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The American Journal of Human Genetics, Volume *95*
Supplemental Data

Mutations in *GTPBP3* Cause a Mitochondrial Translation Defect Associated with Hypertrophic Cardiomyopathy, Lactic Acidosis, and Encephalopathy

Robert Kopajtich, Thomas J. Nicholls, Joanna Rorbach, Metodi D. Metodiev, Peter Freisinger, Hanna Mandel, Arnaud Vanlander, Daniele Ghezzi, Rosalba Carrozzo, Robert W. Taylor, Klaus Marquard, Kei Murayama, Thomas Wieland, Thomas Schwarzmayr, Johannes A. Mayr, Sarah F. Pearce, Christopher A. Powell, Ann Saada, Akira Ohtake, Federica Invernizzi, Eleonora Lamantea, Ewen W. Sommerville, Angela Pyle, Patrick F. Chinnery, Ellen Crushell, Yasushi Okazaki, Masakazu Kohda, Yoshihito Kishita, Yoshimi Tokuzawa, Zahra Assouline, Marlène Rio, François Feillet, Bénédict Mousson de Camaret, Dominique Chretien, Arnold Munnich, Björn Menten, Tom Sante, JoélSmet, Luc Régal, Abraham Lorber, Asaad Khoury, Massimo Zeviani, Tim M. Strom, Thomas Meitinger, Enrico S. Bertini, Rudy Van Coster, Thomas Klopstock, Agnès Rötig, Tobias B. Haack, Michal Minczuk, and Holger Prokisch

Figure S1

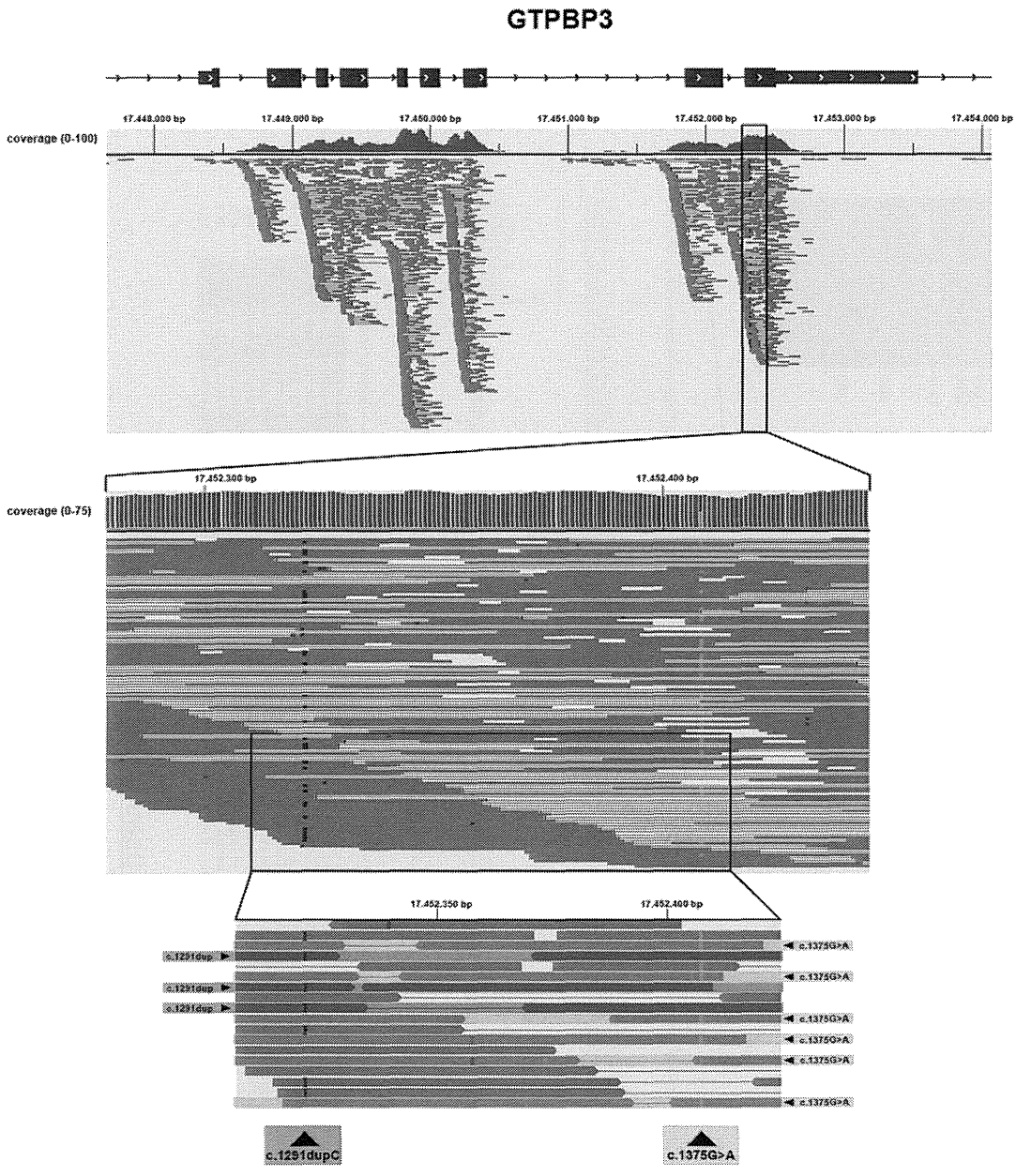

Figure S1) Segregation analysis in family F1 in WES data

The two mutations identified in family F1 (c.1291dupC and c.1375G>A) are only separated by 97 bp which allowed analysis of both alleles despite the lack of parental material. 13 paired sequence reads were identified which covered the region of both variants. All reads contained either of the two mutations demonstrating a compound heterozygous status of the two variants. Figure S1 shows three sequence reads containing the c.1291dupC variant and five reads containing the c.1375G>A variant.

Figure S2

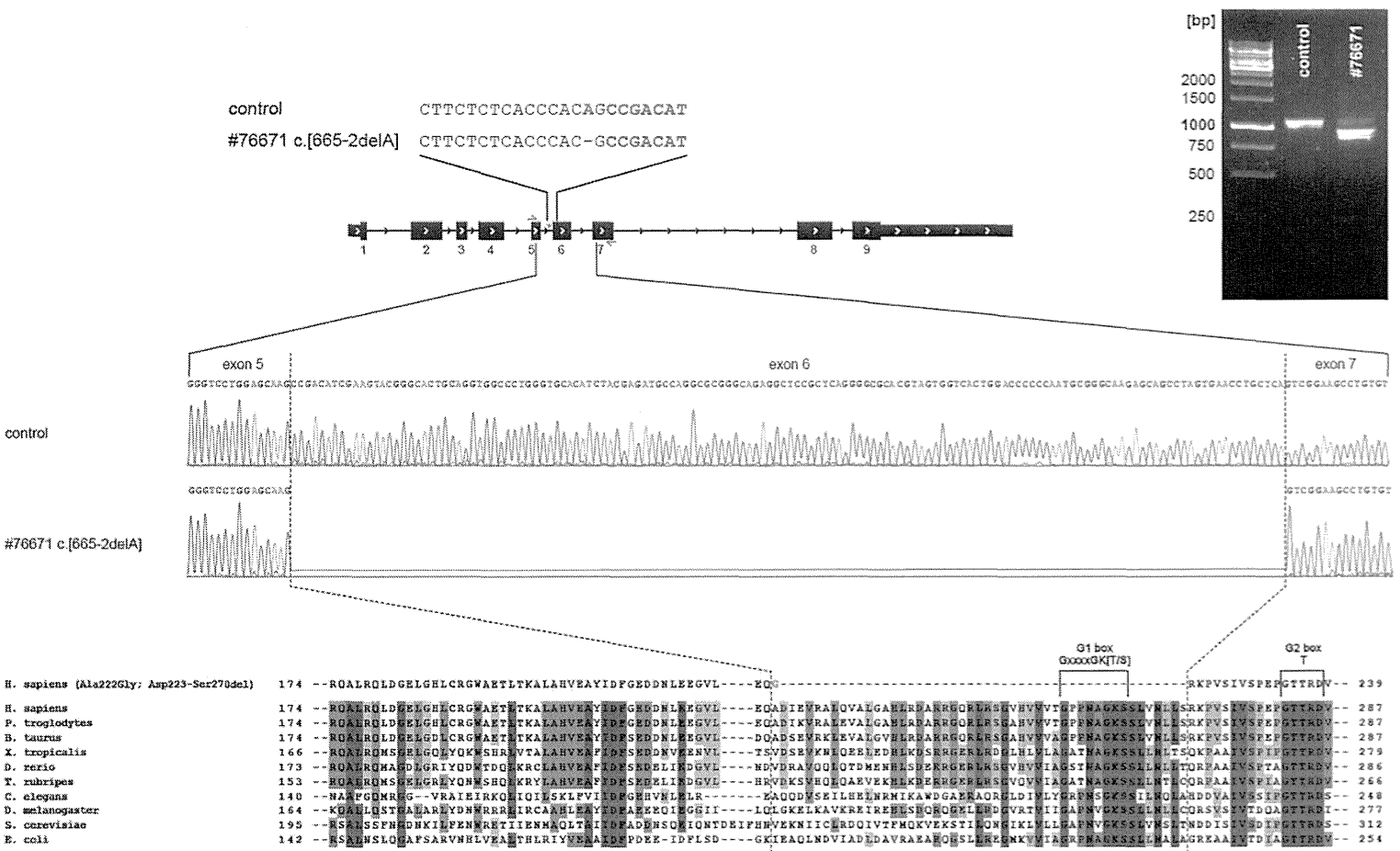

Figure S2) Splice site mutation in individual #76671 causes skipping of exon 6

Analysis of cDNA derived from fibroblasts of individual #76671 yielded a smaller than expected PCR product, indicating alternative splicing. Sanger sequencing revealed that the c.665-2delA mutation affects the conserved splice acceptor site. The splice acceptor upstream of exon 7 is alternatively used, yielding a mature mRNA that lacks exon 6. The resulting protein product is predicted to contain a 1 amino acid exchange followed by a 48 amino acid deletion (p.Ala322Gly; Asp223-270del).

