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Identification of a new mtDNA mutation (14724G>A) associated with mitochondrial leukoencephalopathy

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Abstract

We report a novel 14724G>A mutation in the mitochondrial tRNA glutamic acid gene in a 4-year-old boy with myopathy and leukoencephalopathy. A muscle biopsy showed cytochrome *c* oxidase-negative ragged-red fibers and biochemical analysis of the respiratory chain enzymes in muscle homogenate revealed partial complex I and complex IV deficiencies. The mutation, which affects the dihydrouridine arm at a conserved site, was nearly homoplasmic in muscle and heteroplasmic in blood DNA of the proband, but it was absent in peripheral leukocytes from the asymptomatic mother, sister, and two maternal aunts, suggesting that it arose *de novo*. This report proposes to look for variants in the mitochondrial genome when dealing with otherwise undetermined leukodystrophies of childhood.

Keywords: mtDNA; Mutation; Transfer RNA

Mitochondrial encephalomyopathies are a heterogeneous group of disorders of oxidative phosphorylation (OXPHOS) in which patients exhibit symptoms starting at any age and often include neurological and muscular dysfunctions that can be complicated with cardiac, renal, hepatic, or endocrine involvements [1,2]. The spectrum of brain disorders is highly pleiomorphic and includes symptoms affecting the cortical and subcortical structures, and the white matter. In this respect, astrocyte or oligodendocyte functions are highly dependent upon ATP production through the OXPHOS [3] and so might presumably be their interactions with neurons. Therefore, it is no surprise if mtDNA variants are recognized as causative in otherwise undetermined leukodystrophies (UL) [4–6].

For the synthesis of its proteins, mitochondria are dependent on the nuclear and mitochondrial genomes that operate a twin control on the complex assembly machinery [7,8]. Of the almost 200 pathogenic mtDNA mutations reported to date [2], www.mitomap.org, about 75% are in the 22 mitochondrial transfer RNA (mt-tRNA) genes, although their sequences represent only a small part of the mitochondrial chromosome. We identified a child carrying a diagnosis of UL in whom we detected a novel mutation in the $tRNA^{Glu}$ gene. The molecular consequences of this new variant were studied in skeletal muscle using immunohistochemical, spectrophotometric, and single-muscle fiber techniques.

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Methods

This 4-year-old-boy, the first child born to healthy, unrelated parents achieved the normal developmental milestones until he showed unsteady gate and hypotonia at age 3 months. A brain MRI showed the presence of periventricular white matter hyperluciencies along with basal ganglia calcifications (Fig. 1a). Careful clinical and neurophysiological examinations revealed a hypotonia, macrocephaly, and cerebellar ataxia at age 1 year. Serum lactate level was elevated (3.60 mmol/L, normal: 0.63–2.44) but lactate/pyruvate ratio was normal. Family history was unremarkable for neurological and neuromuscular disorders. The 2-year-old sister is asymptomatic.

A skeletal muscle biopsy specimen was taken at age 2.5 years and analyzed for abnormal mitochondrial function using standard morphological methodologies, as well as spectrophotometric measurement of respiratory chain complexes and citrate synthase [9,10]. Total DNA was extracted from muscle and peripheral blood and the most commonly encountered mtDNA point mutations where looked for by established diagnostic, PCR-based strategies, as reported elsewhere [11,12]. Direct sequencing of the whole mitochondrial genome, including the 22 mt-tRNAs was performed on an ABI 310 (Applied Biosystems). Quantification of mutant mtDNA used a mispairing PCR method and employed the endonuclease *Rsa*I. Single muscle fibers were selected, isolated, and analyzed by PCR using a modification of the laser capture microdissection (LCM-PCR) technique which allows the selective sampling of tissue from histological sections [13,14]. The aforementioned PCR- RFLP strategy allowed us to quantify levels of mutant mtDNAs in single myocytes.

Comparison of quantitative parameters between groups was performed using the Mann–Whitney test. A p value below 0.05 was considered significant.

