SHORT REPORT

A novel mutation in the mitochondrial *tRNA^{Pro}* gene associated with late-onset ataxia, retinitis pigmentosa, deafness, leukoencephalopathy and complex I deficiency

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We present a patient with ataxia, retinitis pigmentosa, dysarthria, neurosensorial deafness, nystagmus and leukoencephalopathy. A novel heteroplasmic G to A transition at nucleotide 15975 was found, affecting the T arm of the mitochondrial (mt) *tRNA^{Pro}* gene. A biochemical analysis of respiratory chain enzymes in muscle revealed isolated complex I deficiency. This is the fourth pathogenic *tRNA^{Pro}* point mutation to be associated with an mt disorder. The result highlights the importance of molecular dissection of mtDNA in patients with defined mt disorder and confirms the clinical and biochemical heterogeneity associated with *tRNA^{Pro}* mutations.

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Introduction

Mitochondrial (mt) disorders are a vast group of clinical phenotypes characterised by genetically or biochemically defined abnormalities of oxidative phosphorylation (OXPHOS), the terminal component of aerobic energy metabolism. They may be caused by mutations in the nuclear genome or mt DNA.¹ In the last 20 years, over 150 pathogenic mtDNA mutations have been described in association with different human disorders.² More than half of these disease-related mutations are in *tRNA* genes,

representing only one-tenth of the whole mt genome. This can be explained by the essential role of tRNAs in the synthesis of proteins involved in energy metabolism.³ The tRNA-linked disorders cover a wide range of syndromes with heterogeneous clinical presentation. The most common tRNA mutation associated with mt encephalomyopathy, lactic acidosis and stroke (MELAS) is located in the *tRNA^{Leu(UUR)}* gene. Similarly, mutations in *tRNA^{Ile}*, *tRNA^{Lys}* and *tRNA^{Ser(UCN)}* are known to cause different mt encephalomyopathies or non-syndromic deafness. The remaining 18 *tRNA* genes are less reported as mutant in mt disorders.² The *tRNA for proline (tRNA^{Pro})* gene is one of the less polymorphic mt-tRNA genes, with only four mutations reported as pathogenic in the reference databases www.mitomap.org and www.genpat.uu.se/mtDB.

Here, we report an adult patient with severe ataxia, retinitis pigmentosa and bilateral hypoacusia. Morphological,

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biochemical and molecular studies led us to the identification of a novel heteroplasmic point mutation in the *mt-tRNA*^{Pro} gene.

Case report

A 56-year-old woman with a history of progressive walking difficulty that developed when she was 40-years-old was admitted to our department. At 43 years, degenerative pigmentary retinopathy and bilateral neurosensorial deafness were diagnosed. Familial history revealed little of note. Neurological examination showed severe muscular weakness, bilateral nystagmus, dysarthria, bilateral neurosensorial deafness, altered motor coordination on the right side, ataxic gait and brisk tendon reflexes. Cognitive functions were normal. Routine serum chemistry, including pyruvate, lactate, vitamin E, vitamin B12 and leukocyte lysosomal enzymes, was normal. Brain MRI showed, T2-weighted, multiple bilateral periventricular white matter hyperintense signal alteration in the periventricular region, dentate nuclei, and in correspondence of middle and superior cerebellar peduncles. The ventricular system was enlarged. Electroretinogram showed diffuse retinal dysfunction. EEG and ECG were normal. Electrophysiological examination, including motor and sensory conduction velocities and needle electromyography, was normal, whereas sensitive-evoked potentials indicated afferent long tract lesions, prevalently to the left.

Materials and methods

Morphological and biochemical analyses

Morphological analysis of skeletal muscle and biochemical assays of the individual respiratory chain complexes on muscle homogenate were carried out as described.^{4,5} Specific activities of each complex were normalised to that of citrate synthase, an index of mt mass.

Molecular analysis

Total DNA was extracted from skeletal muscle, blood, hair roots, fibroblasts, mouth and urinary epithelium of the proband, and from blood, hair roots, mouth and urinary epithelium of maternal relatives using QIAmp DNA Mini kits (Qiagen). Appropriate PCR-RFLP analysis was used to rule out the presence of the T8993C and T8993G mutations associated with NARP (neuropathy, ataxia, and retinitis pigmentosa).⁶ Direct sequencing of the whole mt

genome from skeletal muscle was performed to search for pathogenic mtDNA mutations, as reported earlier.⁷ The presence of the G15975A mutation in various tissues of the patient and in 130 controls was investigated by mismatch restriction fragment length polymorphism (RFLP)-PCR analysis, using the following oligonucleotide primers: forward 15 863–15 883: 5'-GAAAACAAAATACTCAAATG G-3' and reverse 16 003–16 025: 5'-AACAGAGAATAGTTTA AAGCAGA-3' (mismatch nucleotides shown in bold and underlined). Mismatch in the reverse primer combined with mutant A at position 15 975 creates a unique restriction site for *Tsp509I*. The 162-bp PCR product from the wild-type mtDNA is uncut; the presence of the G15975A transition results in restriction fragments of 112 and 50 bp. The cleaved fragments corresponding to mutant mtDNA were differentiated from the uncut 'wild-type' fragment by 3% MS agarose gel electrophoresis and visualised with UV light after staining with ethidium bromide. The proportion of mutant *versus* total mtDNA was calculated by densitometry using a Biorad Gel Doc 2000 image analyzer.