Figure S3

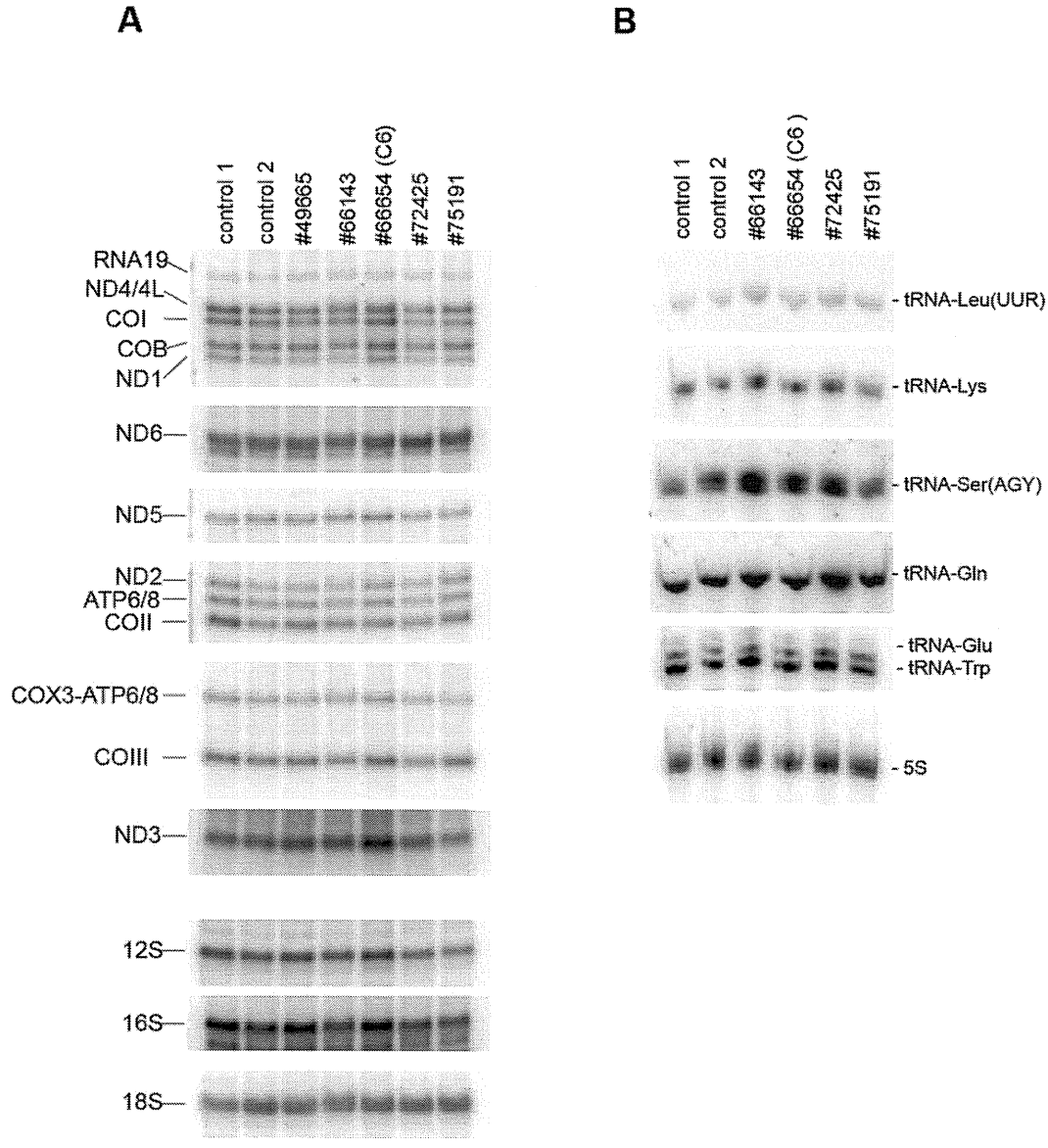

Figure S3) Northern blot analysis of the steady-state levels of mitochondrial transcripts in GTPBP3 patient fibroblasts.

A) Northern blot analysis of total RNA isolated from the *GTPBP3* patient or control primary fibroblasts. The blots were probed with the mt-mRNA- and mt-rRNA-specific probes as indicated. The cytosolic 18S rRNA was used as a loading control.

B)) High-resolution Northern blot analysis of total RNA isolated from the *GTPBP3* patient or control primary fibroblasts. The blots were probed with the mt-tRNA -specific probes as indicated. The cytosolic 5S rRNA was used as a loading control.

Figure S4

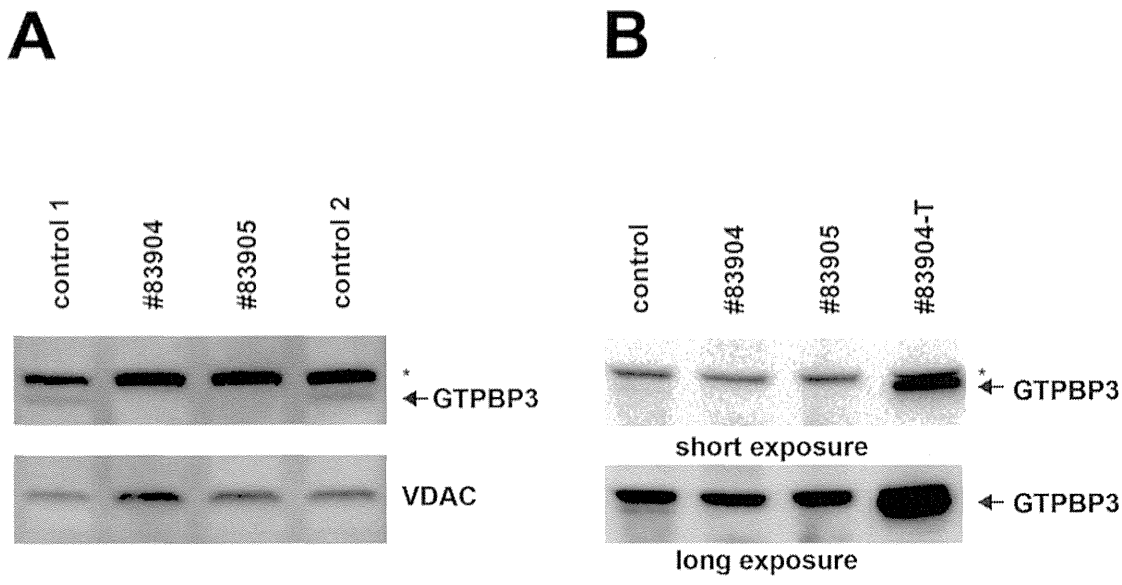

Figure S4) Analysis of GTPBP3 protein levels in patient fibroblasts

A) Immunoblot analysis of GTPBP3 protein levels in fibroblasts from affected individuals #83904 and #83905 from family F9. VDAC served as a mitochondrial loading control. (Asterics indicates a non-specific band.)

B) Comparison of the electrophoretic migration of GTPBP3 in un-transfected cells (lane control) and cells derived from one of the affected individuals transfected with a plasmid (pIRES2-EGFP) for *GTPBP3* cDNA expression (lane #83904-T). (Asterics indicates a non-specific band.)

