

# Mild clinical manifestation and unusual recovery upon coenzyme Q<sub>10</sub> treatment in the first Chinese Leigh syndrome pedigree with mutation m.10197 G>A

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**Abstract.** The Leigh syndrome (LS), characterized by psychomotor retardation, seizures, nystagmus, ophthalmoparesis, optic atrophy, ataxia, dystonia, or respiratory failure, is one of the most severe mitochondrial diseases. In the majority of cases, the disease is fatal and patients die before age 5. Mutation m.10197 G>A was found to relate to the severe phenotype of the Leigh syndrome. Here, we describe the first Chinese Leigh syndrome pedigree with this mutation. The proband had the characteristic brain lesions of the Leigh syndrome and presented a decrease in exercise tolerance and mild face paralysis. Sequencing the NADH dehydrogenase, subunit 3 (*ND3*) gene in the pedigree, revealed that the proband, as well as her unaffected brother, have a high mutant load in the *ND3* gene, compared to their mother. Following one-year treatment with the coenzyme Q<sub>10</sub>, an obvious improvement in clinical features was observed by magnetic resonance imaging (MRI) in the proband. Our study and previous reports highlight the variability of phenotypic expression of the m.10197 G>A mutation, and suggest that pathogenesis of the syndrome may be affected by a number of factors. This is the first report on successful treatment of an LS patient carrying the mutation m.10197 G>A with the coenzyme Q<sub>10</sub>, indicating that Q<sub>10</sub> may attenuate the mitochondrial dysfunctions caused by the m.10197 G>A mutation.

## Introduction

Complex I is the first and largest component of the mitochondrial respiratory chain. Impairment of complex I accounts for most cases of respiratory chain deficiency, and results in a wide range of clinical manifestations (1,2), including Leigh syndrome (LS) (3). Complex I deficiency is also the most common cause of LS (4). In LS patients with complex I deficiency, complex I activity may be reduced up to 66% (4), whereas the activity of other complexes is normal.

LS is a subacute, necrotizing encephalopathy, characterized by bilateral symmetrical necrotic lesions of gray matter nuclei in the basal ganglia, diencephalon, cerebellum, or brainstem (5). Onset of LS typically occurs at early infancy. Patients present psychomotor retardation, seizures, nystagmus, ophthalmoparesis, optic atrophy, ataxia, dystonia, or respiratory failure, due to the progressive decline of the central nervous system functions. In most cases, LS patients die before age 5 (6).

The mutation m.10197 G>A in the mitochondrial NADH dehydrogenase, subunit 3 (*ND3*) gene was found to cause LS. This mutation, causing the typical manifestations of LS, is considered to lead to a severe phenotype. In this study, we describe the clinical and molecular features of the first Chinese LS pedigree with the mutation m.10197 G>A, and compare the phenotype of these LS patients to those previously described for LS patients with the same mutation. We also describe follow-up results of treatment with the coenzyme Q<sub>10</sub>.

## Materials and methods

**Pedigree and ethics.** The study was approved by the Ethics Committee of Fujian Medical University Union Hospital. Informed consent was obtained from all patients included in the study.

A two-generation Han Chinese family living in south-east China near the Min River with 4 family members was studied (Fig. 1A). The proband, a 16-year-old girl (40 kg weight, 157 cm height), was admitted in the Union Hospital

