Original Paper

Journal of Biomedical Science

J Biomed Sci 2001;8:328–335

Received: November 9, 2000
Accepted: January 31, 2001

A Novel Mutation in the Mitochondrial 16S rRNA Gene in a Patient with MELAS Syndrome, Diabetes Mellitus, Hyperthyroidism and Cardiomyopathy

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Key Words

MELAS · Mitochondrial DNA · 16S rRNA · RNase protection · Cybrid

Abstract

Using RNase protection analysis, we found a novel C to G mutation at nucleotide position 3093 of mitochondrial DNA (mtDNA) in a previously reported 35-year-old woman exhibiting clinical features of mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome together with diabetes mellitus, hyperthyroidism and cardiomyopathy. The patient also had an A3243G mutation in the $tRNA^{Leu(UUR)}$ gene and a 260-base pair duplication in the D-loop of mtDNA. The fibroblasts of the patient were cultured and used for the construction of cybrids using cytoplasmic transfer of the patient's mtDNA to the mtDNA-less ρ^0 cells. RNA isolated from the cybrids was subjected to RNase protection analysis, and a C3093G transversion at the 16S rRNA gene and a MELAS-associated A3243G mutation of mtDNA were detected. The novel C3093G mutation together with the A3243G transition were found in muscle biopsies, hair follicles and blood cells of this patient and also in her skin fibroblasts and cybrids. The proportion of the C3093G mutant mtDNA in muscle biopsies of the patient was 51%. In contrast, the mutation was not detected in three sons of the proband. To characterize the impact of the mtDNA mutation-associated defects on mitochondrial function, we determined the respiratory enzyme activities of the primary culture of fibroblasts established from the proband, her mother and her three sons. The proportions of mtDNA with the C3093G transversion and the A3243G transition in the fibroblasts of the proband were 45 and 58%, respectively. However, the fibroblasts of the proband's mother and children harbored lower levels of mtDNA with the A3243G mutation but did not contain the C3093G mutation. The complex I activity in the proband's fibroblasts was decreased to 47% of the control but those of the fibroblasts of the mother and three sons of the proband were not significantly changed. These findings suggest that the C3093G transversion together with the A3243G transition of mtDNA impaired the respiratory function of mitochondria and caused the atypical MELAS syndrome associated with diabetes mellitus, hyperthyroidism and cardiomyopathy in this patient.

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Introduction

Mitochondrial encephalomyopathies are a group of neuromuscular diseases that are frequently associated with mutations of mitochondrial DNA (mtDNA). They have been demonstrated to impair mitochondrial electron transport and ATP production in affected tissues. Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome has been associated with a point mutation at nucleotide position (np) 3243 or 3271 in the mitochondrial $tRNA^{Leu(UUR)}$ gene [14, 34]. Recently, specific mutations in mtDNA have been identified to associate with a variety of endocrine dysfunctions and metabolic disorders, including diabetes mellitus [4, 13, 21, 31] and short stature [32]. The A3243G mutation has also been detected in a small percentage of patients with diabetes mellitus [20, 30, 37]. A heteroplasmic 260-base pair (bp) tandem duplication in the D-loop of mtDNA was first identified in the target tissues of several patients with mitochondrial myopathies [7, 27]. The duplicated region was approximately 260 bp in size, starting at a poly C stretch (np 302–308) and ending at another poly C stretch (np 567–573). Both the L- and H-strand promoters were duplicated and it was suggested that this duplication might predispose to large-scale deletion of mtDNA through slip replication [7]. The proband of the present report and her mother and three sons also harbor the 260-bp tandem duplication [25].

King et al. [23] found incompletely processed RNA in the cultured cells from a MELAS patient harboring a high proportion of mtDNA with the A3243G mutation. These findings have prompted us to search for abnormal transcripts of $tRNA^{Leu(UUR)}$ and other genes responsible for the atypical MELAS syndrome associated with diabetes mellitus, hyperthyroidism and cardiomyopathy in one of our patients. In this study, the fibroblasts of a woman with MELAS syndrome who was the subject of a previous report [25] and those of her mother and three sons were cultured for the construction of cybrids, which were then subjected to molecular and biochemical analyses.