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ORIGINAL ARTICLE

A Japanese case of cerebellar ataxia, spastic paraparesis and deep sensory impairment associated with a novel homozygous *TTC19* mutation

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Mitochondrial complex III (CIII) deficiency comprises a group of complex and heterogeneous genetic disorders. *TTC19* mutations constitute a rare cause of CIII deficiency and are associated with neurological disorders in childhood and adulthood. Herein, we describe a 27-year-old Japanese man with cerebellar ataxia, spastic paraparesis, loss of deep sensation, mild frontal lobe dysfunction and transient psychiatric symptoms. Brain magnetic resonance imaging showed cerebellar atrophy and bilateral high-intensity signals in the inferior olives and regions adjacent to periaqueductal gray matter, on T2-weighted images. On whole-exome sequencing, we detected a novel homozygous frameshift mutation c.157_158dup [p.Pro54Alafs*48] in *TTC19*. Mitochondrial enzyme assays confirmed mild impairment of CIII enzymatic activity in lymphoblasts, which was consistent with *TTC19*-related CIII deficiency. His symptoms and radiological findings demonstrated an early stage or mild form of this disease, and further clarify the characteristics of patients with rare *TTC19* mutations.
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INTRODUCTION

Mitochondrial complex III (CIII) deficiency is a rare disease that belongs to a heterogeneous group of neuromuscular and multisystemic disorders. CIII is a multiprotein enzyme complex located in the mitochondrial inner membrane, which catalyzes the transfer of electrons from reduced coenzyme Q to cytochrome *c* with a concomitant pump of protons to the inner membrane.[1] CIII is composed of 10 nuclear-encoded subunits and 1 mitochondrial-encoded subunit.[2] Mutations in either nuclear or mitochondrial genes can be the cause of CIII deficiency. Among these genes, mutations in nuclear genes, such as *UQCRB* [MIM 191330],[3] *UQCRQ* [612080],[4] *UQCRC2* [191329],[5] *CYC1* [123980],[6] *BCS1L* [603647],[7] *TTC19* [613814],[8] *UQCC2* [614461],[9] *UQCC3* [no MIM number][10] and *LYRM7* [615831],[11] and in the mitochondrial gene *MT-CYB* [MIM 516020][12] are currently known to cause CIII deficiencies. *UQCRB*, *UQCRQ*, *UQCRC2* and *CYC1* encode components of CIII itself, whereas *BCS1L*, *TTC19*, *UQCC2*, *UQCC3* and *LYRM7* produce mitochondrial assembly factors. Recently, homozygous or compound heterozygous mutations of *TTC19*, which encodes tetratricopeptide repeat domain 19, have been found to be the cause of a neurological disorder presenting with various degrees of progressive encephalopathy and ataxia.[8,13–15] Herein, we report a 27-year-old Japanese man presenting with progressive cerebellar ataxia, spastic paraparesis, loss of deep sensation, mild frontal lobe dysfunction and transient psychiatric symptoms, along with a previously undescribed homozygous frameshift mutation in *TTC19*.

SUBJECTS AND METHODS

Clinical information and a blood sample were taken from the man after obtaining written informed consent. The experimental protocols were approved by the Institutional Review Board of Yokohama City University School of Medicine.

To identify a causative mutation, whole-exome sequencing was performed on the patient's DNA. Three micrograms of genomic DNA were processed using the SureSelect Human All Exon Kit (51 Mb; Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The captured DNA was sequenced using a HiSeq2000 sequencer (Illumina, San Diego, CA, USA).

Mitochondrial fractions were prepared from lymphoblasts derived from the patient and control subjects. Activities of respiratory chain complexes CI, CII, CIII and CIV were assayed as described previously.[16–18] Enzyme activities are expressed as % of mean normal control activity relative to the levels of protein or to the activities of citrate synthase (CS) or CII.

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RESULTS

Case report

The present case is a 27-year-old man, who is the second of two children from non-consanguineous healthy parents originating from the same area. Other than showing a slight delay in the acquisition of gait, his psychomotor development was normal with no clinically relevant complaints, until the age of 17 years. It was then that he became aware of a slight speech difficulty. At 23 years of age, he started to experience difficulty in walking. One year later, because his gait disturbance had progressed and he was falling frequently, he visited a local doctor. Brain magnetic resonance imaging (MRI) indicated subtle cerebellar atrophy. Around that time he became unable to walk unaided and he also gradually developed psychiatric symptoms such as depression and irritability. At the age 25, his parents had him admitted to a psychiatric hospital because of his violent behavior. Following short-term treatment with carbamazepine and nitrazepam, his psychiatric symptoms were well controlled and he was transferred to our hospital. After withdrawal of the medications, neurological examination revealed a disturbance of smooth pursuit eye movements, slight dysarthria, mild limb ataxias, pronounced truncal ataxia, spasticity of the lower extremities and increased deep tendon reflexes. He had a moderate loss of vibratory sense and a severe loss of position sense in the lower extremities, with *pes cavus* and hammer toe deformities. He exhibited attentional impairment and psychomotor deteriorations. His Mini-Mental State Examination (MMSE) score was 28/30, and his Frontal Assessment Battery (FAB)[19] score was 13/18, indicating mild frontal lobe dysfunction. Laboratory biochemistry results were normal, including levels of vitamin B_1, vitamin B_{12}, vitamin E and very long chain fatty acids, as was a cerebrospinal fluid study. His lactate and pyruvate levels in blood were 8.7 mg dl^{-1} (normal range: 4–16 mg dl^{-1}) and 0.71 mg dl^{-1} (normal range: 0.3–0.9 mg dl^{-1}), respectively. Serum antibody for Human T lymphotropic virus type 1 was negative. Activity of galactocerebrosidase, glucosidase and β-hexosaminidase in leukocytes was normal. Blood amino-acid analysis revealed no apparent abnormality. Neoplastic, autoimmune, thyroidal and rheumatic diseases were excluded by the appropriate tests. Brain MRI showed mild cerebellar atrophy (Figure 1a), and symmetrical high-intensity signals in the inferior olives and lesions adjacent to periaqueductal gray matter, on T2-weighted images (Figures 1b and c). Supratentorial lesions, such as white matter changes, basal ganglia lesions, cortical brain atrophy or structural brain anomalies were not detected by MRI. Magnetic resonance spectroscopy (MRS) could not detect a clear lactate doublet in the basal ganglia (Figure 1d). MRIs of the whole spine showed no abnormality. ^{99m}Tc-ethyl cysteinate dimer single-photon emission computed tomography demonstrated reduced perfusion in the cerebellum and bilateral occipital cortices (Figure 1e). Nerve conduction studies indicated a motor axonal neuropathy of the lower extremities, but no apparent sensory neuropathy. The patient was negative for genetic alterations associated with Friedreich ataxia, spinocerebellar ataxia (SCA) 1, SCA2, SCA3 (Machado–Joseph disease), SCA6, SCA7, SCA12, SCA17 and dentatorubro-pallidoluysian atrophy. We diagnosed him as having a rare form of SCA or a complicated form of spastic paraparesis. At 27 years of age, his MMSE score was 30/30, indicating that his cognitive functions remained stable without medications. At this point, his verbal intelligence quotient (IQ) was 85, performance IQ was 71 and total IQ was 76 on the Wechsler Adult Intelligent Scale-Third Edition. Other neurological findings also showed no apparent progressions.

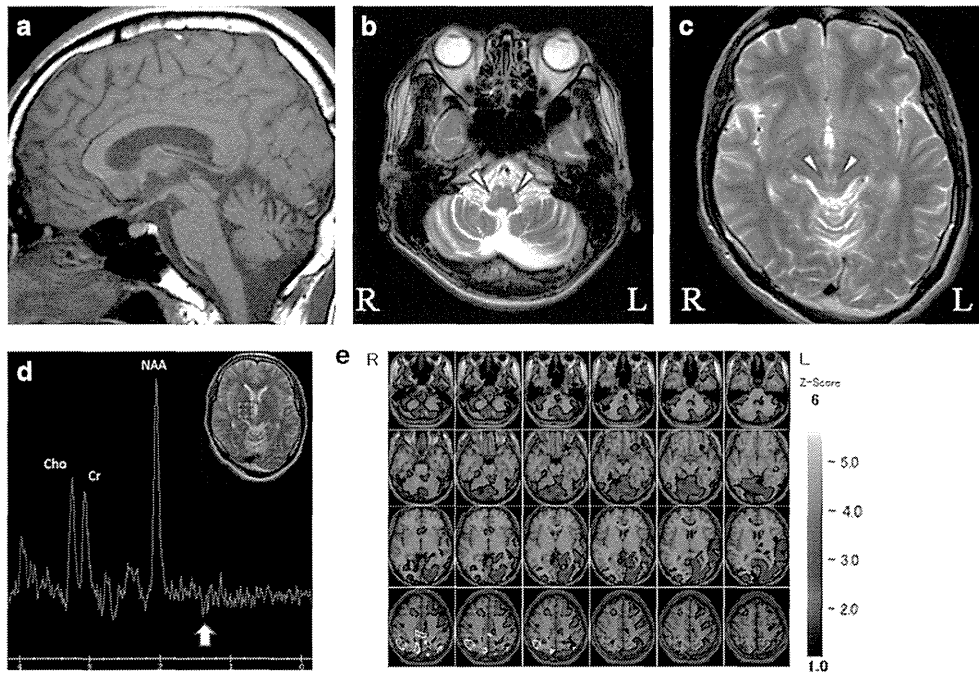

Figure 1 (**a**–**c**) Brain MRI of the patient at 25 years of age. A midsagittal section of brain on a T1-weighted image (**a**), and axial sections of medulla oblongata (**b**) and midbrain (**c**) on a T2-weighted image are shown. Arrowheads indicate hyper-intense signal in the inferior olives (**b**) and in the midbrain (**c**). (**d**) Proton MRS obtained from the thalamic area, at a magnetic field of 3 Tesla (echo time 144 ms). A clear lactate peak, which should be present at 1.3 p.p.m. (arrow), could not be detected. Cho, choline; Cr, creatine; NAA, *N*-acetyl aspartate. (**e**) ^{99m}Tc-ECD SPECT images at 25 years of age. The *Z*-score maps displayed on an anatomically standardized MRI template are shown.[27]

His elder sister has not recognized any difficulties, and has not undergone any radiological and electrophysiological examinations. However, on neurological examination, she showed slight truncal ataxia and a slight impairment of position sense in the lower extremities, with *pes cavus* deformity (Table 1).