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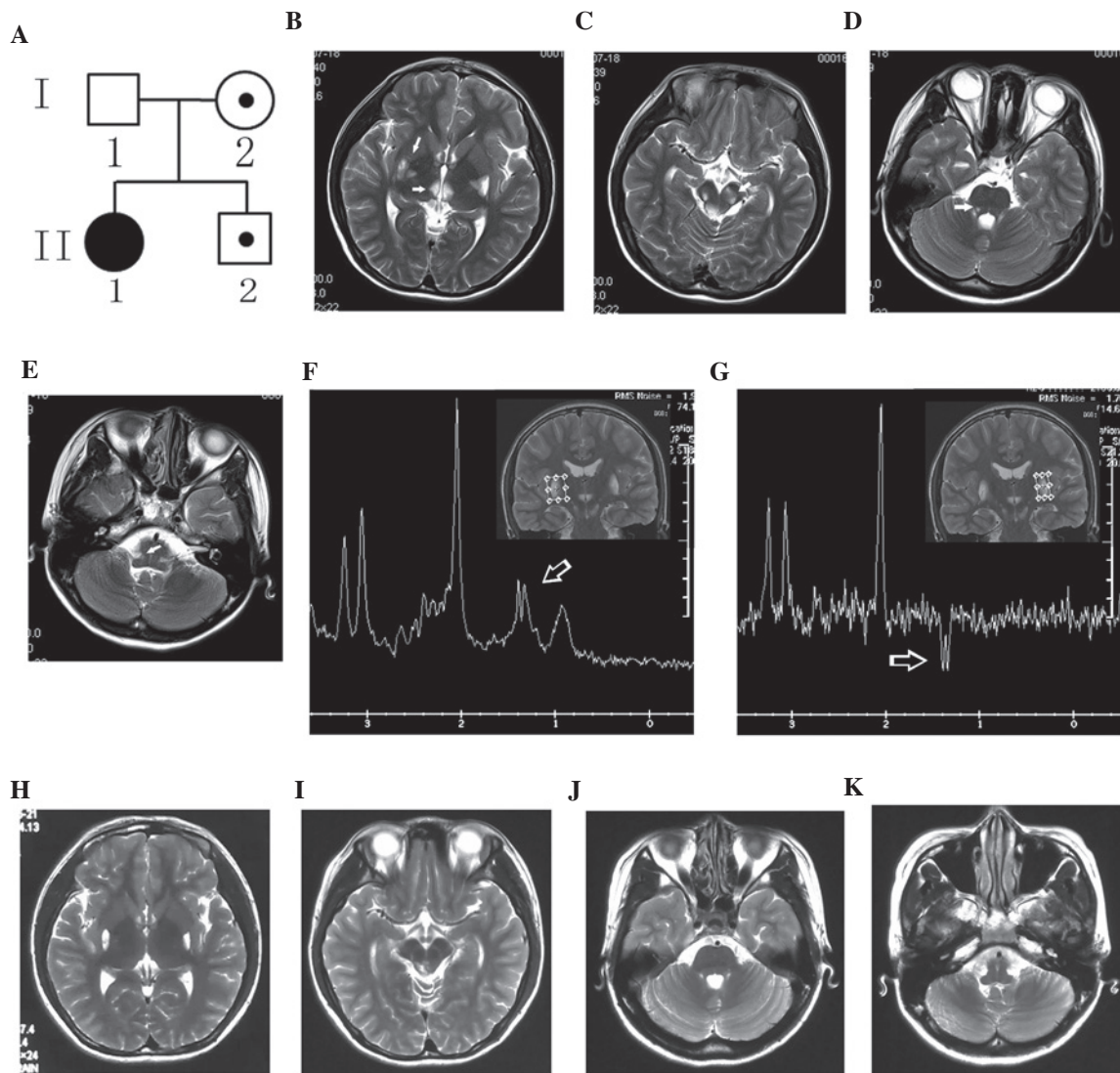


Figure 1. Pedigree chart of the family with the Leigh syndrome (LS) and neuroimaging of the proband. (A) Pedigree chart. I and II denote generation number, and 1-2 individual number; the black circle denotes the proband. (B-E) T2-weighted images (T2WI) from magnetic resonance imaging (MRI) of the proband show prolonged signals (arrows) in the (B) basal ganglia and thalamus, (C) midbrain, (D) pons, and (E) medulla oblongata. (F and G) As shown in the magnetic resonance spectra, there is a markedly increased lactate doublet (arrows) in the prolonged signal region in the bilateral basal ganglia. Following one-year treatment, the lesions disappeared in the (H) thalamus, (I) bilateral cerebral peduncle, (J) pons and (K) medulla oblongata, and partially reduced in the (H) basal ganglia and (I) the dorsal midbrain, as shown by T2WI.

