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ORIGINAL ARTICLE

New mitochondrial DNA mutations in tRNA associated with three severe encephalopamyopathic phenotypes: neonatal, infantile, and childhood onset

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Abstract The reported cases showed clinical, biochemical, histopathological, and molecular features lending support to the hypothesis of a pathogenic effect of the detected mutations. Case 1 was a neonatal presentation who showed multiple mitochondrial respiratory chain enzyme defects in muscle associated with a new homoplasmic m.5514A > G transition in the $tRNA^{Trp}$ gene. Case 2 was a late infantile presentation who also showed mitochondrial respiratory chain enzyme deficiencies in muscle together with a new m.1643A > G $tRNA^{Val}$ mutation in homoplasmy. Case 3 showed a MERRF phenotype presented in childhood associated with the once previously reported m.15923A > G mutation in heteroplasmy in all the tissues studied.

Keywords Mitochondrial DNA · tRNA mutations · Homoplasmic mutations · Mitochondrial respiratory chain · Encephalopamyopathic phenotypes

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Introduction

Mitochondrial DNA (mtDNA) point mutations affect protein-coding or protein-synthesis machinery genes (2 rRNA and 22 tRNA) of the oxidative phosphorylation system. tRNA are responsible for more than half of pathogenic mtDNA point mutations [1–3].

Canonical pathogenic criteria for mtDNA point mutations were reported [4]. However, determining the pathogenicity of novel mutations in mitochondrial tRNA genes can be difficult, particularly when the mutations are associated with unusual characteristics such as dominance, skewed segregation within a tissue, or homoplasmy [5–7]. Many clinical phenotypes have been attributed to homoplasmic mtDNA mutations [3, 5, 6, 8–10]. These reports support the concept that homoplasmic mutations in tRNA genes

may be responsible for mitochondrial disorders characterized by extremely variable penetrance [2].

We report two new homoplasmic mtDNA mutations in the $tRNA^{Trp}$ (HGNC *MT-TW*; mitochondrially encoded tRNA tryptophan) and $tRNA^{Val}$ (HGCN *MT-TV*; mitochondrially encoded tRNA valine) genes associated with different phenotypes and a new instance of the previously reported $tRNA^{Thr}$ (HGNC *MT-TT*; mitochondrially encoded tRNA threonine) gene mutation in a case, presenting a MERRF (OMIM 545000) phenotype.

Material and methods

Screening for inborn errors of metabolism including lactate, pyruvate, amino acids, and organic acids in blood, and urine was performed by standard procedures. Muscle biopsies were collected and processed as previously reported [11].

Histopathological investigations in muscle included haematoxylin and eosin stains, modified Gomori trichrome, reduced nicotinamide adenine dinucleotide dehydrogenase, succinate dehydrogenase (SDH), cytochrome c oxidase, and black Sudan stain on frozen sections. Ultrastructural examination was carried out following fixation with 2 % glutaraldehyde, postfixation with 2 % osmium tetroxide, araldite embedding, and staining of ultrathin sections with uranyl acetate and lead citrate.

Mitochondrial respiratory chain (MRC) enzyme activities and coenzyme Q_{10} content were measured in muscle biopsies with spectrophotometric and HPLC-ED procedures, as previously reported [11].

Sequencing of the whole mtDNA from muscle was carried out in all patients using a set of primers available upon request. The percentage of the detected mutations was analyzed by last-cycle radioactive polymerase chain reaction and restriction fragment length polymorphism in different tissues from patients and their mothers using the following primers and enzymes: For the m.1643A > G mutation, the oligonucleotide primers HmtL1578 (ACTGGAAAGTG CACTTGGAC) and HmtH1680 (TAGGTTTAGCTCA GAGCGGTCAAGTTAAGTTGA**G**ATC) were used. HmtL1680 contains a mismatch (C–G) at nucleotide position 1,647. The amplicon size is 103 bp and the PCR conditions were 94 °C, 2 min (95 °C, 30 s/62 °C, 30 s/72 °C, 1 min and 30 s) 35 cycles and 72 °C, 5 min. The restriction enzyme *BspPI* (GGATC (4/5)) digests the amplicon in two fragments of 74 and 29 bp in the presence of the mutation. The wild type DNA lacks the restriction cutting site, with only one fragment of 103 bp remaining. For the m.15923A > G mutation, we used the oligonucleotide primers HmtL15591 (TTCGCCTACACAATTCTCCG) and HmtH112 (ACAGA TACTGCGACATAGGG). The amplicon size is 1,091 bp and the PCR conditions were 94 °C, 2 min (94 °C, 30 s/56 °C, 30 s/72 °C, 1 min and 30 s) 35 cycles and 72 °C, 5 min. The restriction enzyme *AccI* (GT/MKAC) cuts the amplicon into two fragments of 760 and 331 bp in the presence of the mutation. The wild type at position removes the cutting site, with a band of 1,091 bp remaining.