Results

Histochemical analysis of muscle biopsy showed over 20% ragged-red fibers (RRF) in skeletal-muscle biopsy of the patient, with most fibers being cytochrome *c* oxidase (COX) negative (Fig. 1b). Fewer than 10% were RRF/COX(+). Spectrophotometric measurement of respiratory chain complexes in skeletal muscle showed that the residual activities of NADH-ubiquinone oxidoreductase (complex I) and cytochrome *c* oxidase (complex IV) were 46% and 23%, respectively, when referred to the activity of citrate synthase, an index of mitochondrial mass.

Immunohistochemical staining of the patient's muscle using a specific antibody against a mtDNA-encoded subunit of complex IV (COII) showed a mosaic pattern whereas staining for complex II (Ip subunit), a nuclear-DNA encoded subunit of complex IV (COIV) and complex III (core 2) demonstrated normal patterns. Routine molecular analyses identified no classic mutations, deletion or duplication. However, a thorough investigation of the tRNA genes identified a novel G>A transition at nucleotide position 14724 (14724G>A) in $tRNA^{Glu}$ gene (Fig. 2a and b). The mutation was nearly homoplasmic (94%) in muscle and heteroplasmic (62%) in peripheral leukocytes, it was absent in over 300 haplotype-matched control samples, and it alters a highly conserved nucleotide of the $tRNA^{Glu}$. The 14724G>A mutation was not found in

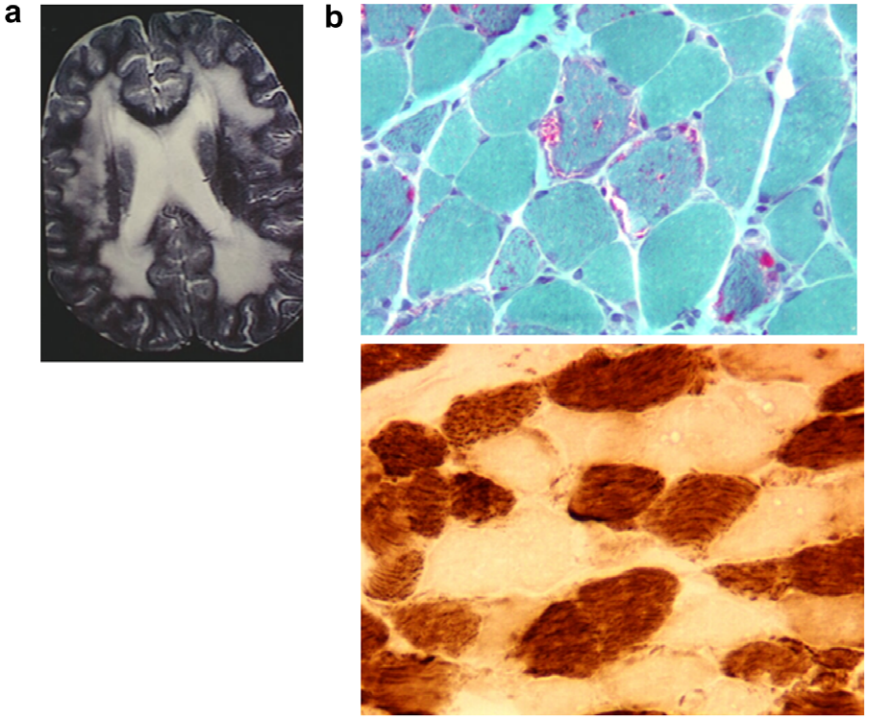

Fig. 1. (a) Brain MRI in a child harboring the novel 14724G>A mutation. Axial T2 weighted images demonstrated diffuse periventricular white matter hyperlucencies. (b) Morphological investigations in muscle sections: Gomori trichrome staining showing numerous ragged-red fibres (top), cytochrome *c* oxidase (COX) staining demonstrating COX-negative muscle fibers (bottom).