Materials and Methods

Patient

The MELAS patient (II-2, fig. 1) had a history of diabetes mellitus and hyperthyroidism and had received a subtotal thyroidectomy at the age of 26 years [25]. She experienced the first episode of disorientation and epileptic seizures when she was 30 years old. Upon admission, neurologic examination revealed inattention, incoherent speech and psychiatric symptoms. A computed tomography (CT) scan of her brain showed subacute infarctions over the right frontal lobe and the temporo-parieto-occipital region. An old infarct in the right posterior temporal region was noted. A subsequent CT scan and magnetic resonance imaging of the brain also showed multiple infarcts. The lactate levels in the serum (33.1 mg/dl) and cerebrospinal fluid (109.7 mg/dl) and fasting plasma glucose (158 mg/dl) were well above the normal ranges. A muscle biopsy showed ragged-red fibers on a modified Gomori's trichrome stain. Her mental state gradually deteriorated. Recently, we performed a further clinical study and found that the proband also had dilated cardiomyopathy in addition to the above-mentioned multisystem disorders. One of the proband's brothers (II-3, fig. 1) experienced episodic migraine-like headaches, vomiting, loss of vision and repeated focal seizures at the age of 24 years and died at home of unknown causes. He apparently had MELAS syndrome based on the clinical findings and genetic study of his family members, although blood lactate and muscle histology were not examined in his lifetime. Subjects I-2, II-4, III-1, III-2 and III-3 are live members of this three-generation MELAS family (fig. 1). They were all asymptomatic carriers of the A3243G mutation of mtDNA at the time of diagnosis. Muscle biopsies, hair follicles and blood cells were obtained from the proband with the consent of the patient and her mother. Muscle biopsies were quickly frozen and cryopreserved in liquid nitrogen after excision of the muscle. Hair follicles and blood samples were also obtained from her relatives with their consent.

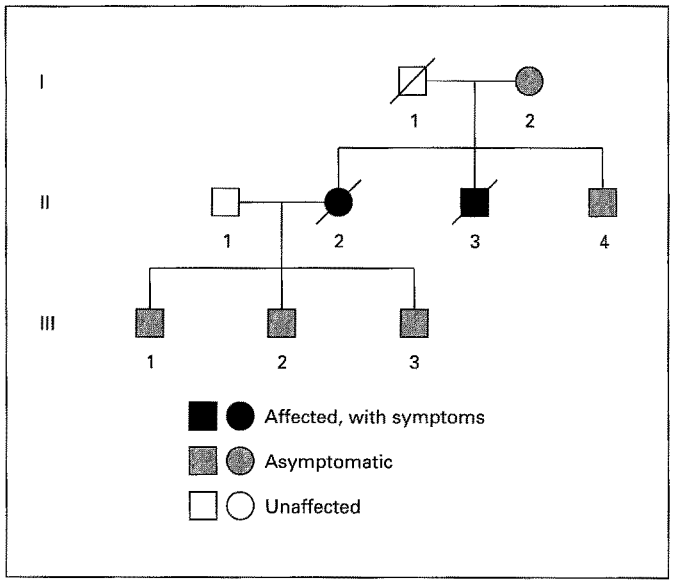

Fig. 1. The three-generation pedigree of the MELAS family. The proband is II-2 in the pedigree. Asymptomatic: asymptomatic members with the A3243G mutation of mtDNA; unaffected: asymptomatic members without this mtDNA mutation.

Primary Culture of Fibroblasts and Construction of Cybrids

The fibroblasts were cultured from skin biopsies of the proband and her relatives in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 μg/ml uridine and 100 μg/ml pyruvate. Fibroblasts from the proband and the proband's mother

were enucleated by cytochalasin B, and the cytoplasts thus obtained were then fused with an excess of mtDNA-less ρ^0 cells according to the method of King and Attardi [22]. After fusion, the hybrid cells (cybrids) were grown in a selective culture medium lacking uridine and pyruvate but containing 5-bromo 2′-deoxyuridine. We also constructed cybrids from the fibroblasts of a patient with myoclonic epilepsy with ragged-red fibers (MERRF) syndrome, who harbored the A8344G mutation but did not have the A3243G mutation of mtDNA.

RNase Protection

An mtDNA segment containing the sequence from the 3′-end of the 16S rRNA and $tRNA^{Leu(UUR)}$ genes and the 5′-end of the ND1 gene was amplified by a forward primer (np 2440–2459, 5′-GCTCATAAGGAAAGGTTAAA-3′) and a reverse primer (np 3435–3416, 5′-GTAGGGGCCTACCACGTTGG-3′). The polymerase chain reaction (PCR) product was cloned into the pGEM-T vector (Promega, Madison, Wisc., USA), which contains the transcriptional promoters of SP6 and T7 phage RNA polymerase. The plasmid DNA was digested with *Eco* RV, which cuts at a site immediately downstream of the probe sequence. The following solutions were added in order at room temperature to a final volume of 100 µl: 20 µl of 5 × transcription buffer (200 m*M* Tris-HCl, 30 m*M* $MgCl_2$, 10 m*M* spermidine-HCl, 25 m*M* NaCl), 10 µl of 100 m*M* dithiothreitol, 100 units of RNasin, 200 µ*M* NTPs, 2 µl of linearized plasmid DNA (2–5 µg) and 20 units of phage RNA polymerase. The mixture was incubated for 2 h at 40 °C for SP6 RNA polymerase. Hybridization was performed by mixing 2 µg of RNA probe with 30 µl of hybridization buffer containing 10 µg of the RNA sample. The solution was heated at 85 °C for 3 min, and then placed at 50–55 °C for at least 8 h. After hybridization, 0.3 ml of RNase A (40 µg/ml) and RNase T1 (2 µg/ml) in 300 m*M* NaCl/5 m*M* EDTA/10 m*M* Tris-HCl (pH 7.5) were added. After incubation at 37 °C for 30 min, the digestion was terminated by adding 20 µl of 10% SDS and 5 µl of 10 mg/ml proteinase K, and the mixture was further incubated at 37 °C for 15 min. The RNA species were analyzed by electrophoresis on a piece of 1.5% agarose gel, and then stained with 1 µg/ml ethidium bromide. The pattern of RNA bands in the gel was photographed and analyzed under UV light transillumination.

