Letters to the Editor

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De Novo mtDNA nt 8993 (T→G) Mutation Resulting in Leigh Syndrome

To the Editor:

Recently, Blok et al. (1997) analyzed mtDNA in oocytes from an asymptomatic mother of three children exhibiting heteroplasmic expression of the mtDNA nt 8993 (T→G) (T8993G) mutation associated with Leigh syndrome (MIM 516060). The mother had 50% mutant mtDNA in her blood. It was striking that one of the seven oocytes analyzed showed no evidence of the mutation, while the remaining six had a mutant load of >95%. Blok et al. suggested that this observation reflected preferential amplification of the mtDNA variant during oogenesis. During formation of the zygote, mtDNA is derived exclusively from the oocyte (Giles et al. 1980). Thus, it is possible that a de novo mutation may arise during oogenesis. A first carrier of a de novo mutation may be a mother who exhibits mosaicism for the mutation restricted to oocytes. However, it has been shown by previous investigators that the mothers of patients with Leigh syndrome associated with the mutation usually have substantial levels of the mutant mtDNA (Tatuch et al. 1992; Santorelli et al. 1993; Tulinius et al. 1995). The proportion of the mutant mtDNA in the lymphocytes from such mothers has been reported to be 38%–76%. Here we report the case of a 1-year-old boy with Leigh syndrome associated with the T8993G mutation, whose mother did not have the mutant mtDNA in her blood or urine sediment cells. It was shown that a de novo T8993G mutation in mtDNA may occur spontaneously at a high level in oocytes, thereby causing Leigh syndrome in the second generation.

The present patient was a Japanese boy born at term after an uncomplicated pregnancy. He was the second child of a 25-year-old mother and a 23-year-old father, who were healthy and unrelated. A 3-year-old sister was also healthy. His birth weight was 2,842 g, and the occipito-frontal circumference (OFC) was 33.2 cm (50th percentile). Generalized hypotonia was noted at birth. He developed apnea attacks and altered consciousness, after upper respiratory infections at the ages of 2 and 4 mo. From the age of 7 mo, he showed symptoms of brain-stem dysfunction, such as irregular respiration and swallowing difficulty. At the age of 9 mo, growth retardation (height 72.0 cm; body weight 6.84 kg, −2.3 kg SD) and microcephaly (OFC 43.0 cm, <10th percentile) were obvious. Although he could follow objects with his eyes and could respond to auditory stimuli, his head control was poor, because of severe generalized hypotonia.

Laboratory examination revealed increased lactate and pyruvate levels in blood (lactate 28 mg/dl; pyruvate 2.0 mg/dl) and cerebrospinal fluid (lactate 50 mg/dl; pyruvate 2.7 mg/dl) with high lactate/pyruvate and β-hydroxybutyrate/acetoacetate (2.9; normal range 1.84 ± 0.96) ratios. Plasma amino acid analysis revealed an increased level of alanine (62.9 μmol/dl; normal range 16.9–48.3 μmol/dl). On arterial blood gas analysis, metabolic acidosis was noted with respiratory compensation, i.e., pH 7.37; carbon dioxide tension 26.8 mmHg; partial pressure of oxygen 113.3 mmHg; bicarbonate 15.4 mEq/liter; and base excess −8.0 mEq/liter. Brain magnetic-resonance images revealed symmetrical necrotic foci in the striatum and periaqueductal gray matter, which are characteristic of Leigh syndrome. Histological examination of biopsied quadriceps femoris muscle showed variation in fiber size, but ragged-red fibers were not seen. Electron microscopy, however, showed a marked increase in the number of variably sized mitochondria with aberrant cristae. The activities of mitochondrial respiratory chain enzymes in the biopsied muscle were normal (NADH cytochrome c reductase 162.9 nmol/min/mg mitochondrial protein, control 27.3 ± 11.6; succinate cytochrome c reductase 139.8, control 76.6 ± 17.7; cytochrome c oxidase 63.9, control 33.0 ± 16.1).

After informed consent had been granted, blood and urine samples for DNA extraction were obtained from the patient and from his parents, sister, and maternal grandmother. In addition, the patient's DNA was also extracted from muscle and skin fibroblasts. Because the ratio of mutant to nonmutant mtDNA found in mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes associated with pathogenic point mutations is not constant in lymphocytes and muscle (Poulton and Morten 1993), we extracted mtDNA from