Exome sequencing

Among the detected variants, 2873 were located in exons or splice sites (within 2 bp of the boundary), and were unregistered or registered as uncommon single-nucleotide polymorphisms with minor allele frequency <1% in dbSNP135. We examined whether these mutations were present in known SCA or hereditary spastic paraplegia-related genes.[20] A novel homozygous frameshift mutation, c.157_158dup [p.Pro54Alafs*48] (NM_017775.3), was identified in *TTC19* (17p12, [MIM 613814]). Sanger sequencing with an ABI 3500xL (Life Technologies, Carlsbad, CA, USA) confirmed c.157_158dup homozygosity in the patient and his sister, and heterozygosity in the parents (Figure 2a). No mutations were detected by Sanger sequencing in the

Table 1 Clinical and laboratory findings in patients with *TTC19* mutations

Reference	*Ghezzi et al.*[8]					*Nogueira et al.*[13]			*Atwal*[15]	*Morino et al.*[14]	*The present report*	
Ethnic origin	*Italian*					*Portuguese*			*Hispanic*	*Japanese*	*Japanese*	
Sex	F	M	F	M	M	M	M	F	M	F	M	F
Age at onset	5	10	5	43	27	12	15	34	1	31	25	29
Nucleotide changes		c.656T>G		c.517C>T		c.963_966del			c.964_967del/ c.577G>A	c.829C>T	c.157_158dup	
Amino-acid changes		p.Leu219*		p.Gln173*		p.Ala321Alafs*8			p.Trp186*/ p.Gly322-Metfs*8	p.Glu277*	p.Pro54Alafs*48	
Symptoms at onset	Learning disability, gait ataxia	Learning disability, gait ataxia	Regression of language, gait ataxia	Weakness of the four limbs	Mood disorder, gait ataxia	Compulsive lying	Aggressive behavior	Avoidance behavior	Developmental delay, regression of language	Dysarthria	Mood disorder, gait ataxia	No subjective symptoms
Clinical symptoms												
Cognitive impairments	NA	+	NA	NA	+	+	+	+	NA	+	+	NA
Psychiatric disturbances	NA	NA	NA	NA	+	+	+	+	NA	NA	+	–
Gait ataxia	+	+	+	+	+	+	+	+	NA	+	+	+
Spasticity	NA	NA	NA	+	+	+	+	NA	NA	NA	+	–
Enhanced tendon reflexes	+	NA	NA	NA	+	+	+	+	NA	+	+	–
Deep sensory impairment	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	+
Peripheral neuropathy	NA	NA	+	+	+	NA	+	NA	NA	NA	+	NA
Pes cavus	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	+	+
MRI												
Cerebellar atrophy	+	NA	+	NA	+	+	+	+	NA	+	+	NA
Supratentorial lesions	+	NA	NA	+	+	+	+	NA	+	NA	–	NA
Hyperintense inferior olives	+	NA	+	NA	+	+	+	+	NA	+	+	NA
Hyperintense mid brain	NA	NA	+	NA	NA	+	NA	NA	NA	NA	+	NA
MRS												
Lactate peak	+	NA	NA	+	NA	NA	NA	NA	NA	NA	unclear	NA
Hypoperfusion in SPECT	NA	NA	NA	Frontoparietal lobe	NA	NA	NA	NA	NA	NA	Cerebellum and occipital lobe	NA

Abbreviations: F, female; M, male; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NA, no data available; SPECT, single-photon emission computed tomography.

other exons of *TTC19*. The mutation was undetected in 408 'in-house' Japanese control exomes.

Mitochondrial enzyme activity

As shown in Table 2, his residual CIII activity relative to CS activity was 74.6% in cultured lymphoblasts. CIII activity relative to CII activity was reduced to 48.5%, indicating impairment of his CIII activity. A previous report showed that residual CIII activity relative to CS activity was 46–89% in cultured cells from patients with a homozygous *TTC19* mutation, whereas in muscles from the same patients, CIII activity relative to CS activity was 14–19%.[8] A milder defect of CIII enzymatic activity was also observed in cultured cells (for example, lymphoblasts or fibroblasts) compared with that in solid tissues (for example, muscle or liver) in other mitochondrial disorders, such as CIII deficiency with *BCS1L* mutations.[2,7] Thus, the mild decrease of CIII activity in lymphoblasts of the patient was consistent with *TTC19*-related CIII deficiency.

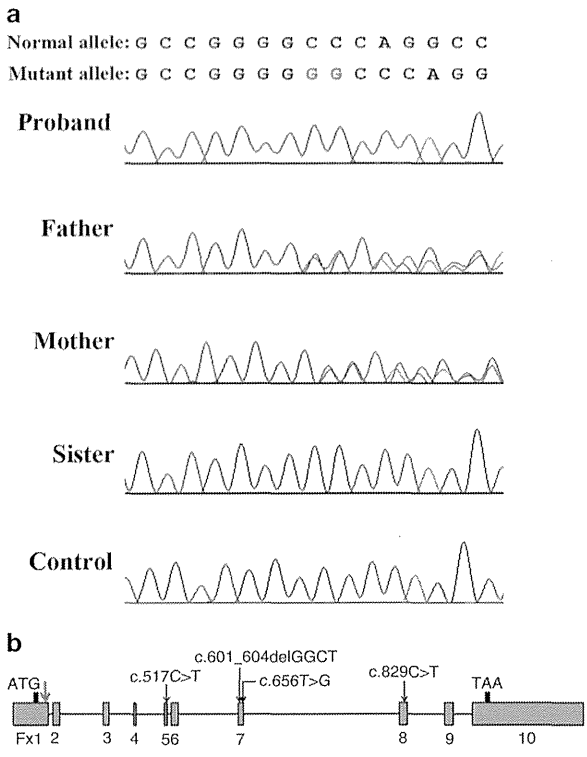

Figure 2 (**a**) Electropherograms of the patient (proband), his parents, his sister and normal control showing the mutation. Red letters indicate the inserted sequence. The patient and his sister are homozygous for the mutation, whereas the parents are heterozygous for the mutation. (**b**) Schematic presentation of *TTC19* mutations. The red arrow indicates the location of the mutation in the patient.

Table 2 Activities of respiratory chain complexes in lymphoblasts

	Co I	*Co II*	*Co II+III*	*Co III*	*Co IV*	*CS*
% of normal	84.6	145.2	153.7	70.4	102.7	94.4
CS ratio (%)	89.5	153.8	162.8	74.6	108.8	
Co II ratio (%)	58.2		105.8	48.5	70.7	

Abbreviations: Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthase.
Enzyme activities are expressed as % of mean normal control activity relative to protein levels (% of normal), CS activities (CS ratio) and Co II activities (Co II ratio).

DISCUSSION

In this report, we describe in detail the clinical symptoms of a patient with a novel homozygous frameshift mutation, c.157_158dup [p.Pro54Alafs*48], in *TTC19*. As three unrelated Italian kindreds with homozygous *TTC19* mutations were first reported in 2011,[8] 10 patients from six families with *TTC19* mutations have been identified.[13–15] The age of disease onset is variable, ranging from 13 months to 42-year old. Progressive cerebellar ataxia and cognitive impairment (mental retardation and/or intellectual deterioration) are the common symptoms, whereas the severity, progression and prognosis of the disease vary even between patients from the same family.[8,13] When the progression of symptoms was slow, patients were diagnosed as possibly having SCA, spastic paraplegia or psychiatric disorders.[13,14] When the progression was relatively rapid, patients were diagnosed as having metabolic disorders, even Leigh syndrome.[15] Our case showed essentially similar clinical manifestations to cases diagnosed as having SCA. In previous reports, all patients with *TTC19* mutations showed cognitive impairment to a greater or lesser extent. Our patient had transient psychiatric symptoms, but cognitive decline was relatively mild compared with the previous cases. Brain MRI of the patient showed mild cerebellar atrophy and bilateral high-intensity signals in the inferior olives, and lesions adjacent to periaqueductal gray matter on T2-weighted images, but no apparent supratentorial lesions. In previous reports, the patients with *TTC19* mutations showed cerebellar atrophy with abnormal high-intensity signals in the inferior olives, caudate, putamen, cerebellar dentate nucleus and/or medial midbrain, on T2-weighted imaging.[8,13–15] Supratentorial brain lesions appeared to be detected in the cases with severe cognitive impairment. Among these abnormalities, cerebellar atrophy and bilateral T2 high-intensity lesions in the inferior olives were detected in all of the reported cases, including in our case. Because an abnormal lactate peak was detected in two out of two cases with *TTC19* mutations, whose ^{1}H-MRS information was available,[13] we performed ^{1}H-MRS in our patient but no lactate peak was apparent. ^{1}H-MRS detection of lactate in the brain parenchyma is regarded as a more precise indicator of cerebral lactic acidosis than blood or cerebrospinal fluid lactate levels.[21,22] The result may indicate that cerebral lactic acidosis in the present case was relatively milder than that in the previous cases. Although not previously described in patients with *TTC19* mutations, the current case showed severe loss of deep sensation in the lower extremities; however, a spondylotic myelopathy or sensory neuropathy was not detected. Disturbance of deep sensation is possibly a symptom of this disease, but might not have been fully recognized because of severe cognitive impairment in the previous cases. Our patient, as in previous cases, had pronounced truncal ataxia and gait disturbance despite relatively mild cerebellar atrophy on MRI, which may be explained by severe loss of deep sensation in the lower extremities. Alternatively, lesions in the inferior olives, which are known to produce truncal ataxia,[23] might have contributed to the exacerbation of his gait disturbance. Considering the clinical symptoms and radiological findings, we consider that our patient may be in the early stage of this disease or have a mild form. It will be necessary to monitor his symptoms and examination findings over time.

