Available online at www.sciencedirect.com

ScienceDirect

Neuromuscular Disorders 29 (2019) 693–697

www.elsevier.com/locate/nmd

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

Debby M.E.I. Hellebrekers[a,*], Emma L. Blakely[b,c], Alexandra T.M. Hendrickx[a], Steven A. Hardy[b,c], Sila Hopton[b,c], Gavin Falkous[b,c], Irenaeus F.M. de Coo[d,e,f], Hubert J.M. Smeets[e,g], Nadine M.E. van der Beek[d], Robert W. Taylor[b,c]

[a] *Department of Clinical Genetics, Maastricht University Medical Centre (MUMC), P. Debyelaan 25, P.O. Box 5800, 6202 AZ Maastricht, the Netherlands*
[b] *Highly Specialised Mitochondrial Diagnostic Laboratory, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK*
[c] *Wellcome Centre for Mitochondrial Research, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne NE2 4HH, UK*
[d] *Department of Neurology and Department of Pediatrics, Erasmus MC, Doctor Molewaterplein 40, 3015 GD Rotterdam, the Netherlands*
[e] *Department of Genetics and Cell Biology, Maastricht University, PO Box 616, 6200 MD Maastricht, the Netherlands*
[f] *Department of Neurology, Medical Spectrum Twente, Koningsplein 1, 7512 KZ Enschede, the Netherlands*
[g] *MHeNs School for Mental Health and Neuroscience, Maastricht University, PO Box 616, 6200 MD Maastricht, the Netherlands*

Received 24 June 2019; received in revised form 13 August 2019; accepted 15 August 2019

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.

Keywords: Mitochondrial disease; Chronic progressive external ophthalmoplegia; Myopathy; mtDNA variant; m.4414T>C; *MTTM*.

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.

* Corresponding author.
E-mail address: debby.hellebrekers@mumc.nl (D.M.E.I. Hellebrekers).

https://doi.org/10.1016/j.nmd.2019.08.005

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 opthalmoplegia. At that time, there was no weakness in the extremities. Later on, she developed an opthalmoparesis 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-μm 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