at least two different somatic cells. DNA in lymphocytes, muscle, and skin fibroblasts was extracted by the phenol/chloroform method, although the procedure involving proteinase K digestion/boiling treatment was required for urine sediment cells. mtDNA encompassing the ATPase 6 gene was amplified by PCR, by use of a pair of primers, 5′-ccg act aat cac cac cca ac-3′ (nt 8648–8665) and 5′-tgt cgt gca ggt aga ggc tt-3′ (nt 9180–9199). The PCR conditions were as follows: 30 cycles, each consisting of denaturation for 0.5 min at 95°C, annealing for 0.5 min at 55°C, and extension for 0.5 min at 72°C. One extra cycle was then performed, after addition of 10 μCi of α-[^{32}P]dATP (3,000 Ci/mmol), 10 pmol of each primer, and 1.0 unit of *Taq* polymerase. Condition for this last cycle were 5 min at 95°C, 0.5 min at 55°C, and 5 min at 72°C. Adding radioactive dATP in only the last PCR cycle avoids the formation of heteroduplex DNA molecules, which would cause underestimation of the mutant mtDNA levels after restriction-enzyme digestion (Schoffner et al. 1990). Five microliters of the PCR product were digested in a final volume of 20 μl for 1 h at 37°C with 10 units of *Ava*I (Toyobo). The digestion products were electrophoresed through a 10% nondenaturing polyacrylamide gel. The gel was dried and autoradiographed at room temperature for 1 h by use of Kodak x-ray film. PCR restriction-digestion analysis of mtDNA from the patient revealed a T→G change at nt 8993, creating a new *Ava*I restriction site (fig. 1). The proportion of the mutant mtDNA was calculated as the ratio between the intensity of the 551-bp band and that of the 345-bp + 206-bp bands, by use of a scanning densitometer. The sensitivity of the PCR assay for the mutation was 0.2%, which was determined by analyzing serial dilutions of mutant mtDNA. The patient had >95% mutant mtDNA in all examined samples, including lymphocytes, urine sediment cells, skeletal muscle, and skin fibroblasts, but no mutant mtDNA was detected in lymphocytes or urine sediment cells from the mother, sister, or maternal grandmother.

Cases have been reported in which novel mtDNA mutations in sporadic patients with mitochondrial encephalomyopathy appeared to be confined only to skeletal muscle (Fu et al. 1996; Weber et al. 1997). It is speculated that this phenomenon may reflect loss of the mutation by random genetic drift in mitotic tissues and proliferation of mitochondria containing the mutant mtDNA in postmitotic cells. Although muscle for DNA analysis was obtained only for the patient, it has been reported that the proportion of the mutant mtDNA was fairly constant in muscle and lymphocytes in patients carrying the T8993G mutation (Mäkelä-Bengs et al. 1995). Furthermore, the de novo occurrence of the T8993G mutation has previously been described, but in these families the amount of mutant mtDNA gradually increased during several generations, before reaching

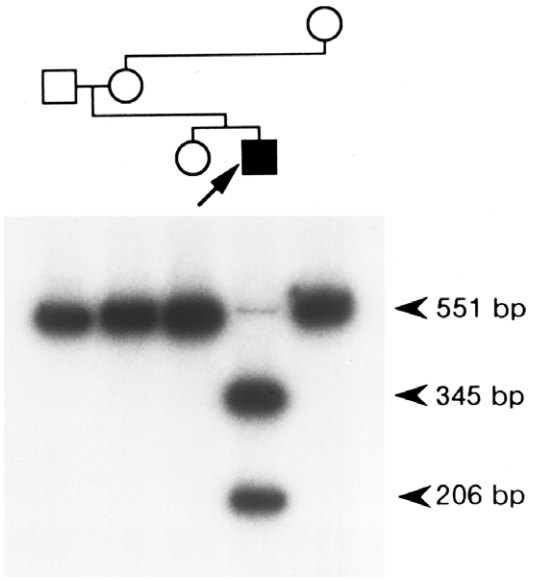

Figure 1 Pedigree of the family and autoradiograph of *Ava*I-digested ^{32}P-labeled PCR products of mtDNA encompassing the ATPase 6 gene. Template DNA was prepared from lymphocytes of family members. In the proband, the 551-bp PCR product harboring the mtDNA nt 8993 (T→G) mutation was cleaved into 345-bp and 206-bp fragments. No mutant mtDNA was detected for the proband's parents, sister, or maternal grandmother.

levels sufficiently high to cause Leigh syndrome (Santorelli et al. 1993; Tulinius et al. 1995). The finding that the mutation was present at high percentages in all samples from the patient but not in the mother or sister may be interpreted as follows: the mutation occurred spontaneously in some, but not all, oocytes, and consequently the mother's ovary may have had a mosaic status for the mutation, although it is not clear when the mutation arose. The germ-line mosaicism for the T8993G mutation identified by Blok et al. (1997) supports our interpretation. The recurrence risk cannot be estimated correctly, whereas the germ-line mosaicism may lead to familial clustering of affected individuals. We believe that this observation is useful for genetic counseling for families with affected patients.

Satoru Takahashi ,[1] Yoshio Makita,[1]
Junichi Oki,[1] Akie Miyamoto,[1]
Junichi Yanagawa,[2] Etsuo Naito,[3]
Yu-ichi Goto,[4] and Akimasa Okuno,[1]
[1]*Department of Pediatrics, Asahikawa Medical College, and* [2]*Department of Pediatrics, Haboro Hospital, Hokkaido;* [3]*Department of Pediatrics, School of Medicine, University of Tokushima, Tokushima; and* [4]*Department of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo*

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Address for correspondence and reprints: Dr. Satoru Takahashi, Department of Pediatrics, Asahikawa Medical College, Nishikagura 4-5-3-11, Asahikawa 078, Japan. E-mail: satoru5p@asahikawa-med.ac.jp

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