CIII deficiencies are rare, but they cause a broad spectrum of symptoms and display tissue-specific lesion formation in humans.[24] Although the functions of TTC19 are still poorly characterized, TTC19 is thought to be an important factor in early CIII assembly. Mutations of *BCS1L*, another factor involved in CIII assembly, are known to cause a wide range of phenotypes, from highly restricted pili torti and sensorineural hearing loss (Björnstad syndrome) to profound

multisystem organ failure (GRACILE syndrome and CIII deficiency).[25] Although a molecular basis for the phenotypic differences was identified in these cases,[25] disease severity varied even within the family members harboring the same mutation,[26] suggesting that alternative unknown determinants of disease severity may exist. Because all of the reported *TTC19* mutations were nonsense or frameshift mutations, loss of function of *TTC19*, which leads to impairment of CIII assembly, is thought to be the cause of the disease. Given that the p.Pro54Alafs*48 mutation in our case was the most N-terminally located of all the reported mutations (Figure 2b), and that the clinical manifestations are mild in comparison with previous cases,[8,13–15] genotype–phenotype correlations cannot be explained simply by the retaining of partial *TTC19* function. Moreover, in the cell lines, we could not detect a clear difference in CIII activity between the present case and severe cases.[8] We speculate that phenotypic determinants, other than *TTC19* genotype (for example, environmental factors or genotypes of other genes), exist in patients with *TTC19* mutations. We assessed exome data of the patients for single-nucleotide variants (SNVs) in nuclear-encoded CIII component genes (*CYC1*, *UQCRC1*, *UQCRC2*, *UQCRFS1*, *UQCRB*, *UQCRQ*, *UQCRH*, *UQCR10* and *UQCR11*) and assembly factor genes (*UQCC1*, *UQCC2*, *UQCC3*, *BCS1L* and *LYRM7*), which may modify the disease phenotype, but we found no functional SNVs in these genes.

In conclusion, our case carrying a novel homozygous frameshift mutation in *TTC19* indicated that severe loss of deep sensation might be an unrecognized symptom of this disease. More cases are needed to further understand the crucial factor(s) determining the disease phenotype.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Case Report

Myocerebrohepatopathy spectrum disorder due to *POLG* mutations: A clinicopathological report

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Abstract

We report on the clinical, neuropathological, and genetic findings of a Japanese case with myocerebrohepatopathy spectrum (MCHS) disorder due to polymerase gamma (*POLG*) mutations. A girl manifested poor sucking and failure to thrive since 4 months of age and had frequent vomiting and developmental regression at 5 months of age. She showed significant hypotonia and hepatomegaly. Laboratory tests showed hepatocellular dysfunction and elevated protein and lactate levels in the cerebrospinal fluid. Her liver function and neurologic condition exacerbated, and she died at 8 months of age. At autopsy, fatty degeneration and fibrosis were observed in the liver. Neuropathological examination revealed white matter-predominant spongy changes with Alzheimer type II glia and loss of myelin. Enzyme activities of the respiratory chain complex I, III, and IV relative to citrate synthase in the muscle were normal in the biopsied muscle tissue, but they were reduced in the liver to 0%, 10%, and 14% of normal values, respectively. In the liver, the copy number of mitochondrial DNA compared to nuclear DNA was reduced to 3.3% of normal values as evaluated by quantitative polymerase chain reaction. Genetic analysis revealed compound heterozygous mutations for *POLG* (I1185T/A957V). This case represents the differential involvement of multiple organs and phenotype-specific distribution of brain lesions in mitochondrial DNA depletion disorders.

Keywords: Alpers syndrome; Mitochondrial DNA depletion; Myocerebrohepatopathy spectrum disorder; *POLG*

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1. Introduction

Mitochondrial DNA (mtDNA) depletion syndrome (MDDS), first described in 1991, is defined as a reduction in the mtDNA copy number in different tissues, leading to insufficient synthesis of respiratory chain complexes (RCC) [1]. Clinical manifestations of MDDS involve many organ systems including the central and peripheral nervous system, liver, muscle, and gastrointestinal tract [2]. Human polymerase gamma (*POLG*) is the common causative gene involved in MDDS, whose mutations result in a diverse group of phenotypes, such as Alpers syndrome and myocerebrohepatopathy spectrum (MCHS) disorders, which typically show disease onset during early childhood. Further, several *POLG*-related phenotypes manifesting during adolescence and adulthood are recognized, including progressive external ophthalmoplegia, ataxia-neuropathy spectrum disorders, myoclonus epilepsy myopathy sensory ataxia, and sensory ataxic neuropathy with dysarthria/dysphagia and ophthalmoplegia. Some overlaps in the symptoms between these adult phenotypes exist, and can be additionally accompanied by tremor, parkinsonism, hearing loss, stroke-like episodes, and gastrointestinal symptoms, which are reminiscent of symptoms of mitochondrial diseases with pathomechanisms other than MDDS [3,4].

MCHS, the most severe phenotype of POLG disorders, was recently identified and is defined by the clinical triad of (1) myopathy or hypotonia, (2) developmental delay or dementia, and (3) liver dysfunction [3,5]. Severe, intractable epilepsy is included in the diagnostic hallmarks of Alpers syndrome, but is not characteristic of MCHS. As the number of patients with MCHS disorders is small and detailed clinicopathological findings are unavailable, we herein report the case of a girl with MCHS disorders due to *POLG* mutations. As far as we know, this is the first Japanese case of MCHS disorders with *POLG* mutation.

2. Case report

A girl was born at 40 weeks of gestation to healthy non-consanguineous parents without any abnormalities. The birth weight, height, and head circumference were normative. Early development and growth were unremarkable. At 4 months of age, she developed poor weight gain, emesis, hypotonia, developmental delay, and lethargy. She was admitted to our hospital because of recurrent vomiting at 6 months of age.

On admission, body length was 60.9 cm [−2.2 standard deviation (SD)], body weight was 5600 g (−2.3 SD), and head circumference was 42 cm (+0.2 SD). Hepatomegaly of a hard consistency was observed approximately 3 cm under the costal margin with no associated splenomegaly. She was alert and could establish good eye contact and smile. She showed severe hypotonia and proximal dominant muscular weakness. She could hold neither her head nor limbs up. All deep tendon reflexes were weak.

Although complete blood count and urinalysis were unremarkable, hepatocellular dysfunction was obvious at the time of hospitalization, with the following laboratory test values: aspartate aminotransferase, 390 U/L; alanine aminotransferase, 218 U/L; total bilirubin, 1.6 mg/dL; total bile acids, 172 μmol/L; γ-glutamyl transpeptidase, 179 IU/L; leucine aminopeptidase, 268 IU/L; and cholinesterase, 73 IU/L. Levels of serum creatine kinase and blood glucose were normal. Cerebrospinal fluid (CSF) examination showed elevated protein levels of 304 mg/dL and normal cell count and glucose levels. Lactic acid was elevated in both plasma and CSF, at 15.9 mg/dL and 30.3 mg/dL, respectively. Pyruvic acid was normal in both plasma and CSF. Metabolic screening tests, including urine organic acids, plasma, and urine amino acids, were unremarkable. Initial brain computed tomography (CT) and magnetic resonance imaging performed at 6 months of age were unremarkable. The electroencephalogram showed generalized slow wave activity. Only wave I was identifiable on auditory evoked potentials. Motor nerve and sensory conduction were mildly delayed.

Muscle biopsy findings at 6 months of age showed a variation in fiber type; ragged-red fiber was not observed. Lipid and glycogen storage were not observed. Cytochrome c oxidase staining showed normal findings. Analysis of the RCC enzyme activity revealed no abnormality. No mtDNA mutations were identified.

