

MERRF/MELAS overlap syndrome due to the m.3291T>C mutation

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Abstract We report the case of a 19-year-old Chinese female harboring the m.3291T>C mutation in the *MT-TL1* gene encoding the mitochondrial transfer RNA for leucine. She presented with a complex phenotype characterized by progressive cerebellar ataxia, frequent myoclonus seizures, recurrent stroke-like episodes, migraine-like headaches with nausea and vomiting, and elevated resting lactate blood level. It is known that the myoclonus epilepsy with ragged-red fibers (MERRF) is characterized by cerebellar ataxia and myoclonus epilepsy, while that the mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is characterized by recurrent stroke-like episodes, migraine-like headaches, and elevated resting lactate blood level. So the patient's clinical manifestations suggest the presence of a MERRF/MELAS overlap syndrome. Muscle biopsy of the patient showed the presence of numerous scattered ragged-red fibers, some cytochrome *c* oxidase-deficient fibers, and several strongly succinate dehydrogenase-reactive vessels, suggestive of a mitochondrial disorder. Direct sequencing of the complete

mitochondrial genome of the proband revealed no mutations other than the T-to-C transition at nucleotide position 3291. Restriction fragment length polymorphism analysis of the proband and her family revealed maternal inheritance of the mutation in a heteroplasmic manner. The analysis of aerobic respiration and glycolysis demonstrated that the fibroblasts from the patient had mitochondrial dysfunction. Our results suggest that the m.3291T>C is pathogenic. This study is the first to describe the m.3291T>C mutation in association with the MERRF/MELAS overlap syndrome.

Keywords Point mutation · m.3291T>C(tRNA^{Leu(UUR)}) · MERRF · MELAS · Mitochondrial DNA (mtDNA)

Introduction

Mitochondrial encephalomyopathy is usually caused by point mutations in mitochondrial genes and is presented as a multi-system disease. Myoclonic epilepsy with ragged-red fibers (MERRF, OMIM 545000) and mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS, OMIM 540000) are established phenotypes of mitochondrial encephalomyopathy. MERRF is characterized by myoclonic seizures, cerebellar ataxia, myopathy, and ragged-red fibers (RRFs) on muscle biopsy. Approximately 80 % of MERRF cases are caused by the A8344G mutation in the transfer RNA (tRNA) Lys gene (Yoneda et al. 1990). MELAS is characterized by stroke-like episodes, episodic headache and vomiting, lactic acidosis, and skeletal myopathy; it is commonly caused by the A3243G mutation in the mitochondrial tRNA Leu(UUR) gene (Goto et al. 1990).

The correlations between the genotype and clinical phenotype of mitochondrial encephalomyopathy are complex and imprecise. One type of clinical syndrome can also be associated with different point mutations, whereas the same mtDNA

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point mutations can lead to various clinical phenotypes. The investigation of the correlations between the genotype and clinical phenotype is useful to elucidate the pathogenesis and have implications in the treatment of mitochondrial encephalomyopathy.

In this study, we report the case of a MERRF/MELAS overlap syndrome associated with a T-to-C transition at nucleotide position 3291 in the *MT-TL1* (OMIM 590050) gene encoding the mitochondrial tRNA for leucine (UUR). To date, no other studies have described the association of the MERRF/MELAS overlap syndrome with the m.3291T>C mutation.