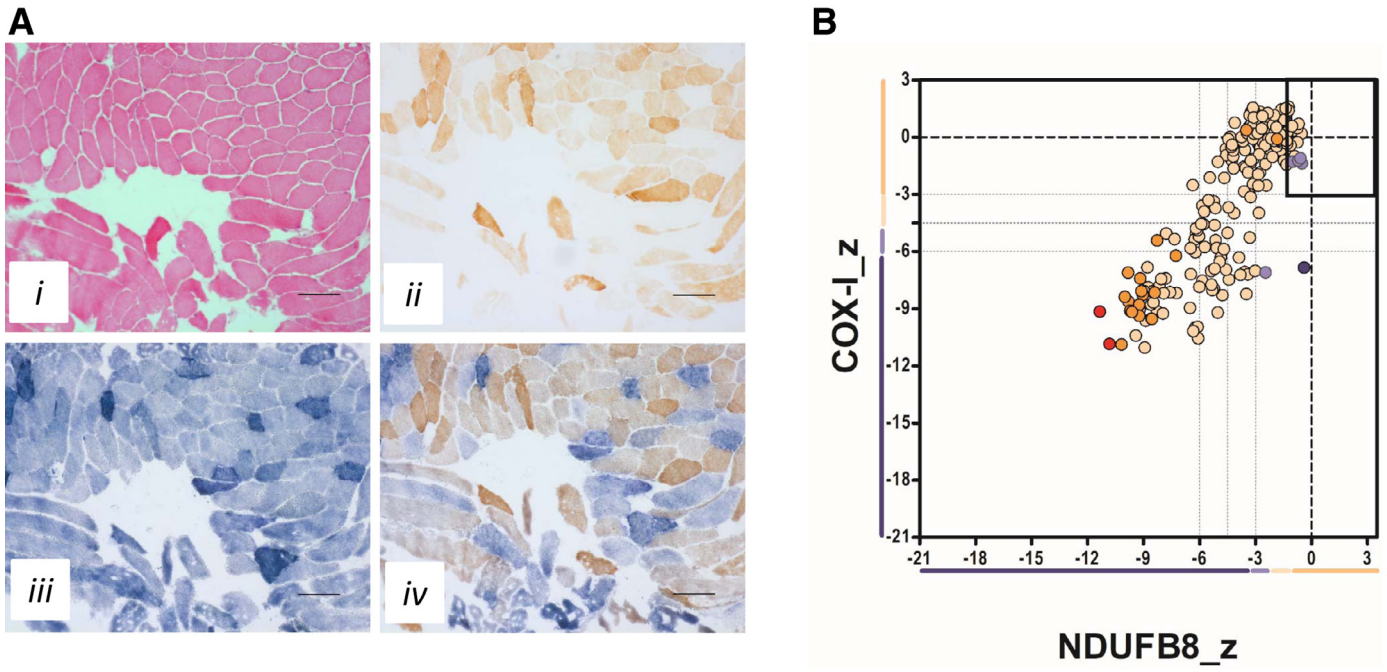

Fig. 1. Histological and histochemical analyses of the patient's skeletal muscle biopsy. (A) Hematoxylin and eosin (H&E) staining (*i*), cytochrome *c* oxidase (COX) histochemistry (*ii*), succinate dehydrogenase (SDH) histochemistry (*iii*) and sequential COX-SDH histochemistry (*iv*). Scale bar = 100 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 ± 1.025%, $n = 13$) compared to COX-positive fibres (13.21 ± 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

