

# Atypical MELAS Associated With Mitochondrial tRNA<sup>Lys</sup> Gene A8296G Mutation

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**We report on a unique patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) presenting optic atrophy, cardiomyopathy, and bilateral striatal necrosis before stroke-like episodes became apparent. Skeletal muscle total mitochondrial DNA analysis identified a heteroplasmic A to G point mutation in the tRNA<sup>Lys</sup> gene at position 8296. Skeletal muscle pathology revealed typical MELAS findings, including ragged-red fibers cytochrome *c* oxidase positive strongly succinate dehydrogenase-reactive blood vessels. Recent reports describe the 8296 mutation identified in patients with diabetes mellitus or myoclonus epilepsy with ragged-red fibers, not MELAS. We conclude that the 8296 mutation is likely to be pathogenic and that it may be not only a mutation responsible for diabetes mellitus or myoclonus epilepsy with ragged-red fibers but also for MELAS. © 2002 by Elsevier Science Inc. All rights reserved.**

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## Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) are characterized by recurrent neurologic deficits including seizures, headache, dementia, and psychomotor retardation that initiate in childhood. Although symptomatic heterogeneity among MELAS patients has been known, the combination of chronic progressive external ophthalmoplegia, hearing loss, and diabetes mellitus is shared by most of them [1]. It is rare to have optic atrophy of early childhood onset with cardiomyopathy and bilateral striatal necrosis before stroke-like episodes become apparent.

We performed direct sequencing of all mitochondrial DNA (mtDNA) and identified the A8296G substitution in the tRNA<sup>Lys</sup> gene. In recent reports the A8296G mutation has been identified in patients with diabetes mellitus and myoclonus epilepsy with ragged-red fibers (MERRF). Herein we report on the first patient with atypical MELAS associated with the A8296G mutation and its causal link to the clinical features.

## Case Report

A 14-year-old Japanese male was born by normal spontaneous delivery at 38 weeks of gestation weighing 2,675 gm. His mother, 39 years old, was healthy, but his 65-year-old maternal grandmother had hearing loss and noninsulin-dependent diabetes mellitus. In his prenatal and perinatal periods and through early infancy, no abnormal characteristics were observed. At 4 years of age, he temporarily lost central vision in both eyes without noticeable pain. However, the loss of vision was transient, lasting for a few days; his visual acuity was limited to perceiving hand movements in both eyes. The optic disc pallor developed after a few months, and he was thus diagnosed as having optic atrophy. His visual acuity was gradually reduced. Hypertrophic cardiomyopathy was found at a school check-up when he was 6 years of age. However, he did not develop heart failure. Then he developed persistent fatigue and weakness of skeletal muscles between 8 and 9 years of age. At that time, he was noted to have short stature, sensorineural hearing loss, a variable speech disorder, poor short-term memory, and clumsiness. A computed tomography brain scan revealed a low-density area in bilateral striatal lesions. At 14 years of age, he had an episode of recurrent headache with nausea accompanied by focal motor and generalized epileptic seizures and lactic acidosis. He was referred to our hospital for further examination, including muscle biopsy, biochemical analysis, and DNA analysis. On admission, physical examination revealed short stature and body weight both below the second percentile. His pubertal development was normal. General examination findings were otherwise unremarkable. Optic atrophy was observed, but there was no retinal pigmentation. He had myoclonus with hypertonicity. There was no objective sensory deficit. Brain magnetic resonance imaging demonstrated diffuse cortical