in 2012 with slight facial paralysis. She had been delivered with mild asphyxia, due to a 6-h-long second stage of labor with an 8-h duration from full dilatation to delivery. She presented symptoms of anorexia and fatigue following physical activities. Therefore, she did not pass any physical agility test at school. With regards to academic performance, she ranked middle in her class. There was no evidence of mental or psychomotor retardation. She scored 28 in both the Mini-Mental State Examination and Montreal Cognitive Assessment. Neurological examination revealed horizontal nystagmus, slightly shallow left nasolabial fold, slight hypalgesia in the left low face, hypotonia, and indications of bilateral Babinski sign. The levels of serum ceruloplasmin, serum creatine kinase, serum creatine kinase-MB, and antinuclear antibody were normal. An elevated plasma lactate level was noted [4.0 mmol/l; normal level (N) <2.7]. Electroencephalogram and electromyography examinations revealed no abnormality. Brain magnetic resonance imaging

(MRI) showed prolonged T1- and T2-weighted signals in the basal ganglia, thalamus, midbrain, pons, and medulla oblongata (Fig. 1B-E). Magnetic resonance spectroscopy showed a markedly increased lactate doublet in the lesions (Fig. 1F and G). The pressure, routine, biochemical, cytological, and IgG index examinations of the cerebrospinal fluid (CSF) were normal, and oligoclonal bands were absent. Histochemical examination of a muscle biopsy specimen stained with hematoxylin and eosin (Fig. 2A), modified Gomori trichrome (Fig. 2B), nicotinamide adenine dinucleotide (Fig. 2C), as well as electron microscopy examination (Fig. 2D) showed no characteristic abnormality. The mother of the proband, and her 8-year-old brother were normal. There were no abnormalities in their physical examination and MRI brain imaging. The proband received treatment with 90 mg/day of the coenzyme Q<sub>10</sub> for a year. Physical examination, MRI brain imaging, and plasma lactate level assessment were performed at the end of the treatment.

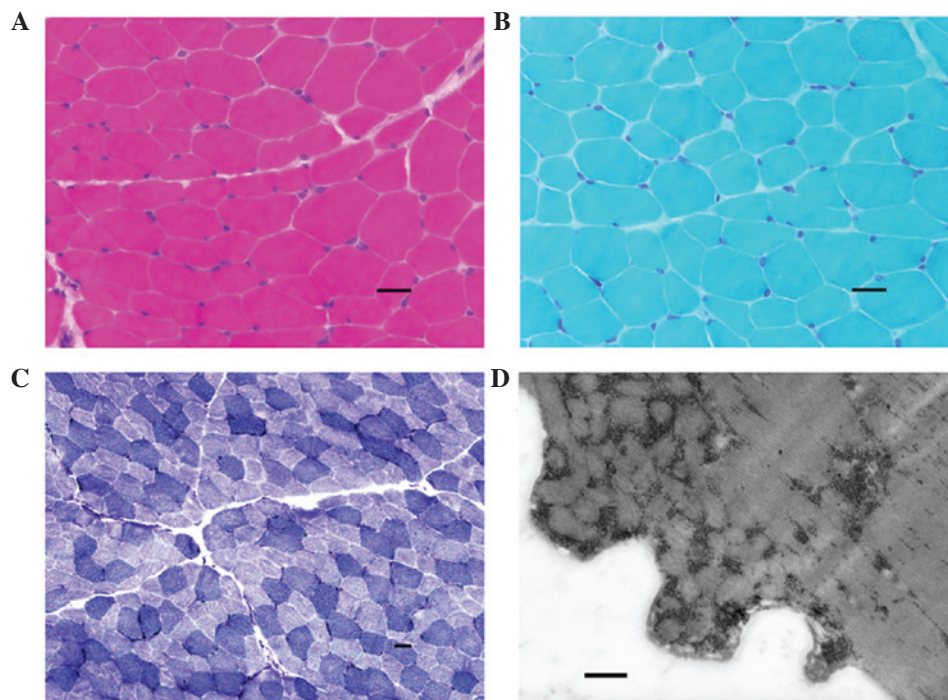


Figure 2. Histochemical and electron microscopy examination of muscle biopsy specimens. No obvious abnormalities are observed in the specimens (A) stained with hematoxylin and eosin, (B) stained with modified Gomori trichrome, (C) stained with nicotinamide adenine dinucleotide (scale bar, 100  $\mu$ m), and (D) examined under the electron microscope (scale bar, 300 nm). Mild atrophy of type II muscle fibers is detected by nicotinamide adenine dinucleotide staining, and mild subsarcolemmal proliferation of the mitochondria is detected by electron microscope examination.