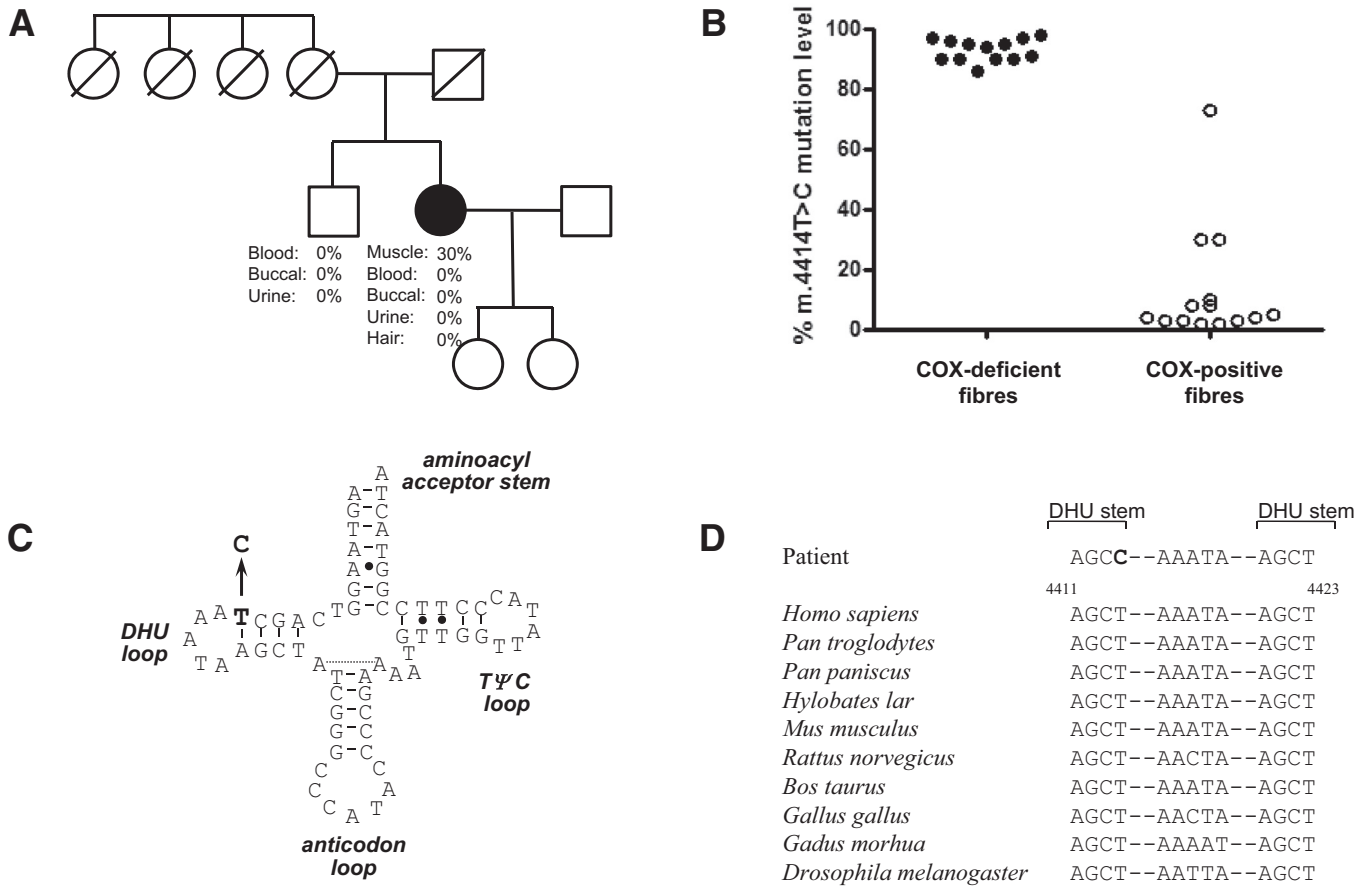

Fig. 2. Mitochondrial DNA studies revealing a pathogenic m.4414T>C variant. (A) Pedigree of the patient's family. The proband is indicated with an arrow. Affected and unaffected status is indicated by black and white symbols, respectively. (B). Mutation load analysis in single COX-deficient and COX-positive 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 varaint 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.

Acknowledgements

RWT is supported by the Wellcome Centre for Mitochondrial Research (203105/Z/16/Z), the Medical Research Council (MRC) International Centre for Genomic Medicine in Neuromuscular Disease, the Mitochondrial Disease Patient Cohort (UK) (G0800674), the Lily Foundation, the UK NIHR Biomedical Research Centre for Ageing and Age-related disease award to the Newcastle upon Tyne Foundation Hospitals NHS Trust, the MRC/EPSRC

Molecular Pathology Node and the UK NHS Highly Specialised Service for Rare Mitochondrial Disorders of Adults and Children (http://www.newcastle-mitochondria.com/). IdC is funded by NeMO.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2019.08.005.

References

[1] Blakely EL, Yarham JW, Alston CL, Craig K, Poulton J, Brierley C, et al. Pathogenic mitochondrial tRNA point mutations: nine novel mutations affirm their importance as a cause of mitochondrial disease. Hum Mutat 2013;34(9):1260–8. doi:10.1002/humu.22358.

[2] Schon EA, DiMauro S, Hirano M. Human mitochondrial DNA: roles of inherited and somatic mutations. Nat Rev Genet 2012;13(12):878–90. doi:10.1038/nrg3275.

[3] Yarham JW, Elson JL, Blakely EL, McFarland R, Taylor RW. Mitochondrial tRNA mutations and disease. Wiley Interdiscip Rev RNA 2010;1(2):304–24. doi:10.1002/wrna.27.

[4] Yarham JW, Al-Dosary M, Blakely EL, Alston CL, Taylor RW, Elson JL, et al. A comparative analysis approach to determining the pathogenicity of mitochondrial tRNA mutations. Hum Mutat 2011;32(11):1319–25. doi:10.1002/humu.21575.

[5] Yarham JW, McFarland R, Taylor RW, Elson JL. A proposed consensus panel of organisms for determining evolutionary conservation of mt-tRNA point mutations. Mitochondrion 2012;12(5):533–8. doi:10.1016/j.mito.2012.06.009.

[6] Old SL, Johnson MA. Methods of microphotometric assay of succinate dehydrogenase and cytochrome c oxidase activities for use on human skeletal muscle. Histochem J 1989;21(9–10):545–55. doi:10.1007/BF01753355.

[7] Rocha MC, Grady JP, Grünewald A, Vincent A, Dobson PF, Taylor RW, et al. A novel immunofluorescent assay to investigate oxidative phosphorylation deficiency in mitochondrial myopathy: understanding mechanisms and improving diagnosis. Sci Rep 2015;5:15037. doi:10.1038/srep15037.

[8] Theunissen TEJ, Nguyen M, Kamps R, Hendrickx AT, Sallevelt SCEH, Gottschalk RWH, et al. Whole exome sequencing is the preferred strategy to identify the genetic defect in patients with a probable or possible mitochondrial cause. Front Genet 2018;9:400. doi:10.3389/fgene.2018.00400.