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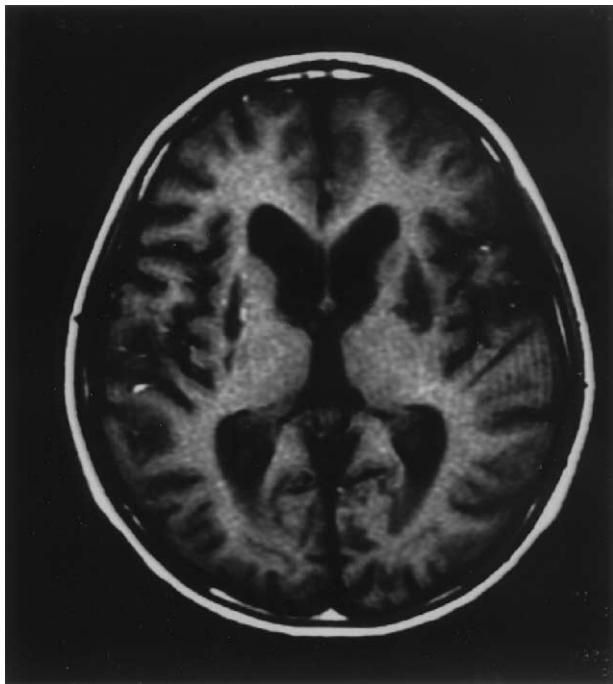


Figure 1. Axial  $T_1$ -weighted brain magnetic resonance image ( $TR = 400$  ms,  $TE = 15$  ms) is presented. Note the diffuse cortical atrophy and bilaterally symmetric lesions (low-intensity area) of the putamen and caudate nucleus.

atrophy and bilaterally symmetric lesions at the putamen and caudate nucleus (Fig 1). Echocardiography revealed hypertrophy of the anterolateral and posterior walls of the left ventricle (Fig 2). We treated the patient with cytochrome *c* and dichloroacetate for mitochondrial dysfunction, without significant effects.

Informed consent from the patient and patient's mother was obtained for all investigations.

### Muscle Biopsy

We obtained biopsied muscle from the left biceps brachii muscle. Serial frozen sections were stained with hematoxylin and eosin, modified Gomori trichrome, succinate dehydrogenase (SDH), cytochrome *c* oxidase (COX), and a battery of histochemical methods.



Figure 2. Echocardiography reveals hypertrophy of the anterolateral and posterior walls of the left ventricle at 14 years of age (parasternal long-axis view).

### DNA Analyses

Total DNA was extracted from a part of the muscle biopsy specimen. DNA sample was analyzed for the mutations previously identified in MELAS or MERRF, as described [2-6]. For sequencing analysis, 12 sets of primers were used to amplify the DNA fragments covering all of the mitochondrial tRNA regions. Polymerase chain reaction products were purified and their nucleotide sequences determined by an ABI 377 Automatic Sequencer (Perkin Elmer Biosystem, Foster City, CA) using the DNA Sequencing Kit and the Dye-Terminator Protocol (Perkin Elmer Biosystem, Foster City, CA).

Based on the sequencing result a simple screening method was devised to detect the A8296G mutation. This method utilizes an *Aci*I restriction site that is only present in the mutated allele. The mtDNA fragment surrounding the tRNA<sup>Lys</sup> was amplified by polymerase chain reaction using a sense primer, 5':-AACCAAACCACTTTCACCGCT-3'; (8123-8143), and an antisense primer, 5':-CATACGGTAGTATTTAGTTGG-3'; (8391-8371). The polymerase chain reaction was performed in a total volume of 5  $\mu$ L containing 5 ng of genomic DNA, 50 nM of two primers, 62.5  $\mu$ M of dNTP, 50 mM KCl, 10 mM Tris-HCl, 2.0 mM MgCl<sub>2</sub>, and 0.25 U of the Taq DNA polymerase. The polymerase chain reaction was performed for 30 cycles consisting of 15 seconds denaturation at 94°C, 15 seconds annealing at 50°C, and 30 seconds primer extension at 72°C in a Thermal Cycler (Perkin-Elmer Biosystem, Foster City, CA).