**Mutational analysis.** Genomic DNA was extracted from blood lymphocytes and skeletal muscle of the pedigree using QIAamp DNA Mini kit (Qiagen, Dusseldorf, Germany). Full-length mitochondrial DNA (mtDNA) was amplified by polymerase chain reaction using 24 pairs of overlapping primers (Sangon Biotech, Shanghai, China). PCR was performed in a total volume of 25  $\mu$ l, containing 100 ng of genomic DNA, 0.25 M of each primer, 10 M deoxynucleotide triphosphates, 1.25 U of Taq enzyme and 2.5  $\mu$ l of 10X buffer. PCR amplification was performed using an Applied Biosystems 9700 thermocycler (Life Technologies, Carlsbad, CA, USA) with an initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 45 sec, and extension at 72°C for 1 min, with a final extension step at 71°C for 6 min. Sequencing was performed on an Applied Biosystems 3730 DNA automatic sequencer (Life Technologies) and resulting sequences were compared to the revised Cambridge reference sequence (rCRS) of the human mtDNA (7,8).

**Quantification of heteroplasmy.** The mutant load of m.10197 G>A was determined by restriction fragment length polymorphism analysis. A 476-bp fragment was amplified using the fluorescent-labeled FAM forward primer 5'-FAM-TTTGTAGCCACAGGCTTCC-3' and the reverse primer 5'-AAGGAGGGCAATTTCTAGAT-3'. The fragment was then digested with the restriction enzyme *Cac8I* (restriction site: GCN\*NGC). This restriction site is present only in the wild-type mtDNA, and is thus expected to give 402- and 74-bp products upon wild-type mtDNA digestion. The digested fragments were subjected to electrophoresis and DNA sequencing, and the fluorescent fragments were detected using the Applied

Biosystems GeneMapper® software V3.3 (Life Technologies). Heteroplasmy or mutant load was calculated by dividing the 476-bp peak area by the sum of the 476- and 402-bp peak areas.

**Haplogroup identification in the pedigree.** MtDNA haplogroup analysis was performed by full-length mitochondrial DNA sequencing, using the same method described above for mutational analysis. The results were confirmed by referring to Kivisild's study (9).

## Results

**Mutational analysis.** Direct sequence analysis revealed our proband with the m.10197 G>A mutation in the ND3 gene (Fig. 3A). This mutation was also found in the proband's mother and young brother, with the exception of the father. Previously reported common mutations such as T8993C, T9176G, and A3243G were not found (10). The mutation m.10197 G>A is a substitution at codon 47 of the ND3 gene, causing an alanine-to-threonine amino acid change. This mutation has been reported (11,12), and based on Ensembl (www.ensembl.org), was not a polymorphism.

**Quantification of heteroplasmy.** Digestion of the mtDNA with the restriction enzyme *Cac8I* and electrophoresis (Fig. 3B) showed that the mutant load of the proband and her brother are higher than that of their mother. Quantification of the dye signal showed that the proband is heteroplasmic with 97% mutant load in the skeletal muscle (Fig. 3C), and 95% in the leukocytes (Fig. 3D). Leukocytes of her mother had 39% mutant load (Fig. 2E) and those of her brother 80% (Fig. 2F).