Materials and methods

Subjects

The proband was a 19-year-old female born from a nonconsanguineous Chinese couple after a regular pregnancy and delivery. She had frequent myoclonic seizures and progressive cerebellar ataxia since the age of 14. At age 17, she had recurrent stroke-like episodes, migraine-like headaches with nausea and vomiting, homonymous hemianopsia, hemiparesis, and hemihypesthesia. She had a height of 168 cm and a weight of 38 kg. Neurological examinations revealed very low visual acuity, constricted visual fields, and nystagmus, but fundoscopic examination showed no abnormalities in the eyeground. External ophthalmoplegia was not observed. Her deep tendon reflexes were exaggerated and muscle strength was reduced. The myoclonus could be induced by sudden buckling limbs. She had dysmetria of the upper and lower limbs with a broad-based gait. She cannot perform a tandem gait. Both her Babinski sign and meningeal irritation signs were negative. Neuropsychological testing showed that she scored 18 on the Mini-Mental State Exam and 13 on the Montreal Cognitive Assessment, indicating cognitive impairment. Laboratory examinations revealed increased levels of resting lactate acid in the blood (17.1 mmol/L, normal <1.55 mmol/L). Fundus photography examination revealed no papillary edema or optic nerve atrophy. Electromyography revealed myopathic pattern in all muscles in the lower limbs and a slight decrease in exercise-induced conduction velocity in the bilateral tibial nerve. When the myoclonus epilepsy attacked the patient causing sudden, quick, small jerks of her arms, the electroencephalogram (EEG) recorded multiple spike-and-wave complexes (3 Hz to 4 Hz) (Fig. 1a). Computed tomography (CT) showed bilateral symmetrical calcification in the region of the basal ganglia (Fig. 1b). Brain magnetic resonance imaging (MRI) showed cerebral and cerebellar atrophy (Fig. 1c and d). Twice stroke-like lesions located in the occipito-parieto-temporal cortexes of bilateral hemispheres were also detected by MRI (Fig. 1c and d).

The proband's mother is 48 years old, and her neurological examination showed no abnormalities except for short stature with emaciation.

The proband's sister is 2 years and 6 months old, who showed normal development and no neurological abnormalities until now.

Muscle histology and histochemistry

The left bicep muscle of the proband was subjected to muscle biopsy, and serial frozen sections (10 µm) were stained with standard hematoxylin-eosin (H&E), modified Gomori trichrome (MGT), cytochrome *c* oxidase (COX), and succinate dehydrogenase (SDH) according to established protocols (Old and Johnson 1989).

Cell culture

Primary fibroblasts were derived from the skin of the proband. This experiment was approved by the local ethics committee and conforms to the Declaration of Helsinki. The cells were plated in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) supplemented with 10 % fetal calf serum (Hyclone, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin and then incubated at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂. The medium was changed twice a week.