[9] Grady JP, Pickett SJ, Ng YS, Alston CL, Blakely EL, Hardy SA, et al. mtDNA heteroplasmy level and copy number indicate disease burden in m.3243A>G mitochondrial disease. EMBO Mol Med 2018;10(6):pii:e8262. doi:10.15252/emmm.201708262.

[10] Moraes CT, DiMauro S, Zeviani M, Lombes A, Shanske S, Miranda AF, et al. Mitochondrial DNA deletions in progressive external ophthalmoplegia and kearne-sayre syndrome. N Engl J Med 1989;320(20):1293–9. doi:10.1056/NEJM198905183202001.

[11] Lott MT, Leipzig JN, Derbeneva O, Xie HM, Chalkia D, Sarmady M, et al. mtDNA variation and analysis using mitomap and mitomaster. Curr Protoc Bioinform 2013;44(1.23):1–26. doi:10.1002/0471250953.bi0123s44.

[12] Helm M, Brulé H, Friede D, Giegé R, Pütz D, Florentz C. Search for characteristic structural features of mammalian mitochondrial tRNAs. RNA 2000;6(10):1356–79. doi:10.1017/s1355838200001047.

[13] Pinós T, Marotta M, Gallardo E, Illa I, Díaz-Manera J, Gonzalez-Vioque E, et al. A novel mutation in the mitochondrial tRNA(Ala) gene (m.5636T>C) in a patient with progressive external ophthalmoplegia. Mitochondrion 2011;11(1):228–33. doi:10.1016/j.mito.2010.08.008.

[14] Alston CL, Lowe J, Turnbull DM, Maddison P, Taylor RW. A novel mitochondrial tRNAGlu (MTTE) gene mutation causing chronic progressive external ophthalmoplegia at low levels of heteroplasmy in muscle. J Neurol Sci 2010;298(1–2):140–4. doi:10.1016/j.jns.2010.08.014.

[15] Jackson CB, Neuwirth C, Hahn D, Nuoffer JM, Frank S, Gallati S, et al. Novel mitochondrial tRNA(Ile) m.4282A>G gene mutation leads to chronic progressive external ophthalmoplegia plus phenotype. Br J Ophthalmol 2014;98:1453–9. doi:10.1136/bjophthalmol-2014-305300.

[16] Cardaioli E, Da Pozzo P, Radi E, Dotti MT, Federico A. A novel heteroplasmic tRNA(Leu(CUN)) mtDNA point mutation associated with chronic progressive external ophthalmoplegia. Biochem Biophys Res Commun 2005;327(3):675–8. doi:10.1016/j.bbrc.2004.11.170.

[17] Cardaioli E, Da Pozzo P, Gallus GN, Malandrini A, Gambelli S, Gaudiano C, et al. A novel heteroplasmic tRNA(Ser(UCN)) mtDNA point mutation associated with progressive external ophthalmoplegia and hearing loss. Neuromuscul Disord 2007;17(9–10):681–3. doi:10.1016/j.nmd.2007.05.001.

[18] Lim AZ, Blakely EL, Baty K, He L, Hopton S, Falkous G, et al. A novel pathogenic m.4412G>A *MT-TM* mitochondrial DNA variant associated with childhood-onset seizures, myopathy and bilateral basal ganglia changes. Mitochondrion 2019;47:18–23. doi:10.1016/j.mito.2019.04.007.

[19] Greaves LC, Yu-Wai-Man P, Blakely EL, Krishnan KJ, Beadle NE, Kerin J, et al. Mitochondrial DNA defects and selective extraocular muscle involvement in CPEO. Invest Ophthalmol Vis Sci 2010;51(7):3340–6. doi:10.1167/iovs.09-4659.

[20] Elson JL, Swalwell H, Blakely EL, McFarland R, Taylor RW, Turnbull DM. Pathogenic mitochondrial tRNA mutations—which mutations are inherited and why? Hum Mutat 2009;30(11):E984–92. doi:10.1002/humu.21113.

[21] Hardy SA, Blakely EL, Purvis AI, Rocha MC, Ahmed S, Falkous G, et al. Pathogenic mtDNA mutations causing mitochondrial myopathy: the need for muscle biopsy. Neurol Genet 2016;2(4):e82. doi:10.1212/NXG.0000000000000082.