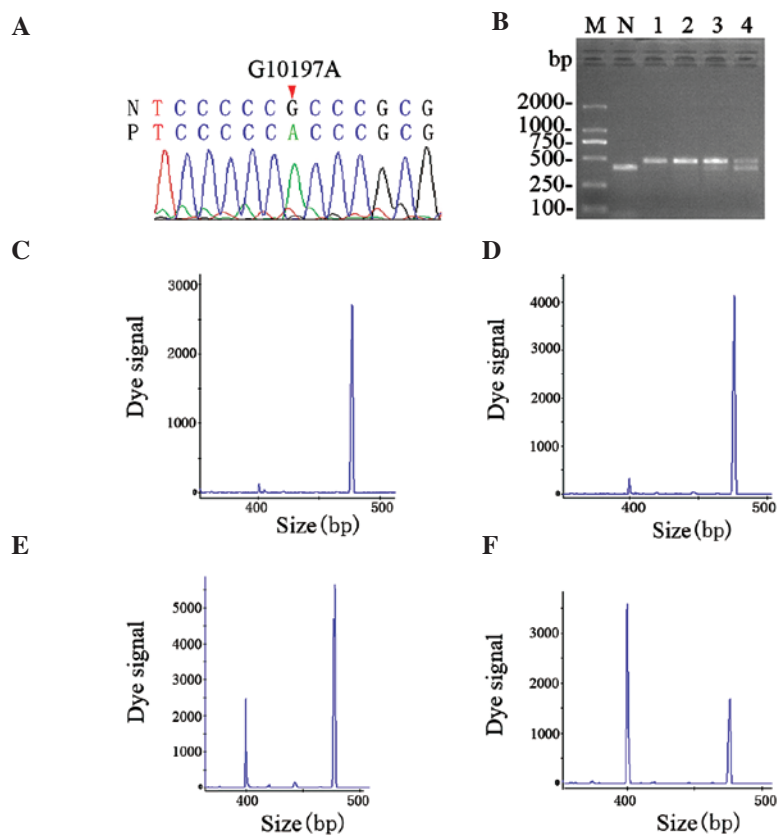


Figure 3. Identification of the mutation and analysis of heteroplasmy. (A) The mutation m.10197 G>A (red arrow) in the NADH dehydrogenase, subunit 3 (*ND3*) gene as shown in the sequencing chromatogram. (B) Gel image of digestion. Only the wild-type mtDNA sequence could be digested into two fragments of 402 and 74 bp. The latter was absent in the gel. The proband and the proband's brother contained a higher proportion of mutant sequence than their mother. M, DNA ladder; N, normal individual; lane 1, proband skeletal muscle DNA; lane 2, proband leukocyte DNA; lane 3, leukocyte DNA of proband's brother; lane 4, leukocyte DNA of proband's mother. (C-F) Quantification of heteroplasmy. The digested segments were separated by sequencer and heteroplasmy was calculated by dividing the 476-bp peak area by the sum of the 476- and 402-bp peak area. The proportion of mutant mtDNA of the (C) proband's muscle, (D) proband's leukocytes, (E) leukocytes of the proband's mother and (F) leukocytes of the proband's brother were 97, 95, 39 and 80%, respectively.

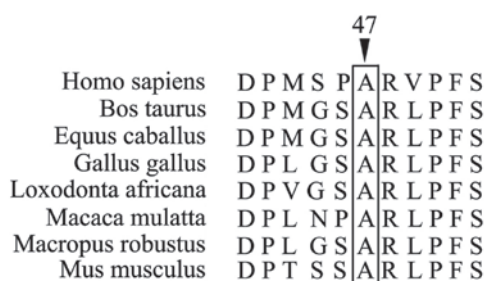


Figure 4. Multiple sequence alignment of the ND3 protein segment containing the mutation. As shown by the arrow, the alanine residue encoded by codon 47 is highly conserved in higher eukaryotes.

**Haplogroup identification in the pedigree.** By analysis of direct sequence, the mtDNA haplogroup for our pedigree was determined. The proband, her mother and young brother belong to the mitochondrial haplogroup N9a.

*Following results of treatment with coenzyme Q<sub>10</sub>.* After a 3-month treatment with the coenzyme Q<sub>10</sub>, neurological examination of the proband revealed no marked alteration. However, the proband's exercise tolerance and anorexia were markedly improved. Her body weight increased by 10 kg, and she passed all the physical agility tests. One year later, the

proband maintained this status. Reexamination by brain MRI demonstrated that certain lesions detected one year earlier had disappeared (Fig. 1H-K). The plasma lactate level was decreased to a normal level (2.5 mmol/l, N<2.7).