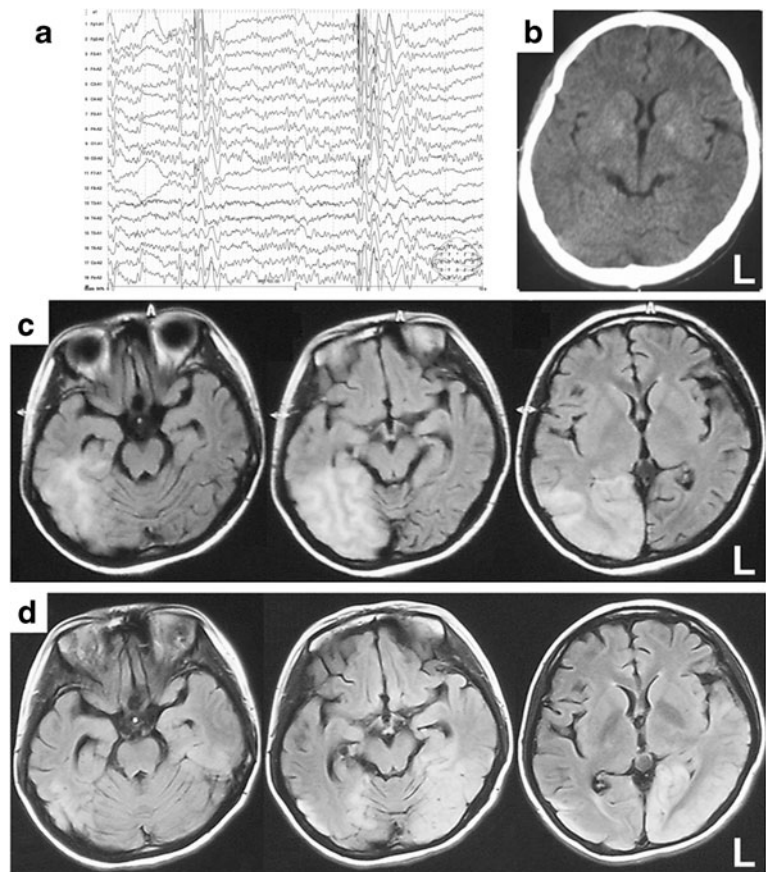
mtDNA mutational analysis

DNA samples were prepared from the proband's skeletal muscle, blood, and fibroblasts. DNA samples from the blood of the proband's mother and sister were also prepared. Total DNA was extracted from tissue samples as previously described (Rieder et al. 1998). Direct sequencing of the complete mitochondrial genome and polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analysis were performed to detect the mtDNA mutation (Rieder et al. 1998). The following sets of primers were used to detect the m.3291T>C mutation: forward primer, 5'-CACCCAAGAA CAGGGTTTGT-3'; reverse primer, 5'-AGGAATGCCATT GCGATTAG-3'. Wild-type mtDNA was cut into two fragments (80 and 74 bp) by the enzyme MluCI. Mutant mtDNA cannot be digested. The digestion products were analyzed in a phospho-imager (Molecular Analyst, BioRad, Hercules, CA) using Image-Quant software (Molecular Dynamics, Sunnyvale, CA) to assess the mutation load.

Measurement of O₂ consumption rate (OCR) and extracellular acidification rate (ECAR)

OCR and ECAR were measured with an XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA) as

Fig. 1 EEG and neuroradiologic findings in the proband. **a** When the myoclonus epilepsy attacked the patient causing sudden, quick, small jerks of her arms, the electroencephalogram (EEG) recorded multiple spike-and-wave complexes (3 Hz to 4 Hz). **b** CT showed bilateral symmetrical calcification in the region of the basal ganglia. **c–d** Twice stroke-like lesions located in the occipito-parieto-temporal cortex of bilateral hemispheres were detected by FLAIR images of brain MRI, which also showed cerebral and cerebellar atrophy. The brain edema in the second stroke-like episode (**d**) is more extensive than that in the first stroke-like episode (**c**). *L* left



previously described (Invernizzi et al. 2012). Fibroblasts were seeded into 24-well Seahorse XF24 plates at a density of 20×10^3 cells/well in 200 μ L of DMEM. The basal OCR of the cells was measured in the absence of an uncoupler for six cycles. The mitochondrial inhibitors oligomycin (1 μ M) and mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (0.7 μ M) were injected into the wells before measuring the OCR. ECAR was measured by determining the change in extracellular pH over time. OCR and ECAR were normalized to the cell number.

Western blot analysis

We used Western blots to estimate the content of respiratory complexes in the proband's muscle (Luo et al. 2013; Patel et al. 2009). Mitochondrial proteins (15 μ g/lane) were resolved electrophoretically in SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA, USA). Membranes were blocked and incubated overnight with primary antibodies (Invitrogen, Carlsbad, CA, USA) against the following subunits of mitochondrial respiratory complexes: OxPhos Complex I subunit NDUF53, OxPhos Complex II 30 kDa subunit, OxPhos Complex II 70 kDa subunit, OxPhos Complex III subunit core 1, OxPhos Complex IV subunit I,

OxPhos Complex V (F_1F_0 -ATPase subunit a). After the membranes were washed, they were incubated for 1 h with secondary antibody and were washed again. Western blots were developed using chemiluminescent HRP substrate. Density of the resultant bands was quantified using Image-Quant software (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

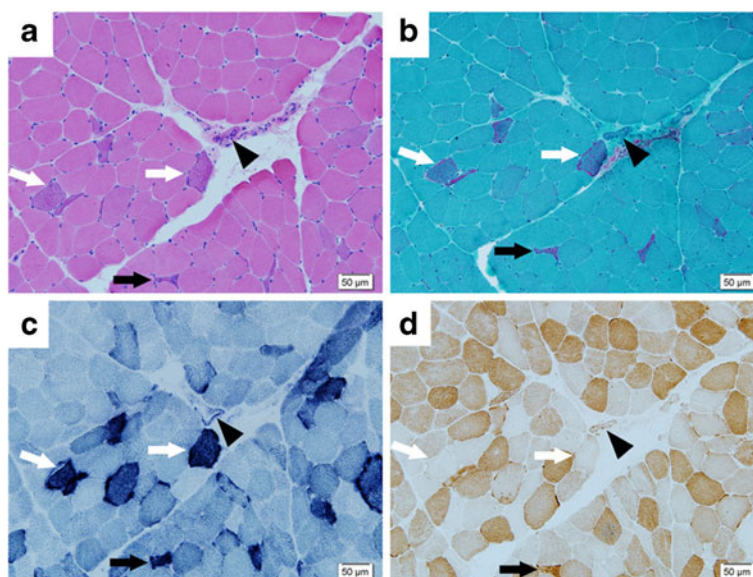
All the data were presented as the mean \pm standard deviation. Student's *t* test was performed to compare the two experimental groups. A statistically significant difference was defined as $p < 0.05$. All the procedures were performed using SPSS 19 software (SPSS Inc., Chicago, IL, USA).