### Results

#### Biochemical and Histologic Characteristics

Routine hematologic and biologic investigations were indistinguishable from normal. Glucose tolerance test was normal. However, lactate levels of fasting blood and cerebrospinal fluid were elevated to 40 mg/dL and 80 mg/dL (normal range = 3.3-14.9 mg/dL), respectively.

The muscle fibers varied in size from 20 to 80  $\mu$ m in diameter. Some ragged-red fibers (RRFs) and strongly SDH-reactive blood vessels (SSVs) were recognized. All the muscle fibers were COX positive. RRFs and SSVs demonstrated increased levels of COX activity (Fig 3). Enzymes involved in mitochondrial respiratory chain were not analyzed.

#### DNA Analyses

The DNA sample was screened for the mutations in mitochondrial tRNA genes that were previously found to be associated with MELAS or MERRF. They included the 3243, 3271, 3291 mutation of the tRNA<sup>Leu(UUR)</sup> gene, and the 8344 and 8363 mutation of the tRNA<sup>Lys</sup> gene [2-6]. However, none of these were detected. Sequence analysis identified a number of nucleotide substitutions, A1438G, T2626C, A2706G, C2772 torr, G3423 torr, A4769G, A4958G, G4985A, C7028 torr, A8296G, A8701G, A8860G, T9540 torr, T11335C, C12705 torr, and G14365C, in comparison to the Cambridge sequence. With the exception of the A8296G mutation, these substitutions have been viewed as polymorphisms without negative effects on function. Cleavage of the 268-bp polymerase chain reaction product containing the mutated region by *Aci*I revealed a heteroplasmic proportion (Fig 4). With this simple and specific screening method, the

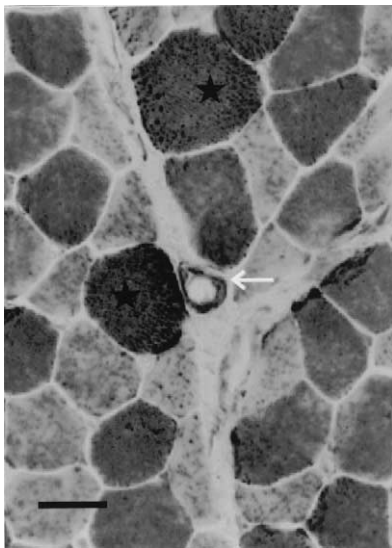


Figure 3. Frozen cross-sections are illustrated with cytochrome c oxidase (COX) stain. There was a mild variation in fiber size. Some ragged-red fibers (RRFs)(★) and strongly SDH-reactive blood vessels (SSVs)(←) were recognized. All the muscle fibers were COX positive. RRFs and SSVs demonstrated increased levels of COX activity (bar = 50  $\mu$ m).

A8296G mutation was not detected in 100 normal control DNA samples.

## Discussion

The diagnosis of MELAS was determined for this patient based on the following: (1) stroke-like episodes with recurrent headache and vomiting, (2) high lactate level presenting both in blood and cerebrospinal fluid, (3) muscle biopsy findings including RRFs and COX-positive SSV. The presence of COX-positive SSV is a characteristic vascular finding for MELAS. In MERRF, SSVs also appear in muscle specimens, but COX activity in SSVs is almost negative [1].

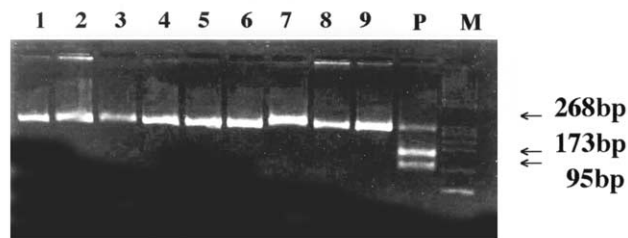


Figure 4. The A8296G mutation and normal alleles of the tRNA<sup>Lys</sup> gene detected by polymerase chain reaction followed by AclI digest are depicted. The DNA fragments were stained with ethidium bromide and visualized using an ultraviolet light transilluminator. The polymerase chain reaction products from the mutant mtDNA of the patient (lane P) and wild type of mtDNA in control subjects (lane 1-9) were fractionated by electrophoresis through a 3% Nusieve agarose gel. The larger DNA fragment of 268-bp in size is derived from the nonmutated allele. The mutated allele contains an AclI site and thus generates two smaller fragments (173 and 95 bp) by an AclI cleavage. The proportion of the two alleles in the patient DNA sample indicates a characteristic of a heteroplasmic mutation. M lane is DNA size marker.