Results

Morphological, histochemical and biochemical investigations

Histochemical examination of the muscle biopsy revealed slight variation in fibre size with rare splitting; nuclear internalisation was normal. A few fibres (2%) were ragged red when stained with modified Gomori's Trichrome, and a slightly higher percentage (about 5%) were negative to the histochemical reaction for cytochrome *c* oxidase (COX). Biochemistry revealed isolated complex I deficiency (37% of mean control) of the respiratory chain (Table 1).

Molecular analysis

The most common pathogenic mtDNA point mutations described in NARP/Leigh diseases were ruled out in muscle from the proband. As clinical features and biochemical and histopathological findings indicated an mt disorder, analysis of the entire mtDNA was performed by direct sequencing. Sequence analysis showed eight variations of the revised Cambridge Reference Sequence (rCRS):⁸ A750G, A1438G, A4769G, G5262A, T6776C, G13708A, A15326G and G15975A. These variations were compared with the public 'MITOMAP' and 'Uppsala mtDB' databases, which indicated that they were known polymorphisms, except for G–A at

Table 1 Respiratory chain activities^a in muscle

	NADH-CoQ ₁ red (complex I)	Succ-CoQ ₁ red (complex II)	DBH ₂ Cyt c red (complex III)	COX (complex IV)	ATPase (complex V)	CS
Patient	6.9	18	98.8	122.1	303.4	69
Controls' mean (n = 35)	18.5 ± 5.5	21.5 ± 6.5	127.5 ± 39.5	170 ± 50	205 ± 75	145 ± 65

^aThe activities are expressed as nmol per min per mg protein and normalised to that of citrate synthase (CS). Control values ± SD.

nucleotide 15975 in the *tRNA^{Pro}* gene found in heteroplasmic state (Figure 1a). PCR/RFLP analysis and densitometry confirmed that this mutation was heteroplasmic in multiple tissues from the patient, with a mutant load of 40% in muscle, 40% in urinary epithelium, 30% in mouth epithelium and <10% in blood (Figure 1b). Mutant load in hair follicles and fibroblasts were hardly detectable. This mutation was not detected in any of the four accessible tissues (blood, urine, mouth epithelium and hair follicles) from the patient's healthy sister and daughter (Figure 1c). The G15975A point mutation was neither detected in 100 patients with different neurological disorders nor in 30 patients with various mt diseases with or without known point mutations. The mutation is located in the T-Ψ-C stem of the proposed *tRNA^{Pro}* cloverleaf (Figure 2a). Unfortunately, there was insufficient material to perform single muscle fibre PCR analysis.

Discussion

The proband had a late-onset phenotype characterised by ataxia, retinitis pigmentosa, dysarthria, neurosensorial

deafness, nystagmus and leukoencephalopathy. These clinical features are typical of an mt disease, and suggest an NARP-like syndrome; however, typical sensory neuropathy, dementia and seizures are absent in our patient. Seizures are usually rare in adult onset cases.⁹ The mt abnormality was confirmed by muscle biopsy, which revealed scattered ragged red fibres and COX-negative fibres, and by an isolated defect of complex I of the respiratory chain. The combination of clinical, biochemical and morphological findings led us to suspect an mtDNA mutation as the cause of the disease. Direct sequencing of the entire coding sequence of the mt genome revealed a novel G15975A point mutation in the *tRNA^{Pro}* gene. We believe that this mutation is pathogenic for several reasons. First, it has never been reported as a neutral polymorphism and was not detected by us in more than 100 controls. Second, it is heteroplasmic, and heteroplasmy is a common feature of pathogenic mtDNA mutations. Third, the point mutation that we found in a *tRNA* gene is consistent with the histochemical observation of the scattered COX-negative ragged red fibres and with

Figure 1 (a) Direct sequencing of mitochondrial *tRNA^{Pro}* gene from the patient's muscle and blood and from the muscle of a control. Electropherograms of nucleotide position 15 967–15 982 showing heteroplasmic C–T transition at position 15 975 in muscle mtDNA and its presence at low levels from blood mtDNA. (b) PCR-RFLP analysis of DNA amplified from the patient's skeletal muscle (M), blood (B), urinary epithelium (U), mouth epithelium (ME), hair root (H) and fibroblasts (F). UC, uncut PCR; MVIII, DNA molecular weight marker VIII. In the presence of the mutation, a 162-base pair (bp) fragment is digested by *Tsp509I* into two fragments of 112 and 50 bp (see Materials and methods for details). (c) Family pedigree. Mutant load is shown for various tissues in the patient and family members. ND, not detected; trace, mutant bands detectable, but too faint to be quantified (<5%).