Results

Histological and histochemical analyses

H&E-stained muscle biopsies showed a moderate variation in fibre size (Fig. 2a). Numerous scattered RRFs (approximately 10 %) (Fig. 2b) were observed with MGT stain. Several strongly succinate dehydrogenase-reactive vessels (SSVs) were scattered on SDH stain (Fig. 2c). Staining for COX

Fig. 2 Pathology observed in left bicep muscle biopsies in the proband. **a**, **b**, **c** and **d** are pairs of adjacent sections stained as indicated. **a** H&E stain showed a moderate variation in fibre size. **b** Numerous scattered RRFs were observed with MGT stain. **c** SDH stain showed SSVs (arrowhead). **d** COX stain showed scattered COX-deficient fibers, occasional RRFs with (the black arrows) or without (the white arrows) COX activities, and the SSVs with COX activities (arrowhead)



activity revealed the presence of scattered COX-deficient fibers, and showed that some RRFs (approximately 35 %) and most of the SSVs (approximately 85 %) possessed COX activity (Fig. 2d). These results are suggestive of mitochondrial disease.

mtDNA mutation analysis

Direct sequencing of the complete mitochondrial genome from blood and muscle of the proband revealed no mutations other than the T-to-C transition at nucleotide position 3291 in the *MT-TL1* gene (Fig. 3a). PCR-RFLP revealed the

heteroplasmy of m.3291T>C mutation in the proband, which were at a level of 93 % in muscle sample, 67 % in blood, and 62 % in fibroblasts (Fig. 3b). This mtDNA mutation was also detected and was heteroplasmic in the blood of her mother and sister, the proportions of the mutations in her mother (46 %) and her sister (50 %) were clearly less than those in the proband (Fig. 3c).

Functional measurements of mitochondrial activity in fibroblasts

OCR and ECAR values are indicators of aerobic respiration and glycolysis (Invernizzi et al. 2012). Both OCR and ECAR values were normalized to the cell number. The results demonstrated that the basal ($p < 0.05$, Fig. 4a) and maximum ($p < 0.01$, Fig. 4b) oxygen consumption of the fibroblasts from the proband was significantly lower than those of the control fibroblasts. OCR defects can be associated with compensatory stimulation of ECAR, which reflects an increased utilization of the glycolysis pathway. Coincident with this variation in OCR, the fibroblasts from the proband were found to generate more lactic acid than the control fibroblasts ($p < 0.01$, Fig. 4c). These results suggest that the fibroblasts from the proband have mitochondrial dysfunction.

Content of mitochondrial respiratory complexes

Consistent with the mitochondrial dysfunction of the fibroblasts, Western blot demonstrated that the contents of complexes I and IV were significantly lower in the proband's muscle mitochondria compared with the normal control group: $24.9 \pm 3.4 \%$ and $14.8 \pm 5.3 \%$ of the contents measured in the normal muscle mitochondria, respectively ($p < 0.01$, Fig. 5). While the contents of complexes II,