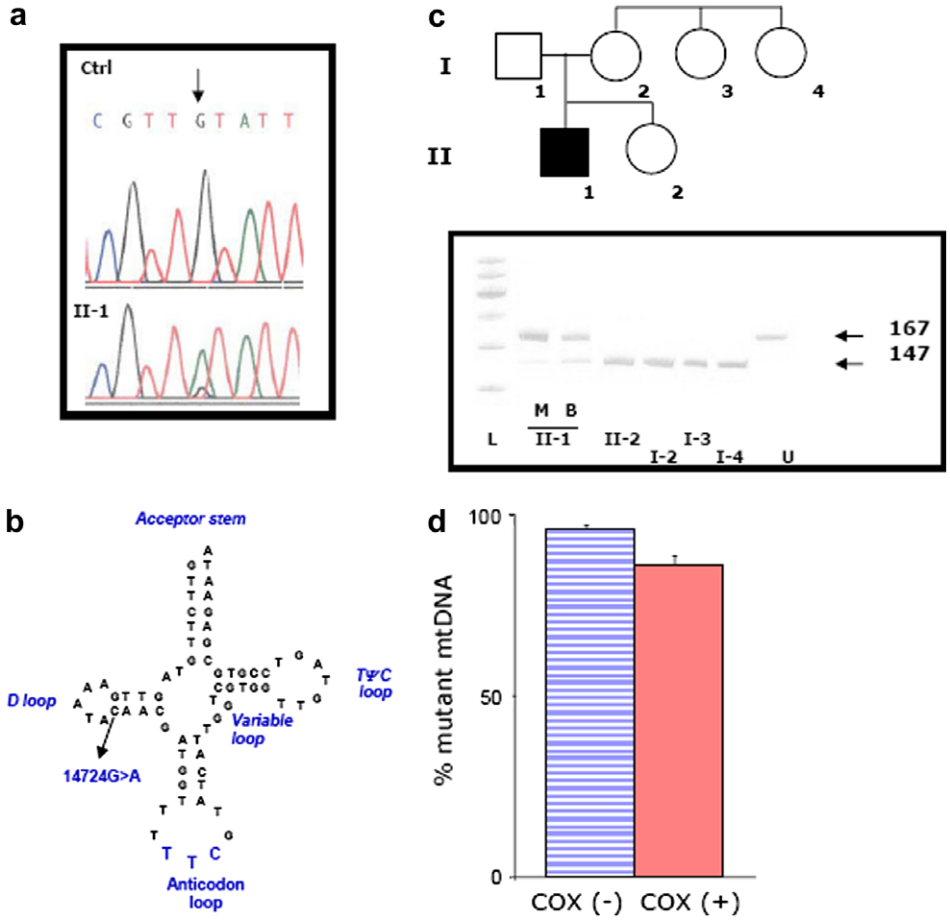

Fig. 2. Mutation analysis of the mitochondrial tRNA^{Glu} gene. (a) Electropherogram of the polymerase chain reaction–amplified blood DNA of a control (Ctrl) and of patient II-1; an arrow indicates the mutation. (b) Proposed tRNA^{Glu} secondary structure showing the mutation at position 14724 in the DHU stem (arrow). (c) Restriction fragment length polymorphism analysis of the 14724G>A mutation. M, patient skeletal muscle; B, patient blood; U, uncut; L, DNA 100-bp ladder. The family pedigree is superimposed. (d) Quantification of the 14724G>A mutation in the tRNA^{Glu} in single muscle fibers. Cytochrome *c* oxidase-negative fibres (COX−): means ± SD 95.84 ± 1.21%; cytochrome *c* oxidase positive fibres (COX+): 85.83 ± 2.71% ($p < 0.01$).

peripheral blood from the patient' healthy sister, mother, and in two additional maternal relatives (Fig. 2c). Single muscle fiber PCR analysis was performed to correlate levels of abnormal mtDNA with the morphological phenotype. The mean proportion of the mutant mtDNA in COX-negative fibers was 95.8 ± 1.2 ($n = 20$), and that in the COX-positive fibers was 85.8 ± 2.7 ($n = 17$). This finding was significant ($p < 0.01$) (Fig. 2d).

Discussion

In a 4-year-old child with encephalomyopathy, combination of clinical data with biochemical and morphological impairment of oxidative metabolism strongly indicated a defect in the mitochondrial genome. The presence of a multiple respiratory chain enzyme deficiency proposed an impairment of the protein synthesis machinery within mitochondria [15]. Accordingly, mtDNA sequence identified a novel G>A point mutation at position 14724 in the tRNA^{Glu} gene.