Results and discussion

Case 1: Neonatal onset

First patient is the third child of healthy non-consanguineous parents. No history of familial neurological diseases was documented except epilepsy in her maternal aunt. At 32 weeks of pregnancy, intrauterine growth retardation was evident. Cesarean delivery was carried out at 39 weeks, with the infant showing very low weight and height (below 3 rd percentile), hypotonia, and reduced spontaneous movements. From the first day of life, she presented poor feeding and frequent vomiting. When she was 4 months old, hypotonia had persisted with no head control. She presented weakness and failure to thrive with weight, height, and head circumference all below 3 rd percentile. Congenital infections were ruled out. Brain magnetic resonance imaging (MRI) at 8 months showed global delay in white matter myelination. At 11 months of age, laboratory analysis disclosed increased plasma lactate (4.2 mmol/L; control values: 0.5–2.2) and CK (282 U/L; control values 100–190). At 18 months of life, persistent markedly axial hypotonia and proximal muscle atrophy and weakness, with preserved deep tendon reflexes were observed. These phenotypical features are frequently associated with oxidative phosphorylation defects in the neonatal period [12]. For example, a global delay in myelination pattern early in brain MRI is the most common non-specific abnormality in patients with mitochondrial disease [13] and also one of the most frequent anomalies in a group of patients with a definite mitochondrial disease [14]. Due to these findings, a muscle biopsy was collected.

Mitochondrial respiratory chain enzyme analyses supported the mitochondrial origin of the disease as activities were clearly decreased (Table 1). Normal activities of complex II and SDH, encoded by nuclear DNA, may arouse suspicion of mtDNA mutation as causing the disease. These results (Table 1) fulfilled the diagnostic criteria for mitochondrial disease [15–17]. Histopathological analysis revealed subsarcolemmal accumulation of mitochondria. Electron microscopy was normal.

The multienzymatic defect suggested a problem in the mtDNA-encoded protein synthesis developing the disease. Therefore, we performed an analysis of mtDNA deletions and depletion, and the results were negative. We sequenced the whole mtDNA looking mainly for tRNA mutations. Compared to the revised Cambridge reference sequence,

Table 1 Results of mitochondrial respiratory chain enzyme analysis and genetic findings in the three cases

Cases	1	2	3
MRC enzyme activities			
Citrate Synthase (CS) nmol/min.mg protein	211 (71–200)	201 (71–200)	156 (71–200)
CI + III (mU/U CS)	71 (107–560)	184 (107–560)	n.d.
CII + III (mU/U CS)	33 (75–149)	45 (75–149)	n.d.
CII (mU/U CS)	33 (33–69)	45 (33–69)	n.d.
SDH (mU/U CS)	81 (57–239)	100 (57–239)	n.d.
CIII (mU/U CS)	332 (610–1,760)	393 (610–1,760)	n.d.
CIV (mU/U CS)	403 (590–1,300)	398 (590–1,300)	1,061 (590–1,300)
CoQ10 (nmol/U CS)	2.4 (2.6–8.4)	4.7 (2.6–8.4)	n.d.
Genetic findings			
tRNA and mutation	$tRNA^{Trp}$ m.5514A > G	$tRNA^{Val}$ m.1643A > G	$tRNA^{Thr}$ m.15923A > G
Mutation load (%)	Muscle: 100	Muscle: 100	Muscle: 78
	Blood: 100	Blood: 100	Blood: 10
		Urine:100	Urine:26
		Oral mucosa:100	Oral mucosa:18
Mother's mutation load (%)	Blood: 100	Oral mucosa:60	Urine: 26

MRC results from patients are stated related to citrate synthase activity, and in brackets, control values

n.d. not determined

30 mutations were found that defined the child as an individual belonging to mtDNA U3 haplogroup. The best candidate mutation was an m.5514A > G transition in the $tRNA^{Trp}$ gene (Fig. 1a, middle panel), not found in any of more than 3,000 patients and controls from around the world and www.mitomap.org. The mutation was homoplasmic in muscle and blood and in the mother's blood. This A is conserved in 162 out of 298 (54 %) mammal species. The mutation breaks an A–U base-pair within the acceptor stem of the $tRNA^{Trp}$ (Fig. 2, middle panel). Although this particular nucleotide is poorly conserved, a Watson-Crick base-pair at the position in question is found in 96 % of mammal species.