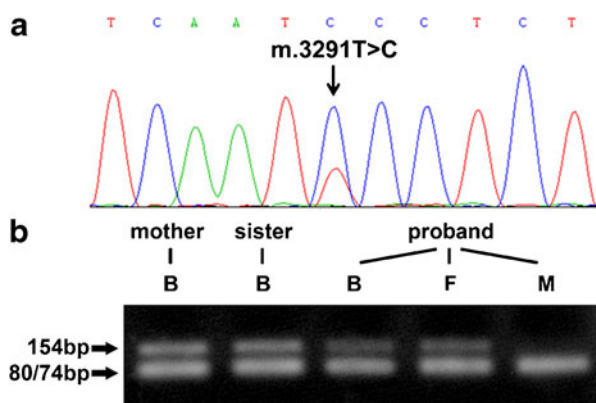
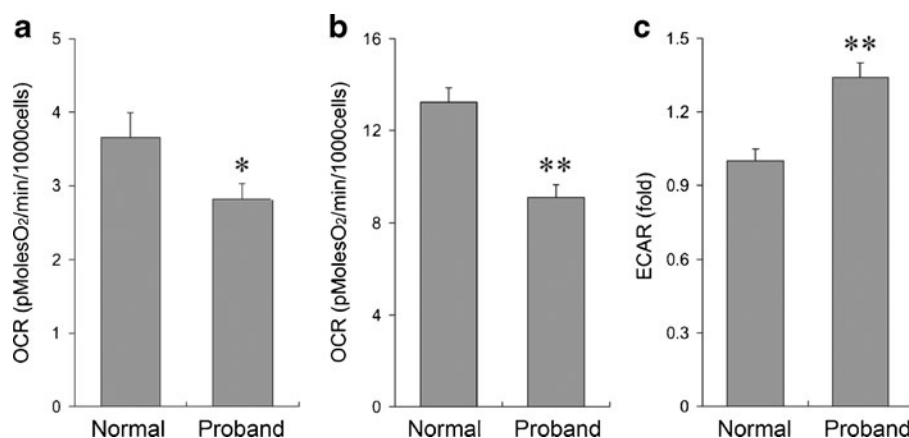


Fig. 3 mtDNA mutation analysis. **a** Direct sequencing of the complete mitochondrial genome from blood and muscle of the proband revealed no mutations other than the m.3291T>C mutation. **b** RFLP analysis showed the variable heteroplasmy level of the m.3291T>C mutation in the pedigree. Uncut 154 bp band represents the wild type (3291 T) while the 80/74 bp band represent the mutant (3291C). Ratio of the mutant in muscle is significantly higher than those in blood and fibroblasts of the proband. The mutation rate in fibroblasts of the proband is higher than those of her mother and her sister. DNA samples are from muscle (M), blood (B), and fibroblasts (F)

Fig. 4 Analysis of the aerobic capacity and glycolysis in fibroblasts from the proband. Both the baseline OCR (**a**) and maximal OCR (**b**) of the fibroblasts from the proband was significantly lower than those of the control fibroblasts. ($n=6$). **c** Relative ECAR showed that the fibroblasts from the proband generated more lactic acid than the normal fibroblasts. ($n=6$). * $p<0.05$ compared with the control group, ** $p<0.01$ compared with the control group



III, and V was not significantly different with the normal control group.

Discussion

This patient's clinical manifestations together with the presence of cognitive impairment on neuropsychological testing, basal ganglia calcification on brain CT, brain atrophy and stroke-like lesions on brain MRI, multiple spike-and-wave complexes on EEG, RRFs, SSVs and COX-deficient fibres on muscle biopsy, and elevated resting lactate blood level, suggest the presence of a MERRF/MELAS overlap syndrome. The MERRF syndrome is characterized by progressive cerebellar ataxia and frequent myoclonus epilepsy, and the EEG finding of multiple spike-and-wave complexes is the characteristic EEG pattern of myoclonus epilepsy. By contrast, the MELAS syndrome is characterized by recurrent stroke-like episodes, migraine-like headaches with nausea and vomiting, homonymous hemianopsia, hemiparesis, and hemihypesthesia.

The MERRF/MELAS overlap syndrome has been reported in seven sporadic cases, which were associated with four mtDNA point mutations, namely, m.8356T>C (Nakamura et al. 2010; Sano et al. 1996; Serra et al. 1996; Zeviani et al. 1993), m.3243A>G (Campos et al. 1996; Nakamura et al.