The current patient is unusual in that the symptoms are accompanied by optic atrophy, cardiomyopathy and bilateral striatal necrosis [7], which appeared in early childhood before the onset of stroke-like episodes. Ophthalmologic impairments usually observed in MELAS are pigmentary retinopathy, ophthalmoplegia, and ptosis [8]. However, optic atrophy observed in this patient has been rarely reported in association with MELAS [9] and thus makes this patient unique. Pulkes et al. reported on a patient having combined syndromes of Leber's hereditary optic neuropathy (LHON) and MELAS [10]. A recent report described a family in which the affected members had late-onset optic atrophy, ataxia, and myopathy associated with a mutation of a complex II gene. These patients did not have stroke-like episodes [11]. LHON cause of permanent blindness in otherwise healthy young adults is more common in men. LHON is mostly associated with mtDNA point mutation in the mitochondrial complex I subunit genes ND1, ND4, and ND6 [12]. Our patient developed blindness in his early childhood; however the loss was only temporary. Because of the disparity of symptoms, our patient was not diagnosed as LHON.

None of the mutations previously associated with MELAS were detected in our patient, and sequencing of all mtDNA was necessary to identify any potentially causal mtDNA mutation. Extensive sequence analysis of mtDNA identified only one nucleotide substitution, A8296G, in the tRNA<sup>Lys</sup> gene of a MELAS patient who presented with an unusual combination of symptoms. More than 80% of MELAS patients are found to have a single nucleotide substitution at position 3243 from A to G (A3243G mutation) in the tRNA<sup>Leu(UUR)</sup> gene of the mitochondrial genome [1]. Mutations at position 3251, 3256, 3271, and 3291 of the tRNA<sup>Leu(UUR)</sup> gene may also be responsible for various forms of MELAS [3,4]. Thus the tRNA<sup>Leu(UUR)</sup> gene may be a genetic hot spot with a specific link to MELAS.

In contrast, many of the mitochondrial DNA mutations in tRNA<sup>Lys</sup> are associated with MERRF [5,6]. Its unique link to and the atypical nature of clinical features imply that the mutation is pathogenic. This notion is supported by the following three additional factors: (1) the mutation is heteroplasmic, (2) the mutated site is located in the aminoacyl acceptor stem of tRNA<sup>Lys</sup>, in which the wild type of nucleotide adenine is highly conserved between a diverse range of species, and (3) the mutation is not present in 100 healthy control subjects.

The A8296G mutation may be a part of the complex cause of diabetes, and 1% of diabetic patients have this particular mutation [13]. Moreover, a recent report documented a double mutation, A8296G and G8363A, being associated with myoclonus epilepsy with MERRF [14]. In this context, because the 8296 mutation was nearly homoplasmic, the authors concluded that G8363A mutation is pathogenic; the co-occurrence of the 8296 mutation is of unclear significance and is likely to be a rare polymorphism. However, this 8296 mutation alters the double

helix of the stem and secondary structure of tRNA. This effect is the impairment of the activities of aminoacyl-tRNA synthase and peptidyl-tRNA hydrolase, which play a central role in the mitochondrial translation process. Thus this study reports the first patient of MELAS associated with the A8296G mutation.

We conclude that the A8296G mutation is likely to be pathogenic and may have a causal link not only to diabetes mellitus and MERRF but also to MELAS.

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