Most of the clinical, histopathological, biochemical, and genetic analysis suggest that this mutation could be responsible for this pathological phenotype. However, this mutation was found to be homoplasmic in the asymptomatic mother. There have been other reports of homoplasmic mt-tRNA pathological mutations in asymptomatic mothers [5, 10]. Thus, other compensatory factors, such as increased levels of valyl and isoleucyl-tRNA synthetases that stabilize the mutant tRNA, must be preventing these individuals from developing of the disease [18, 19]. Therefore, more evidence, such as the discovery of another pedigree with a mitochondrial disease, is required to confirm the pathogenicity of this mutation. It is possible that exome sequencing analysis could provide some clues about this controversial topic.

Case 2: Late infantile onset

The second patient was the first child of non-consanguineous Moroccan parents with no remarkable familial antecedents. Pregnancy, delivery, and postnatal and first psychomotor development were normal. At 2 years and 4 months of age, she presented epileptic status with myoclonic jerks of right limbs that led to admission to intensive care. An infectious disease was ruled out at this time. A first metabolic screening in blood and cerebrospinal fluid disclosed hyperlactacidemia (2.5 mmol/L; control values: 0.7–1.9). Brain MRI disclosed cortical atrophy and T2-weighted hyperintense signal in left thalamus, in both putamen nuclei and parietal cortex. Brain atrophy and basal ganglia involvement were among the most frequent anomalies found in a group of patients with a definite mitochondrial disease [14]. Moreover, the most common specific MRI finding in mitochondrial diseases is a symmetrical signal abnormality of deep gray matter presenting hyperintensity on T2 [13]. EEG presented slow background activity and frequent paroxysms in left frontoparietal region. During the following 5 weeks, seizures remitted, but a global muscular atrophy, some dyskinetic movement in the patient's arms, and left hemiplegia were observed. At 2 years and 9 months of age, she presented a second partial epileptic status with continuous myoclonus in right limbs. Electrophysiological studies revealed a reduction in motor nerve conduction compatible with a peripheral neuropathy [20]. Due to the biochemical findings and the encephalomyopatic epilepsia partialis continua phenotype, we suspected a mitochondrial disease, and muscle biopsy was collected. At 3 years of age, a dystonic tetraplegia with severe muscle atrophy and loss of oral communication were observed. Six months later, during a concomitant illness, the patient presented a third partial continuous epileptic status, and due to the muscle respiratory weakness, she died at home.