In muscle tissue from the propositus, nearly 100% of the mtDNA was mutated, whereas the mutational load was only 62% in blood. The mutation was absent in peripheral leukocytes from the patient's healthy mother and in three additional asymptomatic maternal relatives. No other tissue from the relatives was available for analysis leading to the impression that the mutation originated *de novo* in the germline of the developing embryo. No other change of pathogenic significance was identified when the mtDNA sequences were compared to the revised consensus human sequence [16].

The 14724G>A mutation is an additional disease-related variant in the mitochondrial tRNA^{Glu} gene. Although some of the mitochondrial tRNA genes (e.g., tRNA^{Ile}, tRNA$^{Leu(UUR)}$ and tRNA^{Lys}) are known to be mutational hotspots, only four heteroplasmic mutations in the tRNA^{Glu} gene have been described, so far. Similar to other mt-tRNA mutations the clinical spectrum of patients with tRNA^{Glu} mutations seems to be highly variable. The 14709T>C mutation in the anticodon-loop was identified in several families with mitochondrial myopathy combined with diabetes mellitus [17] whereas the 14696GA>G change in the stem of the pseudouridine loop was recognized in a Finnish patient with delayed psychomotor devel-

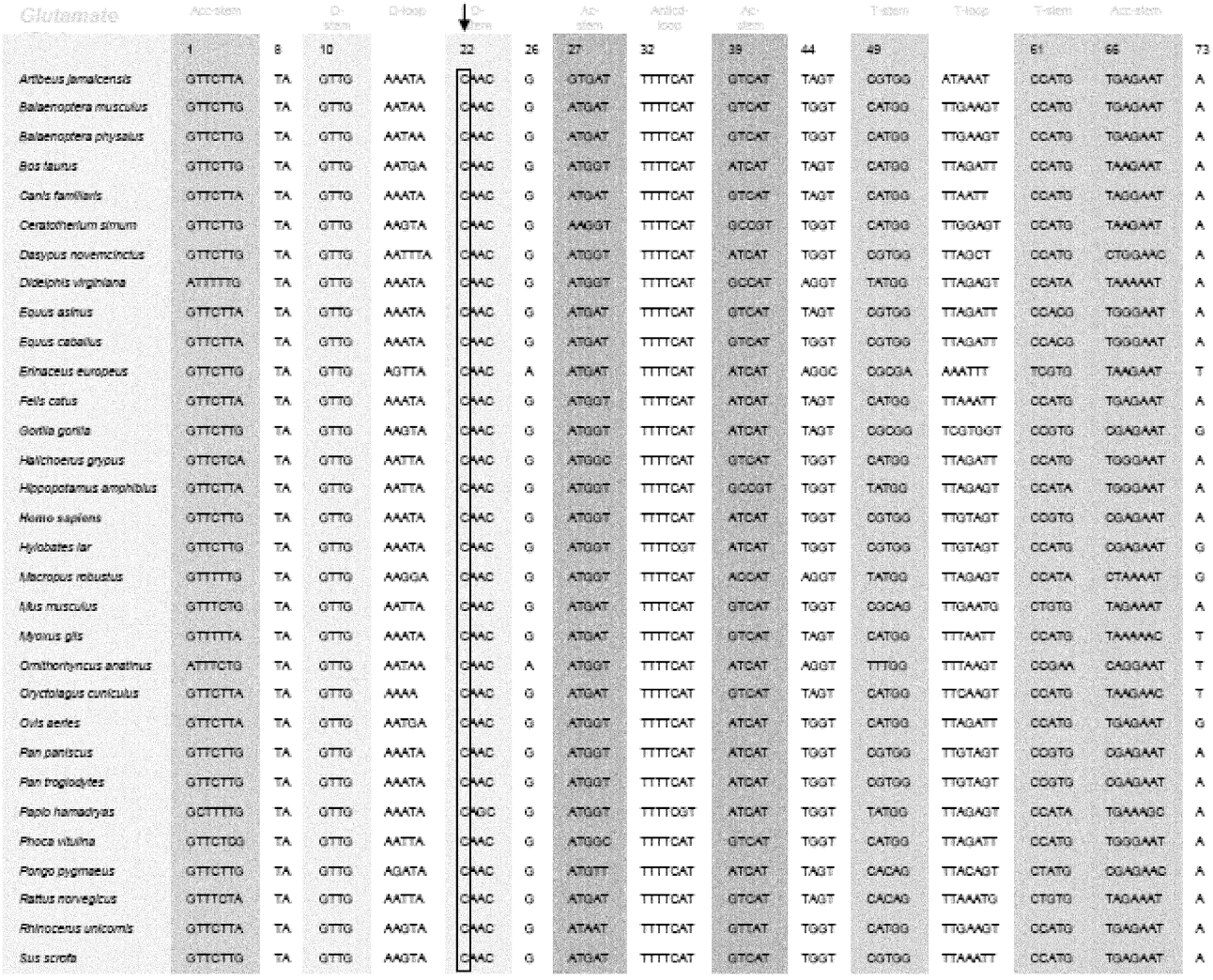

Fig. 3. Compilation of mammalian $tRNA^{Glu}$ genes. Nucleotide position 14724 (arrow) in the human gene is boxed. Alignment is based on the search of common secondary structural domains and follows the structural characteristics included in Ref. [21].

opment and resembling the MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) syndrome [18]. On the other hand, the 14687T>C variant in the TψC loop was detected in a sporadic child with mitochondrial myopathy and respiratory distress [19] while the 14739G>A mutation in the aminoacyl acceptor stem was recently reported in a girl with a predominantly myopathic phenotype [20]. All presented an ample heterogeneity in age of onset—which varied between birth and the forth decade—mutation load (between 70% and 100%), and degree of CNS involvement. The mutation at nt. 14724 lies at position 22 in the dihydrouridine (DHU) stem of the $tRNA^{Glu}$ predicted secondary structure and is distinctively associated with white and grey matter involvement together with a skeletal myopathy. Differences in clinical manifestations and severity of phenotype might only in part be explained by location of the mutated nucleotide within the tRNA predicted cloverleaf [21].