Soon after admission, difficulty in feeding and vomiting aggravated, and tube feeding along with parenteral nutrition was required. She experienced bouts of diarrhea. Consciousness level decreased progressively, and myoclonic jerks of the right and left arms were infrequently observed. Follow-up CT revealed mild cerebral atrophy at 7 months of age. Hepatocellular dysfunction exacerbated progressively, and she died of multiple organ failure caused by hepatic failure at 8 months of age, despite supplementation of multiple vitamins and coenzyme Q 10, and was autopsied. Two years later, another girl was born to the parents. She had the same clinical course and laboratory findings observed in the present patient and died at 7 months of age. Valproic acid was not used in either patient.

2.1. Postmortem examinations

Body weight was 6.0 kg (mean ± SD, 8.0 ± 0.88 kg). The weight of the atrophic liver was 200 g, and the surface was yellowish, irregular, and hard. The lungs were congested and adrenal glands were atrophic. The other visceral organs were unremarkable on macroscopic

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examination. The brain weighed 760 g and showed massive edema and caudal necrosis. Microscopically, hepatocytes and adrenal cortical cells were swollen, and renal tubular cells contained phospholipids and diffuse foam cells. Similar foam cells were also seen in the lungs and cardiac muscle fibers. In the liver, hepatic fibrosis, microvesicular steatosis, and fatty degeneration were observed (Fig. 1). In the central nervous system, a spongy change was noted predominantly in the cerebral white matter, and neuronal loss in the cerebral and cerebellar cortex was mild. Alzheimer type II glia was observed in massive numbers in the cerebral and cerebellar white matter, with a smaller amount in the cerebral cortex and deep gray matter. Neuronal loss, capillary proliferation, and sponginess were prominent in the substantia nigra (Fig. 2). Recent linear necrosis was present in the bilateral caudate nucleus.

2.2. Assay of respiratory chain complex enzyme activity in the liver

The liver samples were immediately frozen at autopsy and stored at −70 °C. Activities of RCC I, II, III and IV were assayed as described previously [6,7]. The percentages of RCC I, II, III and IV activities relative to that of citrate synthase (CS) as a mitochondrial enzyme marker were calculated. Relative enzyme activities of RCC I, III, and IV to CS in the liver were reduced to 0%, 10%, and 14% of normal values, respectively, while that of RCC II was reduced to 29%.

2.3. Analysis of quantitative polymerase chain reaction of mtDNA and DNA sequence of POLG gene

Written informed consent was obtained from the patient's parents in order to perform gene analysis. The quantitative estimation of mtDNA was performed by real-time amplification of fragments of *ND1* in the mtDNA genome, as previously described [7,8]. To determine the overall abundance of mtDNA, we compared the real-time amplification of *ND1* with a single-copy nuclear reference gene (exon 24 of the *CFTR* gene) [7,9]. The ratio of *ND1* to *CFTR* in the liver was reduced to 3.3% (SD, 1.2%) as compared to the control.

Mutation analysis was performed on the genomic DNA using primers designed to amplify the coding exons and the exon-intron boundaries of *POLG* (NM_002693.2). Fragments were analyzed by direct sequencing using ABI 3130XL (Applied Biosystems, Tokyo, Japan). The genetic analysis revealed compound heterozygous mutations in *POLG* (c.2870C>T, p.A957V and c.3554T>C, p.I1185T). The two DNA mutations

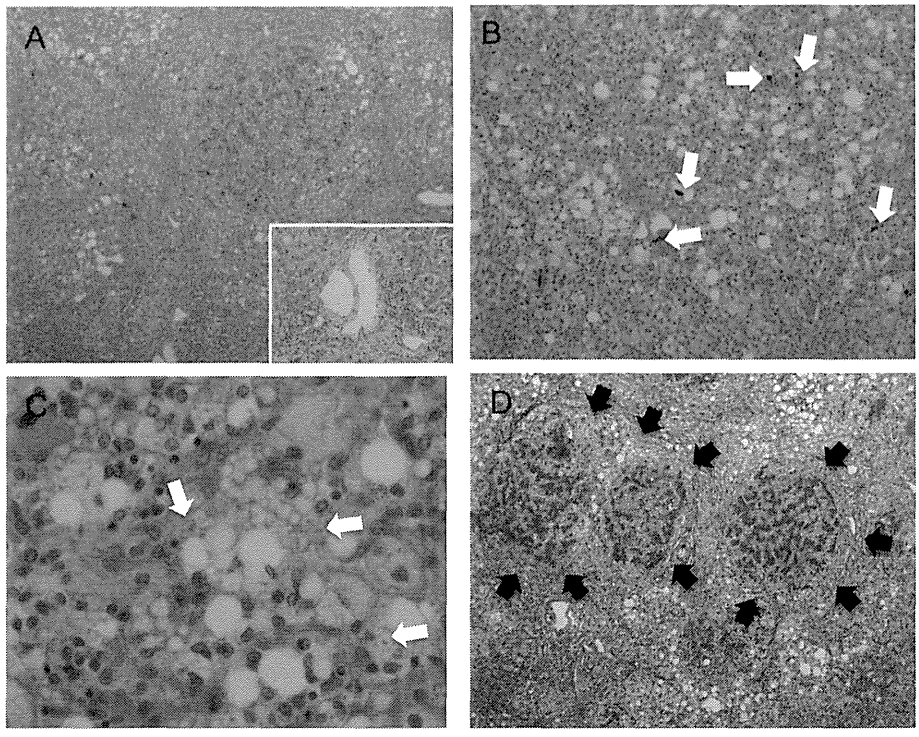

Fig. 1. Pathological findings of the postmortem liver (A–C: hematoxylin & eosin staining, D: Masson trichrome staining). (A) Moderate inflammatory cell infiltration (inset) with destroyed limiting plates and a rather progressive fibrosis with bridging formation in the portal tracts were observed (original magnification, ×40). (B) Swollen hepatocytes containing lipid droplets of various sizes were found. Bile plugs (white arrows in B and C) were noted in the cytoplasm of hepatocytes and dilated canaliculi (×100). (C) Swollen hepatocytes containing lipid droplets of various sizes were found. Bile plugs were noted in the cytoplasm of hepatocytes (×400). (D) A rather progressive fibrosis with bridging formation (arrows) in the portal tracts was found (×40).

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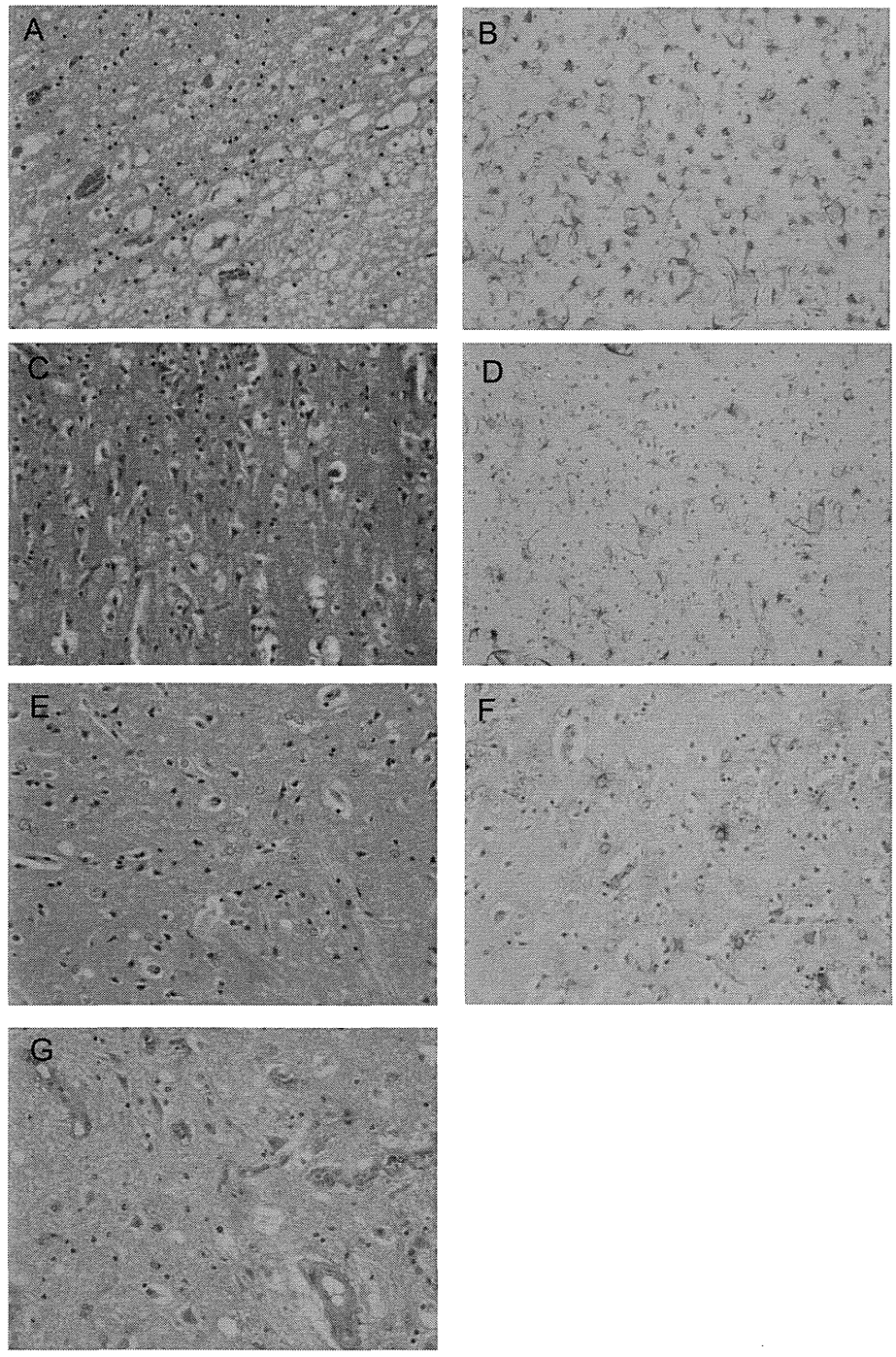