Figure 2 Functional consequence of the G15975A mutation. **(a)** Proposed two-dimensional structure of human mitochondrial *tRNA*^{Pro} (reverse) showing the mutation in the TΨC stem and the affected G–C base pair. **(b)** A closer examination of this region of the *tRNA*^{Pro} gene reveals that the base pair involving the nucleotide at position 15975 is highly conserved throughout the species. The mutated base in the patient is shown underlined in bold.

the biochemical results, showing reduced activity of respiratory chain complex I containing mtDNA-encoded subunits. Fourth, this change disrupts a G–C bond in the TΨC stem of the tRNA, which is conserved throughout evolution, and hence likely to be of functional importance (Figure 2). This change may alter the stability or secondary structure of tRNA, or both. In fact, this mutation introduces a CA mispair into the T-stem region, and transition as well as G–U and C–A mismatches can have strong negative effects on classical tRNA function, especially on their aminoacylation properties.¹⁰ Alternatively, the modified function of *tRNA*^{Pro} can affect intra-mt protein synthesis.

Although single fibre PCR would provide significant evidence of pathogenicity, we were unable to perform it because of insufficient muscle sample.

An analysis of accessible tissues from the proband's relatives showed some interesting features (Figure 1c). In the proband, the mutation occurred in muscle, urinary epithelium, mouth epithelium and, with a lower mutant load, in blood. We could not detect the mutation in any accessible tissues from the patient's healthy sister or daughter. It appears that this mutation arose *de novo* in the proband, probably early in embryogenesis, because it is present in multiple tissues. However, as we could not analyse the patient's mother, maternal transmission of the mutant genome cannot be excluded. The relatively low abundance of mutant mtDNA in skeletal muscle, urinary epithelium and mouth epithelium (40, 40 and 30%, respectively) suggests a low threshold for the phenotypic expression of the G15975A mutation; in other words our mutation acts in a dominant manner. However, the low

percentage of mutant mtDNA in our patient is in line with the late onset of the disease. Moreover, other mutations have been described with very low percentages in association with mt diseases. Two reports have described various degrees of mutational load of the common A3243G transition in different clinical phenotypes.^{11,12} The same is also true of other mt-tRNA mutations, all described in association with chronic progressive external ophthalmoplegia, namely, T5628C in *tRNA*^{Ala}, G12276A in *tRNA*^{Leu(CUN)} and G7506A in *tRNA*^{Ser(UCN)}, with mutant loads of 40, 18 and 30%, respectively.^{13–15}

Although some *mt-tRNA* genes (eg, *tRNA*^{Ile}, *tRNA*^{Leu(UUR)} and *tRNA*^{Lys}) are known to be mutational hotspots, to date, only four mutations in the *mt-tRNA*^{Pro} gene have been described. Like other mt-tRNA mutations, the clinical spectrum of patients with *tRNA*^{Pro} mutations seems to be highly variable. The G15990A mutation in the anticodon has been observed in a 7-year-old girl with myopathy and progressive weakness,¹⁶ whereas the G15995A transition in the anticodon stem associated with another mt mutation in *tRNA*^{Lys} was described in a 21-year-old woman with easy fatigability, dysphagia, lability of mood, eyelid twitching and stimulus-sensitive myoclonus. This patient also developed cystic fibrosis and was found to be heterozygous for two mutations in *CFTR* gene.¹⁷ On the other hand, the A16002G mutation in the DHU arm has been detected in a 30-year-old man who presented exercise intolerance, general muscle weakness, bilateral ptosis and vertical ophthalmoplegia.¹⁸ Finally, the T15965C mutation in the TΨC stem was reported in patients with Parkinson's disease.¹⁹ All cases differed in clinical phenotype, age of onset and mutation load. The new G15975A mutation is

the fifth disease-related variant in the mt-tRNA^{Pro} gene, but the first to be associated with late-onset ataxia, retinitis pigmentosa and leukoencephalopathy. The location of the mutated nucleotide within the tRNA-predicted secondary structure may partly explain the differences in clinical presentation.²⁰

In conclusion, our patient expands the clinical heterogeneity of tRNA^{Pro} mutations and highlights the importance of sequencing whole mtDNA when morphology and biochemistry suggest an mtDNA mutation. In line with a report by Pancrudo *et al*,²¹ this case sustains the concept that accessible tissues, such as urine and cheek mucosa, are preferable to blood for examining the relatives of patients. Finally, this novel point mutation adds to the growing list of mt-tRNA gene mutations with a low level of mutant mtDNA in muscle, confirming that its threshold for biochemical and clinical expression is not always high.

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