When one considers the consensus criteria for novel pathogenic mt-tRNA variants [15,22], the 14724A>G change would score as ''probably pathogenic'' because of indirect arguments. (1) No other mtDNA mutation or rearrangements were detected. (2) The mutation was absent in a large set of haplotype-matched controls. (3) The involved nucleotide within the tRNA displays high phylogenetic conservation through evolution (Fig. 3). (4) The mutation correlates well with the biochemical and morphological defects in single muscle fibers as shown by LCM-PCR analyses. Unfortunately, no tissue was available to generate transmitochondrial hybrids as to render ''probably'' more certain.

The novel variant in mt-$tRNA^{Glu}$ gene affects an extremely conserved G≡C bond in the DHU arm of the tRNA predicted cloverleaf (Fig. 2b). As shown for another pathogenic change affecting the dihydrohuridine arm—namely, the 3256C>T variant in the $tRNA^{Leu(UUR)}$ [23]—the mutation might well cause mitochondrial dysfunction by impairing mitochondrial protein synthesis. For instance, this appears pertinent for ND6 and COII, two mtDNA-encoded polypeptides with the maximum

number of glutamic acid residues in highly conserved positions (www.mitomap.org). This might eventually explain the combined complexes I and IV defect in the patient's muscle.

About 50% of the patients with white matter abnormalities remain without diagnosis [24,25]. Mitochondrial leukoencephalopathy [6] is not uncommon in the Kearns-Sayre syndrome due to large-scale deletion of the mitochondrial genome but in childhood it mostly associates with mutations in nDNA-encoded subunits of complex I or, less frequently, with variants in *SURF1* [26], which encodes an ancillary protein required for correct COX assembly. Moreover, mitochondrial leukodystrophy is mandatory in patients with the MNGIE (myo-neuro-gastrointestinal encephalopathy) syndrome due to mutations in *TP*. Thus, a specific protocol for studying and categorizing these patients should combine data concerning familiarity, onset of symptoms, neurological examination, presence of non-neurological symptoms, neurophysiological studies as well as systematic neuroimaging [27]. MR spectroscopy and new MR techniques will also provide crucial information for defining novel variants. In this respect, our findings not only lend further evidence to the expanding repertoire of mt-tRNA mutations in human diseases but also propose that the finding of a leukoencephalopathy of otherwise unclassified origin in a patient with a complex neurologic picture and multisystem involvement should prompt a thorough mitochondrial evaluation.

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