Fig. 2. Pathological findings of the postmortem brain (A, C, E, and G: hematoxylin & eosin staining; B, D, and F: immunohistochemical staining against glial fibrillary acidic protein; original magnification, ×400). Marked spongy changes (A) with Alzheimer type II astrocytosis (B) was observed in the cerebral white matter, and less prominently in the cerebral cortex (C and D) and striatum (E and F). Neuronal loss, sponginess, and capillary proliferation, which were reminiscent of the findings of Leigh syndrome, were noted in the substantia nigra (G).

were not registered in neither of the 1000 Genomes Project Database (http://www.1000genomes.org/), ESP6500 database (http://evs.gs.washington.edu/EVS/) or HGVD (http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html). The amino acid sequences of these two sites (p.A957V and p.I1185T) are well conserved across species, suggesting their importance (Fig. 3). *In silico* analyses were performed using the prediction algorithms SIFT (http://sift.jcvi.org) and PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/). These mutations are predicted to be deleterious by SIFT (0 and 0, respectively) and PolyPhen2 (0.985 and 0.991, respectively) programs. The results of mutation analysis have been reported previously (patient 6 in Ref. [9]).

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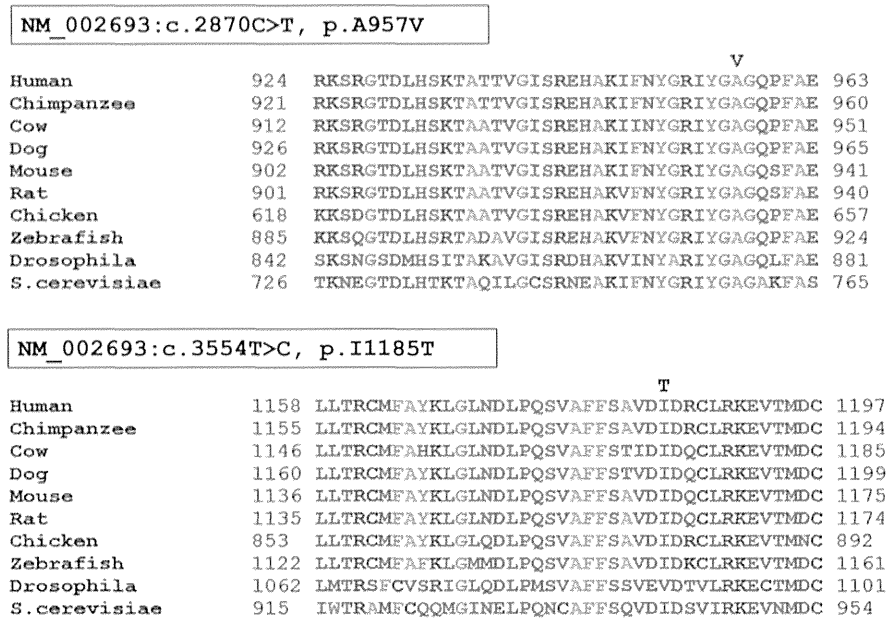

Fig. 3. Conservation analysis of mutation sites in *POLG*. The sites of compound heterozygous amino acid mutations (p.957A and p.1185I) are well conserved across species.

3. Discussion

The hetero compound mutations in *POLG* were not found in either of the 1000 Genomes Project Database, ESP6500 database nor HGVD, suggesting that these are pathogenic mutations. The amino acid sequences of these two sites (p.A957V and p.I1185T) are well conserved across species including *Saccharomyces cerevisiae*, indicating their importance (Fig. 3). *In silico* analyses also predicted that these two amino acid mutations are deleterious. Furthermore A957V has been reported by Tang et al. [10]. They reported A957V allele was shared in three unrelated patients and concluded this mutation is pathogenic. The pathogenic mutations in the flanking region of p.1185I; p.1184D [11,12] and p.1186D [13] have been reported, suggesting this region is also important. Thus, we conclude the compound heterozygous mutations of this patient cause the disease.

Alpers syndrome is defined as the clinical triad of (1) refractory, mixed-type seizures that often include a focal component, (2) psychomotor regression, often triggered by intercurrent infection, and (3) hepatopathy with or without acute liver failure. There is an overlap between the phenotypes of MCHS and Alpers syndrome; however, the former usually shows an earlier onset age and more rapid disease progression, while the latter is characterized by intractable epilepsy. Using the "myo-" prefix in MCHS may be confusing since the pathological findings of muscles in this disorder often shows no evidence of mitochondrial myopathy; instead, the hypotonia observed in the triad can be regarded as a symptom of brain dysfunction. Thus, the clinical features of the patient discussed herein were typical of MCHS.

Although Wong et al. [3] "*...excluded classical Alpers hepatopathy by liver biopsy*" in MHCS, exact pathological findings were not provided by the authors. Differences in the hepatopathy observed in these two phenotypes have not been established; pathological characteristics of the liver in Alpers syndrome include fibrosis, regenerative nodules, hepatocyte dropout, bile duct proliferation, fatty changes, and bile stasis [14]. The findings of the present patient were compatible with those of Alpers syndrome, similar to the case of POLG-related MDDS previously observed [15]. As for the neuropathological findings, Alpers syndrome usually shows a preferential involvement of gray matter, characterized by gliosis, nerve cell loss, spongy degeneration, and accumulation of neural lipids in the cerebral cortex [16]. Alzheimer type II glia, representing hepatic encephalopathy, was also distributed predominantly in the gray matter [17].

A patient exhibiting a clinical evolution from MCHS to Alpers phenotype showed gray matter involvement and microscopic findings similar to those in Leigh syndrome [5], and brain biopsy in another Alpers patient with prominent white matter signal change revealed pathological characteristics typical of Alpers disease with intractable seizures [18]. On the other hand, marked gliosis and sponginess of the white matter without pathological changes in the cerebral cortex was observed in a patient with probable MCHS [17]. Apart from these, we could not find any MCHS cases with a neuropathological description in the literature. The white matter-predominant spongy degeneration with Alzheimer type II astrocytosis in the present patient may therefore be characteristic of MCHS.

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POLG disorders often show elevated levels of lactate both in the serum and CSF as well as elevated levels of hepatic enzymes. However, these findings are not specific for POLG disorders; rather, they are hallmarks of mitochondrial disorders. Analysis of the RCC enzyme activity is the most valuable test for diagnosis of MDDS. However, RCC enzyme activity varies among muscle, liver, kidney, and brain tissues in the same patient [1,19], presumably due to the differential degree of DNA depletion among individual organs. The constituents of complex II are coded by genes in the nuclear, not mitochondrial DNA. In the present patient, the decreased complex II enzyme activity in the biopsied liver may either result from augmented activity of control CS enzyme due to an increase of mitochondria in number, or may be secondary to the damage of hepatocytes with necrotic and fibrotic changes [19]. It is very important to keep in mind that morphological findings and RCC enzyme activities in the muscle are sometimes unremarkable in MCHS patients, even though they show hypotonia or muscular weakness, as in the present case [5,15,20]. Therefore, analysis of RCC enzyme activities in the liver should be considered when Alpers syndrome or MCHS disorders are suspected, even when the morphological findings of muscle or enzyme assay results are unremarkable.

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RESEARCH PAPER

New *MT-ND6* and *NDUFA1* mutations in mitochondrial respiratory chain disorders

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Abstract

Objective: Mitochondrial respiratory chain disorder (MRCD) is an intractable disease of infants with variable clinical symptoms. Our goal was to identify the causative mutations in MRCD patients. **Methods**: The subjects were 90 children diagnosed with MRCD by enzyme assay. We analyzed whole mitochondrial DNA (mtDNA) sequences. A cybrid study was performed in two patients. Whole exome sequencing was performed for one of these two patients whose mtDNA variant was confirmed as non-pathogenic. **Results**: Whole mtDNA sequences identified 29 mtDNA variants in 29 patients (13 were previously reported, the other 13 variants and three deletions were novel). The remaining 61 patients had no pathogenic mutations in their mtDNA. Of the 13 patients harboring unreported mtDNA variants, we excluded seven variants by manual curation. Of the remaining six variants, we selected two Leigh syndrome patients whose mitochondrial enzyme activity was decreased in their fibroblasts and performed a cybrid study. We confirmed that m.14439G>A (*MT-ND6*) was pathogenic, while m.1356A>G (*mitochondrial 12S rRNA*) was shown to be a non-pathogenic polymorphism. Exome sequencing and a complementation study of the latter patient identified a novel c.55C>T hemizygous missense mutation in the nuclear-encoded gene *NDUFA1*. **Interpretation**: Our results demonstrate that it is important to perform whole mtDNA sequencing rather than only typing reported mutations. Cybrid assays are also useful to diagnose the pathogenicity of mtDNA variants, and whole exome sequencing is a powerful tool to diagnose nuclear gene mutations as molecular diagnosis can provide a lead to appropriate genetic counseling.