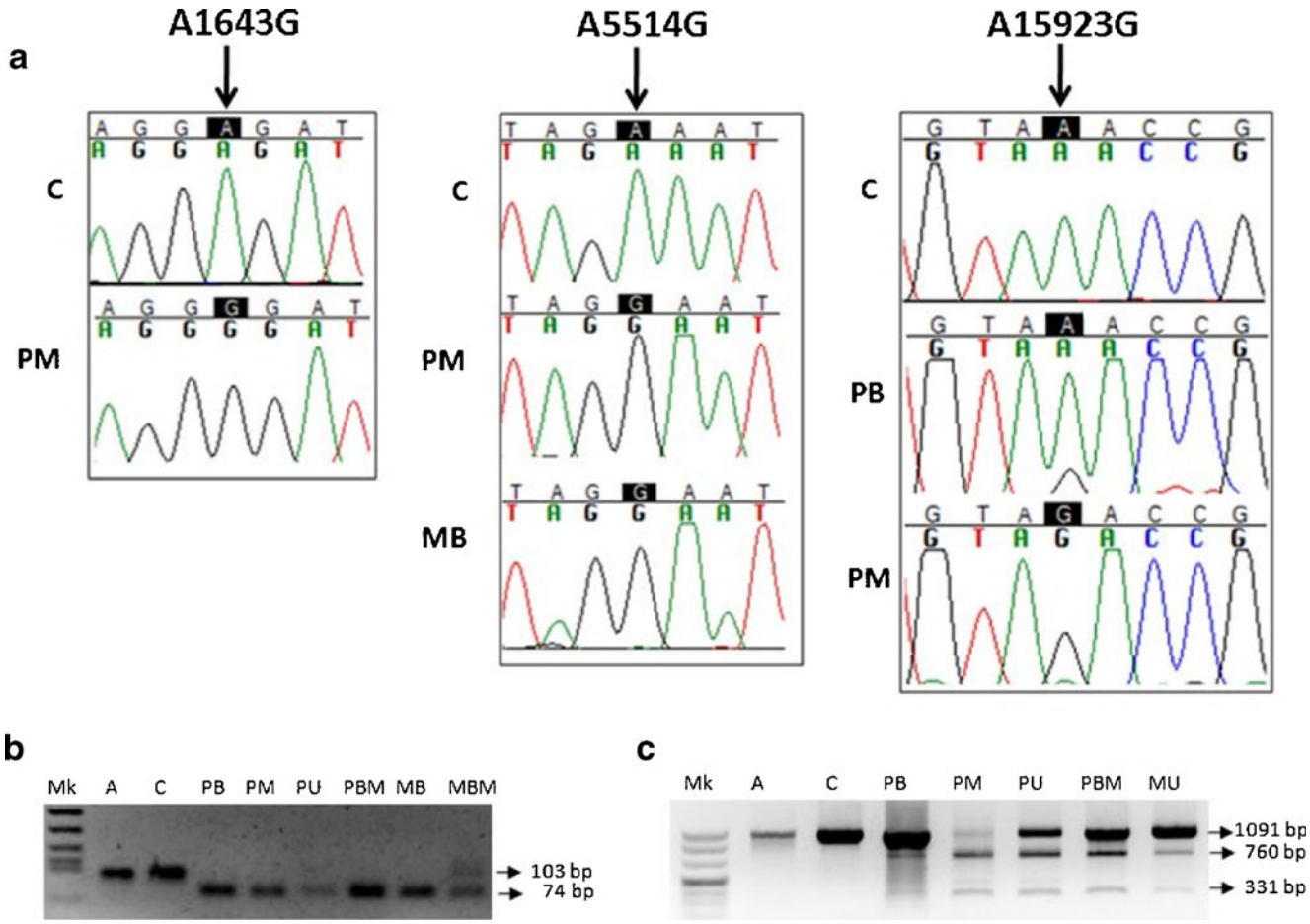

Fig. 1 **a** Mitochondrial genetic analysis of the mutations found in tRNAs (m.1643A > G; m.5514A > G; and m.15923A > G). Electropherograms of a segment of the MT-TV (*left panel*), MT-TW (*middle panel*) and MT-TT (*right panel*) showing the A to G transitions (*C* control, *PM* patient muscle, *PB* patient blood, *MB* mother's blood). **b** and **c** Mutational load determined by PCR/RFLP analysis of the m.1643A > G and m.15923A > G mutations (*MK* molecular weight marker, *A* amplicon, *C* negative control, *PB* patient blood, *PM* patient muscle, *PU* patient urine, *PBM* patient buccal mucose, *MB* mother's blood, *MBM* mother's buccal mucose, *MU* mother's urine)

Mitochondrial respiratory chain enzyme analyses further supported the mitochondrial origin of the disease as activities were clearly decreased when compared with our reference values for several MRC complexes (Table 1). Normal activities of complex II and SDH, encoded by nuclear DNA, may lead to suspicion of an mtDNA mutation as causing the disease. These enzymatic results (Table 1) fulfilled the diagnostic criteria for mitochondrial disease [15–17]. Histopathological analysis revealed subsarcolemmal accumulation of mitochondria and increased sarcolemmal lipid content. Electron microscopy was normal.

