taken together, the m.4414T>C variant scores 13 points with evidence from a gold standard (single-fibre) investigation, thereby fulfilling the accepted criteria for (definite) pathogenicity [4].

Pathogenic variants have been identified in each of the 22 human mt-tRNAs. The D-stem is a rather weak structure of the tRNA and therefore prone to alterations causing disturbance of secondary or tertiary interactions. So far, disease causing D-stem variants are reported for 16 of the 22 mt-tRNA genes, and are associated with a wide range

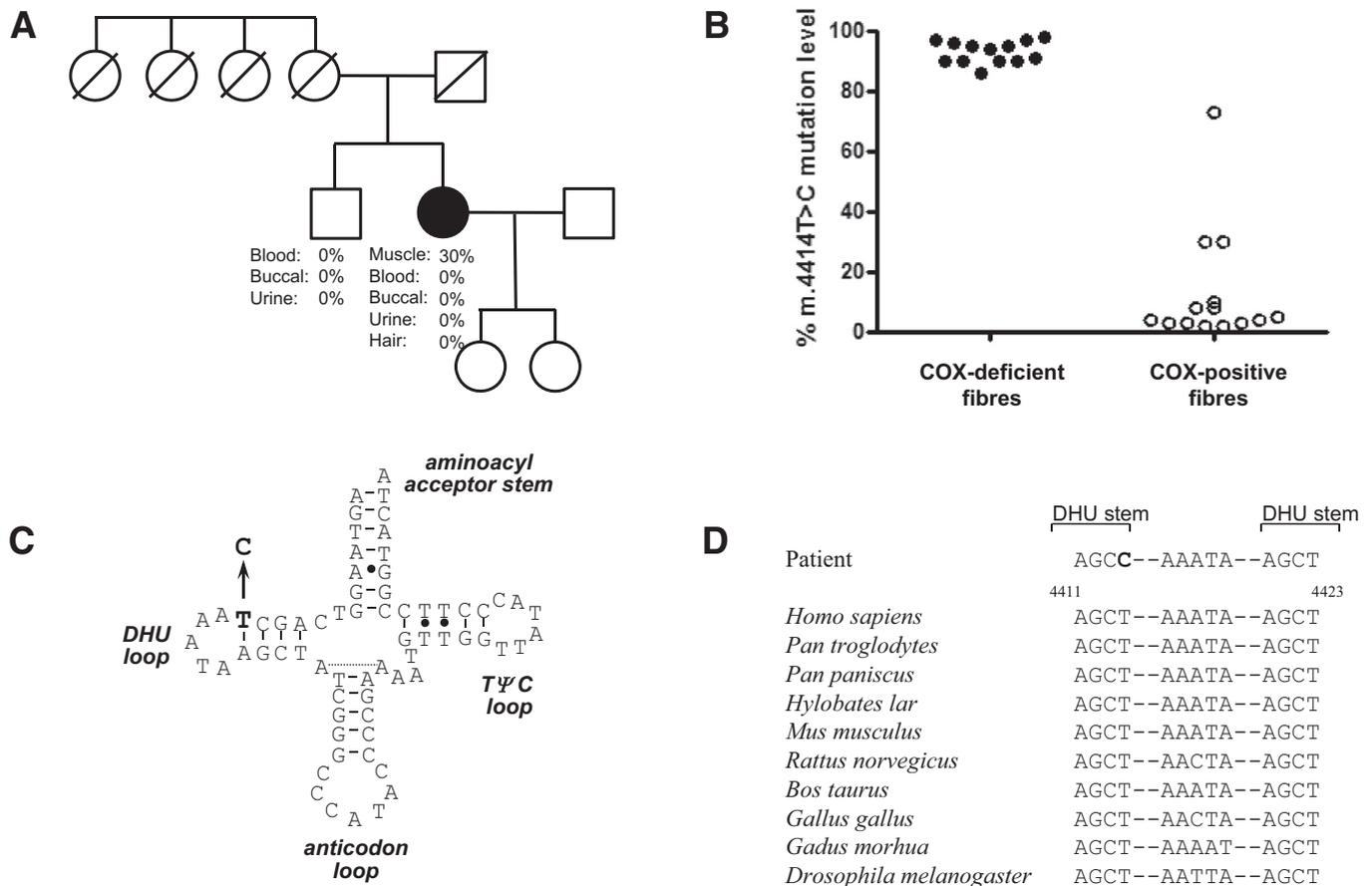


Fig. 2. Mitochondrial DNA studies revealing a pathogenic m.4414T>C variant. (A) Pedigree of the patient's family. The proband is indicated with an arrow. Affected and unaffected status is indicated by black and white symbols, respectively. (B). Mutation load analysis in single COX-deficient and COX-positive muscle fibres. (C) Location of the m.4414T>C variant in the D-stem of the mt-tRNA^{Met}. (D) Evolutionary conservation of the affected base.

of clinical presentations (Supplementary Table 1). Some of these mt-tRNA D-stem cases have a relatively low (18–38%) muscle mutation load and clearly show phenotypical overlap with our patient, displaying relatively mild symptoms (mainly CPEO, ptosis, myopathy) with an age of onset at 26–66 years [13–17]. As compared to other pathogenic variants in the tRNA^{Met} gene (summarized in [18]) our patient has a much lower muscle heteroplasmy level, probably responsible for the milder phenotype and later onset of disease than the other reported *MT-TM* patients.

The heteroplasmy level of the m.4414T>C variant in skeletal muscle of our patient is relatively low, as documented for many mt-tRNA variants causing a CPEO phenotype. It is tempting to speculate that mutation levels might be higher in clinically-affected extraocular muscle tissue as reported by others, although we were unable to test this [19]. Furthermore, CPEO-associated mt-tRNA variants often occur sporadically and are restricted to muscle, which has implications for diagnosis, highlighting the requirement for muscle biopsy to perform mtDNA diagnostic testing, despite the high sensitivity of the current NGS-based mtDNA screening techniques [20,21].

5. Conclusion

In summary, we report a novel pathogenic *de novo* sporadic mtDNA variant in the mitochondrial tRNA^{Met} gene (*MT-TM*), m.4414T>C, in an adult patient with CPEO and myopathy associated with focal COX-deficient and ragged-red fibres in skeletal muscle. The relatively low m.4414T>C mutation levels (30%) in skeletal muscle and absence in non-muscle tissues emphasize that a diagnostic muscle biopsy remains the preferred tissue for mtDNA investigation in patients with a myopathic phenotype.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.nmd.2019.08.005](https://doi.org/10.1016/j.nmd.2019.08.005).

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