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Introduction

The mitochondrial respiratory chain (RC) is a pathway for vital energy generation in which ATP is generated as a form of energy by the substrates generated from glycolysis and β-oxidation. The pathway is composed of five multi-enzyme complexes (complexes I–V), two electron carriers, a quinone (coenzyme Q), and a small hem-containing protein (cytochrome c) that are located in the inner mitochondrial membrane. These RC complexes are formed from subunits encoded by both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), with the exception of complex II, which is entirely encoded by nDNA.

mtDNA is a circular double-stranded DNA molecule ~16 kb in length that encodes 37 genes comprising 13 proteins, 22 mitochondrial tRNAs, and 2 rRNAs.[1,2] Defects in mitochondrial function are associated with numerous neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease, and, in particular with mitochondrial respiratory chain disorder (MRCD). MRCD is genetically, clinically, and biochemically heterogeneous, and it can give rise to any symptoms, in any organs or tissues, at any age and with any mode of inheritance.[3] One in 5000 births is a conservative realistic estimate for the minimum birth prevalence of MRCD.[4] Especially in children, MRCD is an intractable disease and can be regarded as the most common group of inborn errors of metabolism.[5,6]

Some MRCD patients have typical clinical findings that are caused by specific point mutations or large deletions of mtDNA. Typical clinical features include mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), myoclonus epilepsy associated with ragged-red fibers (MERRF), Leber's hereditary optic neuropathy (LHON), and chronic progressive external ophthalmoplegia (CPEO).[2] Although mtDNA mutations or deletions are usually found in adults showing typical clinical findings, they account for only a minority of children with MRCD. Therefore, the diagnosis of MRCD in children by screening known mtDNA mutations is rather difficult.[7] Hence, a combination of general biochemical study, histological study, and genetic analysis is essential for the diagnosis of MRCD, especially in children.[6]

In this study, we performed whole mtDNA sequencing for 90 children diagnosed with MRCD by RC enzyme assay with the aim of identifying causative mtDNA mutations.

Subjects, Materials, and Methods

Patients

Ninety Japanese pediatric patients diagnosed with MRCD and without characteristic clinical syndromes were studied. The primary diagnosis for these patients was definite or probable MRCD based on the criteria of Bernier et al.,[8] and a mitochondrial RC residual enzyme activity of <20% in a tissue, <30% in a fibroblast cell line, or <30% in two or more tissues (Data S1). Informed consent was obtained from the patients and their families before participation in the study.

Patient summaries are shown in Tables 1, 2. The details of the two patients studied in the cybrid assay are as follows: Patient (Pt) 377 is a 1-year-old girl born after a normal pregnancy to non-consanguineous parents. She has a normal brother and sister. She was hospitalized with gait difficulties at the age of 1 year. Blood lactate levels were high. Brain magnetic resonance imaging (MRI)

Table 1. Distribution of mtDNA variants and clinical features.

Characteristics		Non-pathogenic mutations	Low probability variants	New pathogenic deletions	Known variants	Total
Number of subjects		61 (100%)	13 (100%)	3 (100%)	13 (100%)	90 (100%)
No consanguinity		57 (93%)	12 (92%)	3 (100%)	11 (85%)	84 (93%)
Age at onset	≤1 y.o.	54 (89%)	10 (77%)	3 (100%)	9 (69%)	76 (84%)
Status	Alive	33 (54%)	7 (54%)	1 (33%)	11 (85%)	53 (59%)
	Dead	28 (46%)	6 (46%)	2 (67%)	2 (15%)	37 (41%)
Sex	Female	30 (49%)	3 (23%)	2 (67%)	6 (46%)	41 (46%)
	Male	31 (51%)	10 (77%)	1 (33%)	7 (54%)	49 (54%)

y.o., years old.

Table 2. Summary of unreported mutations and deletions.

Patient ID	Age at onset	Clinical diagnosis	Enzyme assay (organ)	mtDNA variation	Locus	Heteroplasmy
377	1 year	LD	1 (Fb)	m.14439G>A	*ND6*	Homo (Fb)
190	1 year 6 months	LD	1,4 (M)	m.11246G>A	*ND4*	73% (fb)
508	0 days	SIDS	1 (Hep,Car)	m.4638A>G	*ND2*	86% (Fb), 0% (Hep, Car)
004	0 months	MC	1 (Fb)	m.5537A>G[1]	*tRNATrp*	27.4% (Fb)
271	0 months	ELBW	1 (Hep)	m.10045T>C	*tRNAGly*	Homo (hep)
312[2]	5 years	LD	1 (Fb) probably	m.1356A>G	*12S rRNA*	66% (Fb)
372	2 days	LIMD	1 (Hep)	Deletion (3424 bp) nt12493-15916		65.7% (Fb), 89.9% (Hep)
336	11 months	HD	1 (Hep)	Deletion (6639 bp) nt7734-14372		9.2% (Fb), 92.6% (Hep)
390	0 days	MC	1,4 (M,Hep)	Deletion (5424 bp) nt8574-13997		44.9% (Fb), 86.4% (Hep)

LIMD, lethal infantile mitochondrial disorder; HD, hepatic disease; LD, Leigh's disease; MC, mitochondrial cytopathy; SIDS, sudden infant death syndrome; ELBW, extremely low birth weight infant; Fb, fibroblast; Hep, liver; Car, heart; M, muscle.

[1]Expected to be causative because of the other reported mutation on the same position.

[2]m.1356A>G was confirmed as non-pathogenic and nDNA mutation was identified in Pt312.

showed bilateral and symmetrical hyperintensity foci in the basal ganglia. She developed progressive motor regression and became bedridden. Pt312 is a 5-year-old boy born after 36 weeks' gestation following a normal pregnancy to non-consanguineous parents. His birth weight was 2154 g. He has a sister who is his fraternal twin. At 5 months of age, his parents noticed hypotonia and nystagmus. At 10 months of age, he had generalized epilepsy and blood lactate and his pyruvate levels were high. A brain MRI revealed symmetrical high T2 signals in the midbrain.

Whole mtDNA sequencing and detection of variants

Genomic DNA (gDNA) was extracted from skin fibroblasts (Data S1), blood, liver, and cardiac muscle using either phenol/chloroform- or column-based extraction. Whole mtDNA was first polymerase chain reaction (PCR)-amplified as two separate large amplicons (LA1 and LA2) avoiding the nonspecific amplifications from nDNA.[9] Second-round PCR was performed using 46 primer pairs (mitoSEQrTM; Applied Biosystems, Carlsbad, CA) and the LA1 and LA2 amplicon mixture from first-round PCR as a template. PCR conditions were as follows: first-round PCR was performed in a reaction mixture containing 0.2 mmol/L of each dNTP, 0.25 U of Takara Ex Taq (Takara Bio, Shiga, Japan), 1× Ex Taq Buffer, 0.3 μmol/L of each primer, and extracted gDNA in a total volume of 50 μL. Initial denaturation was performed at 94°C for 2 min, followed by 30 cycles of 94°C for 20 sec, 60°C for 20 sec, and 72°C for 5 min, with a final extension at 72°C for 11 min. Second-round PCR was performed in a reaction mixture as above except with a 10,000-fold dilution of LA1 amplicon and a 100-fold dilution of LA2 amplicon (total volume of the PCR reaction, 10 μL). Initial denaturation was performed at 96°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 45 sec, and 72°C for 45 sec, with a final extension at 72°C for 10 min.

First- and second-round PCR products were separated by 1% and 2% agarose gels, respectively, then 10 μL of second-round PCR products were incubated with 1 μL of ExoSAP-IT reagent (GE Healthcare UK Ltd., Bucks, U.K.) at 37°C for 30 min to degrade remaining primers and nucleotides. The ExoSAP-IT reagent was then inactivated by incubating at 75°C for 15 min. PCR products were sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI3130xl Genetic Analyzer (Applied Biosystems). Sequence data were compared with the revised Cambridge sequence (GenBank Accession No. NC_012920.1) and sequences present in MITOMAP (http://mitomap.org/MITOMAP) and mtSNP (http://mtsnp.tmig.or.jp/mtsnp/index_e.shtml) using SeqScape software (Applied Biosystems). Whole mtDNA sequencing of seven samples was obtained using an Ion PGM™ sequencer (Life Technologies Corporation, Carlsbad, CA).

Characterization of mtDNA deletions

We searched for mtDNA deletions by focusing on the size of first-round PCR products in agarose electrophoresis. If PCR products were smaller than controls, we suspected mtDNA deletion and performed further analysis. The smaller PCR products were recovered from the gel and amplified by second-round PCR, as described above, and