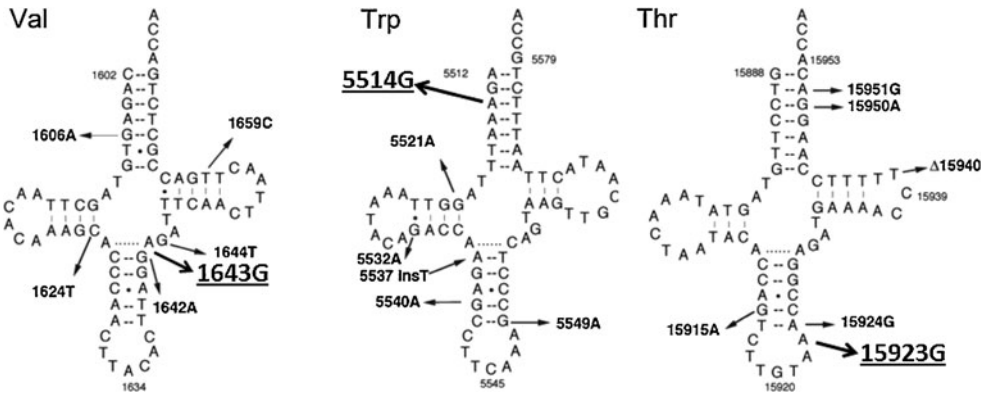

Fig. 2 Wild type $tRNA^{Val}$, $tRNA^{Trp}$, and $tRNA^{Thr}$ secondary structure showing the position of pathologic mutations described in this paper (*underlined*) and other previously described pathologic mutations

We analyzed the mtDNA levels and did not find any mtDNA depletion. Then, we sequenced the whole mtDNA and found 31 other reported polymorphisms that defined the patient as an individual belonging to mtDNA haplogroup U6. She had an m.1643A > G $tRNA^{Val}$ mutation (Fig. 1a, left panel). This mutation was not found in any of more than 3,000 patients and controls from around the world and www.mitomap.org. This mutation was in homoplasmy in muscle, blood, urine, and oral mucosa from the patient and heteroplasmic in her mother's oral mucosa (60 %) (Fig. 1b). The A at position 1,643 is conserved in 260 out of 261 (99.6 %) mammal species. This mutation was located in the extra arm of the $tRNA^{Val}$ (Fig. 2, left panel). According to the criteria proposed for tRNA mutations [21, 22], the findings for this new transition would allow consideration of it as possibly pathogenic.

Case 3: Childhood onset

A Caucasian girl of non-consanguineous parents was born after an uncomplicated pregnancy. Familial history was uneventful except for the presence of deafness diagnosed at 30 years of age in her maternal aunt. During the first 8 years of life, the patient presented exercise intolerance and several episodes of abdominal pain, vomiting, and generalized seizures. EEG showed generalized discharges with normal background activity. From 9 to 11 years old, she presented bilateral neurosensorial deafness, recurrent migraines, and cognitive delay with loss of previously acquired abilities. Brain MRI revealed cortical and cerebellar atrophy. Electroretinogram was impaired, visual evoked potentials showed retarded latencies, and fundus oculi revealed pigmentary retinopathy. In the subsequent 2 years, epilepsy with frequent myoclonic jerks, hypomimic face, muscle weakness, exercise intolerance, ataxia, dysmetry, and mild dysarthria were observed. A second brain MRI disclosed T2-weighted hyperintense signals in globus pallidus. Biochemical analysis showed increased plasma alanine and lactate (5.7 mmol/L; control values 0.5–1.3 mmol/L) with normal CPK values.

Histopathological analysis in muscle biopsy showed the presence of ragged-red and COX negative fibers, and electron microscopy revealed subsarcolemmal aggregates of large mitochondria with alteration of the cristae, supporting the pathogenicity of the detected mtDNA mutation.

No mtDNA deletions were found. We sequenced the whole mtDNA and found 15 reported polymorphisms that defined the patient as an individual belonging to the H3 haplogroup. The m.15923A > G mutation was present in heteroplasmy in all the tissues studied from the patient and her mother (Fig. 1c). The conservation index is 100 % of mammal species. This mutation is localized in the anticodon loop of the $tRNA^{Thr}$, just after the last base-pair of the anticodon stem (Fig. 2, right panel). The mutation could regenerate a new Watson-Crick base-pair, thus increasing the length of this stem. Ten other mitochondrial tRNAs contain C and A nucleotides in the anticodon loop after the anticodon stem and the two mutations observed in the A position (m.5692A > G/*MT-TN*; m.7480A > G/*MT-TS2*), similar to the one reported here, are clearly pathological mutations [1]. This is the second description of a mitochondrial patient harboring the mutation m.15923A > G. However, the previous one showed a lethal neonatal mitochondrial phenotype [23, 24]. By applying the McFarland criteria [21, 22], this mutation reached 12 points and could be considered as pathogenic; it may be responsible for the MERRF phenotype that our patient presented.

Conclusions

We reported two cases harboring new homoplasmic mutations in mtDNA and presenting clinical, biochemical, and molecular features lending support to a pathogenic effect of both mutations. Furthermore, we confirmed the pathogenicity of the previously reported m.15923A > G mutation in a case presenting a different MERRF phenotype.

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