## Discussion

LS is a severe, progressive, metabolic neurodegenerative, mitochondrial disorder. The molecular defects and pathogenetic background of LS would provide considerable information to the patients in terms of management, genetic counseling, and prognosis. In this study, we described the clinical and molecular features of the first Chinese LS pedigree with the m.10197 G>A mutation, which leads to a severe phenotype, and report promising effects of treatment with the coenzyme Q<sub>10</sub>, as assessed by both clinical feature assessment and brain imaging.

The mutation m.10197 G>A was first reported in 2007 in 3 French pedigrees, as present in totally 8 LS patients (11). As shown in Table I, patients generally showed early-onset LS with a severe clinical outcome. Seventy-five percent of the patients died 1 year after the onset. A number of studies have provided evidence for the pathogenic role of the mutation m.10197 G>A. The mutation involves the codon 47 of the *ND3* gene. This codon encodes alanine, a highly conserved residue in higher

Table I. Clinical features of Leigh syndrome patients with the m.10197 G>A mutation, summary of present and literature data. Information on the patient's mother in each pedigree is not shown.

Country (Refs.)	Patient no.	Gender	Age at onset	Manifestation	Mutant load (%)	Duration/ Death age	Cerebral imaging	Lactate level (mM)	Muscle biopsy
France (11)	1	Ma	1 m	Growth retardation, hypotonia, psychomotor regression, seizures	100 (muscle)	4 m/5 m	Brainstem	CSF: 3.5	NA
	2	Ma	2 m	Anorexia, nystagmus, hypotonia	100 (liver)	0 m/2 m	Lentiform nuclei	Blood: 4.27 (N<2.8)	NA
	3	Fe	1 m	Hypotonia, growth retardation, liver enlargement and slight muscle atrophy	100 (muscle)	7 m/8 m	NA	Blood: 2.8	NA
	4	Ma	4 m	Trunk hypotonia, pyramidal syndrome, psychomotor retardation, myoclonic epilepsy	NA	7 m/11 m	Lentiform nuclei, thalamus and red nuclei	Blood: 4.6 CSF: 2.1	NA
	5	Ma	5 m	Trunk hypotonia, peripheral hypertonia, plagiocephalia, severe psychomotor delay, strabismus, epilepsy, dystonia, pyramidal syndrome	100 (muscle)	5 y/No	Basal ganglia	Blood: N CSF: 2.35	NA
Korea (12)	6	Fe	5 m	Seizures and developmental delay	100 (leukocytes)	5 y/No	NA	NA	NA
	7	Ma	NA	Severe progressive encephalopathy, seizures	NA	NA/2 y	NA	Blood: N	NA
	8	Ma	5 m	Motor milestones, hypotonia, seizures	100 (muscle)	6 m/11 m	Basal ganglia, thalamus and brain stems	Blood: N	N
	1	Fe	7 y	Right-hand weakness, emotional lability, progressive gait ataxia, dystonia, poor coordination, dysarthria, cerebellar ataxia	98 (muscle)	NA/No	Globus pallidus	Blood: 1.5 (N<2.5)	Moderate SPM
China (present study)	2	Ma	4 y	Gait abnormality, dysarthria, impaired fine motor coordination, ataxic, dystonia	86 (muscle)	NA/No	Globus pallidus	Blood: 2.5	Mild SPM
	Proband	Fe	16 y	Slight facial paralysis, dystonia	97 (muscle) 95 (leukocytes)	1 y/No	Basal ganglia, thalamus, midbrain, pons and medulla oblongata	Blood: 4 (N<2.7)	N

Ma, male; Fe, female, m, months; y, years; NA, not available; CSF, cerebrospinal fluid; N, normal; SPM, subsarcolemmal proliferation of mitochondria.

eukaryotes such as *Homo sapiens*, *Bos Taurus*, *Equus caballus*, *Mus musculus*, and *Gallus gallus* (Fig. 4). The conservation index of the mutation m.10197 G>A based on 39 species (conservation in 36 species except *Xenopus laevis*, *Drosophila melanogaster* and *Sciurus vulgaris*) is 92.3% (11). This value reached to the average level (93±13%) of conservation index of 22 well-characterized human pathogenic mtDNA mutation studied in the same 39 species (12). The mutation identified in the proband is a substitution from an alanine to a threonine. These two residues are quite different in structure and chemical property. Alanine is a hydrophobic amino acid without any side-chain. By contrast, threonine is a hydrophilic amino acid with a hydroxyl side-chain. These differences in amino acid properties may underlie the pathogenic role of the m.10197 G>A mutation in causing complex I deficiency and a severe LS phenotype.