2010), m.13042G>A (Naini et al. 2005), and m.7512T>C (Nakamura et al. 1995). Previous studies have associated heteroplasmic m.3291T>C mutation with MELAS (Goto et al. 1990), isolated mild myopathy (Uziel et al. 2000), dementia with hearing loss (Salsano et al. 2011), MERRF and Kearns–Sayre overlap syndrome (Emmanuele et al. 2011), cerebellar ataxia with ophthalmoparesis, hearing loss and myopathy (Sunami et al. 2011), and deafness and lipomas (Yarham et al. 2013). In this family, PCR–RFLP analysis detected the m.3291T>C mutation in the *MT-TL1* gene, showing maternal inheritance of the mutation in a heteroplasmic manner.

The asymptomatic mother and sister had the m.3291T>C mutation in their blood but in a significantly lower percentage compared with the proband. These findings indicate that the degree of heteroplasmy is positively correlated with the clinical manifestations. Meanwhile, the amount of mutations varied in the proband's different tissues. That is, the amount of mutations was higher in the muscle and lower in the lymphocytes and fibroblasts. This result is consistent with previous studies, Uziel et al. (Uziel et al. 2000) considered m.3291T>C as a pathogenic mutation with higher amount in muscles than in lymphocytes and fibroblasts. This result can be attributed to the fact that the negative selection for defective mitochondria is possible for rapid turnover cells, such as lymphocytes and fibroblasts, but difficult for highly differentiated, postmitotic syncytial muscle cells.

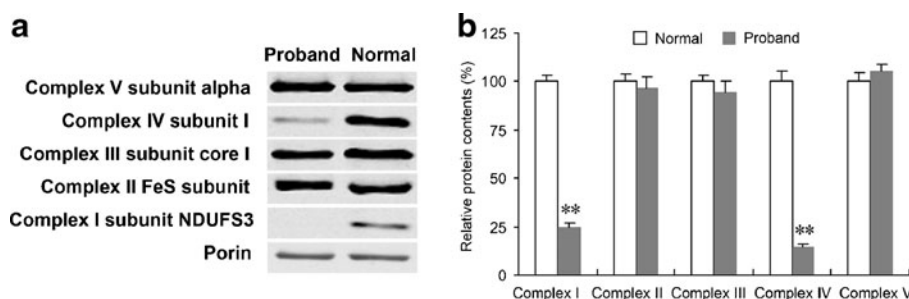


Fig. 5 Western blot analysis of mitochondrial respiratory chain complexes I, II, III, IV, and V. **a** Representative blots from six different experiments. **b** Semi-quantified statistics. The semi-quantified statistics for Western blot

was analyzed and normalized with porin. ($n=6$). ** $p<0.01$ compared with the control group

Furthermore, we performed OCR and ECAR measurements to indicate aerobic respiration and glycolysis of the patient's fibroblasts. OCR and ECAR measurements are considerably more sensitive than spectrophotometric assays in reflecting mitochondrial function and activity, which revealed that mitochondrial function and activity of fibroblasts harboring m.3291T>C mutation were significantly worse than those of the normal control. Consistent with the aerobic respiration capacity of the fibroblasts, Western blot demonstrated that the contents of complexes I and IV were significantly lower in the patient's muscle mitochondria. Therefore, these results also suggest that m.3291T>C is pathogenic.

The m.3291T>C mutation affects a nucleotide in the T-ψ-C loop of the tRNA^{Leu(UUR)} cloverleaf, which might inhibit aminoacylation of the wild-type tRNA (Hao et al. 2004), and also shows partial deficiency of the normal taurine modification at the wobble position (Kirino et al. 2005). However, some scholars had questioned the pathogenicity of m.3291T>C mutation because it only shows moderate conservation according to the consensus panel of species (Ding and Leng 2012). Yarham et al. (Yarham et al. 2013) have recently proven through single-fiber studies that the m.3291T>C mutation is segregated with biochemical deficiency, by which they confirmed the pathogenicity of the m.3291T>C mutation in human mitochondrial disease.

This study is the first to describe the rare *MT-TL1* m.3291T>C mutation in association with the MERRF/MELAS overlap syndrome, further expanding the clinical spectrum associated with the m.3291T>C mutation. Moreover, this case further supports the pathogenicity of the previously reported m.3291T>C mutation in the mitochondrial disease.

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Conflict of interest The authors declare no conflicts of interest.

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