It is notable that the individuals carrying the mutation in our study did not show the typical clinical manifestations, as previously reported (9,14). Certain common hypotheses postulated to explain the phenotypic variability of mtDNA diseases can be excluded based on the results of the Chinese pedigree reported in our study. First, the proband and her brother both presented a rather high mutant load, which has been commonly considered the most important factor affecting the severity of the disease (14). Thus, it is unlikely that the phenotypic variability of the patient may be due to mutant load. Second, the mutation was invariably present in both the blood and muscle tissues of the proband. Thus, it is unlikely that the mild clinical manifestation of the proband might be due to a different tissue distribution of the mutation. Third, the severe pathogenicity associated with certain mutations causing LS is expected to affect the early development of the affected individual. In Leshinsky-Silver's study, brain lesions could be generated even at the fetal stage, before clinical manifestations of the syndrome become apparent (14). However, the normal brain image of the proband's brother suggests that this mutation may not generate visible anatomical changes at the early stage.

Additional factors potentially contributing to the variability of phenotypes of mtDNA diseases include ethnicity, haplogroup and the environment. For instance, in the Leber hereditary optic neuropathy also caused by a mutation, the risk of visual loss is significantly increased in the haplogroups J2 in Europeans and M7b1'2 in Han Chinese, in contrast to the respective H and M8a haplogroups (16,17). As Table I shows, the severity of the disease was different between European patients and Asian ones, although they carried the same mutation m.10197G>A. European patients had an earlier onset than Asian ones (3.3±1.9 month v.s. 9±6.2 year) and the former progressed so rapidly that 75% of them succumbed to the disease within 1 year. The haplogroup of previously reported LS patients presented in Table I is regrettably not available. The Chinese pedigree presented herein was characterized as haplogroup N9a, which is not a common haplogroup in European populations. Individuals of the N9a haplogroup were reported to be resistant or protected against certain metabolic diseases such as type 2 diabetes (18,19) and metabolic syndrome (20). Thus, it is likely that N9a has an additive effect to the insusceptibility to LS. It is notable that another Han Chinese pedigree with the

mutation m.10197 G>A, but in a different genetic background (haplogroup D4b) presented a different phenotype, of Leber hereditary optic neuropathy and dystonia (LDYT) (21).

The prognosis of LS is generally poor, and no effective treatment is available for this syndrome. The coenzyme Q<sub>10</sub> has a crucial role as the electron acceptor in complexes I and II of the mitochondrial electron transport chain. Coenzyme Q<sub>10</sub> affects the apoptosis of mitochondria through a number of mechanisms, including interference with mitochondrial depolarization (22), protection against reactive oxygen species (23), intervention in the production of ceramide (24), and mitochondrial protein uncoupling (25). Q10 is the most common drug used for treatment of mitochondrial diseases. In the present study, the condition of the patient was not serious. However, the typical brain lesions, particularly lower brainstem lesions, indicated that the LS patient would suffer from severe brainstem dysfunction in the near future (26). Low-dose coenzyme Q<sub>10</sub> (2 mg/kg/day) treatment was applied on the proband as an experimental treatment, although patients with LS have rarely shown an improvement following administration of Q<sub>10</sub> (27). Surprisingly, the patient showed a clear improvement in both clinical manifestations and the brain image, while its plasma lactate level also decreased down to a normal level. This is thus the first report on successful treatment of an LS patient with mutation m.10197 G>A. It is likely that the coenzyme Q<sub>10</sub> may attenuate the mitochondrial dysfunctions caused by the m.10197 G>A mutation.

In conclusion, the present study reported the first Chinese LS pedigree with mutation m.10197 G>A, and discussed a number of factors potentially affecting the pathogenic potential of the mutation m.10197 G>A and contributing to the phenotypic variability of LS, such as molecular defects, ethnicity, haplogroup and environment. Our study further indicated that treatment with the coenzyme Q<sub>10</sub> might be useful for LS patients with the mutation m.10197 G>A.

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