Analysis of mtDNA Mutations

A 286-bp mtDNA fragment, encompassing the putative mutation site, was amplified by PCR using specifically designed synthetic oligonucleotide primers. The forward primer was L3058 (np 3058–3092, 5′-TACGTGATCTGAGTTCAGACCGGAGTAATCCAGAT-3′), which had an A→G substitution at np 3091. The reverse primer was H3343 (np 3343–3324, 5′-TGGGTACAATGAGGAGTAGG-3′). A suitable amount (0.5 µCi) of [α-^{33}P]-dATP was added to the reaction system in the last cycle to label the PCR products. With this pair of primers, the PCR products amplified from a normal individual would have the sequence ^{3090}GATC^{3093}, which could be recognized by the restriction endonuclease *Dpn* II, which would digest the amplified DNA segments containing the wild-type sequence into two fragments (250 and 36 bp in size). A C→G transversion at np 3093 of mtDNA would change this sequence to ^{3090}GATG^{3093}, which could not be digested by *Dpn* II, and thus the 286-bp PCR product would remain intact. In order to examine the A3243G mutation in mtDNA, the 286-bp PCR product was also subjected to digestion by *Apa* I as previously described [25]. The restricted DNA fragments were then separated electrophoretically on 1.5% agarose gel. A Hybond-N membrane was then used to blot the DNA bands onto the membrane. Kodak X-ray film was exposed to the blotted membrane at −70 °C for 2 h to 2 days. The relative proportion of mtDNA with each of the two point mutations was determined by scanning densitometry on an autoradiograph of the DNA bands in the agarose gel.

Measurement of Respiratory Enzyme Activities

Mitochondria were isolated from skin fibroblasts of the proband and the proband's mother and three sons, and the submitochondrial particles were used for the assay of various enzyme activities of the respiratory chain. The activities of the respiratory enzymes complex I, complex II, complex II-III, complex III and complex IV were respectively assayed according to the published methods [18]. All assays of the respiratory enzyme activities were conducted on a Hitachi U-3410 spectrophotometer (Hitachi Ltd., Osaka, Japan) using a 1-ml sample cuvette thermostatically maintained at 30 °C.

Mann-Whitney nonparametric analysis was used to determine the statistical significance of the difference in the electron transport activities of respiratory enzymes in fibroblasts between the proband and other members of the MELAS family and the control.

Results

In order to determine the processing of the primary transcripts in the junction region of the 16S rRNA, $tRNA^{Leu(UUR)}$ and ND1 genes, RNAs of three cybrids from the proband, her mother and a MERRF patient were analyzed. The cybrids derived from the proband and her mother harbored 90 and 81% mtDNA with A3243G mutations, respectively. The cybrid derived from a MERRF patient harbored 85% mtDNA with the A8344G mutation but had no detectable A3243G mutation. RNAs prepared from these cybrids and the 143B cell line were used for fine-mapping analysis. The transcript encompassing the 3′-end of 16S rRNA and $tRNA^{Leu(UUR)}$ and the 5′-end of ND1 were analyzed by RNase protection. Gel electrophoretic analysis indicated that the probe protected two major bands (bands a and b, fig. 2). The larger fragments (996 nt, fragment a, fig. 2) corresponded to the unprocessed transcript of 16S rRNA-$tRNA^{Leu(UUR)}$-ND1, which was confirmed by its size and fine mapping. The smaller fragment (790 nt, fragment b, fig. 2) corresponded to the 3′-end of 16S rRNA. There were no variations in the size of these two fragments in the 143B cells and three different cybrids. Significantly, there was a band downstream of the smaller fragment which was only seen in the cybrid constructed from the fibroblasts of the proband (fig. 2, lane 3, band c).

To determine whether the protected band was caused by a point mutation in mtDNA, we used the previous primers to amplify the DNA fragment of interest from

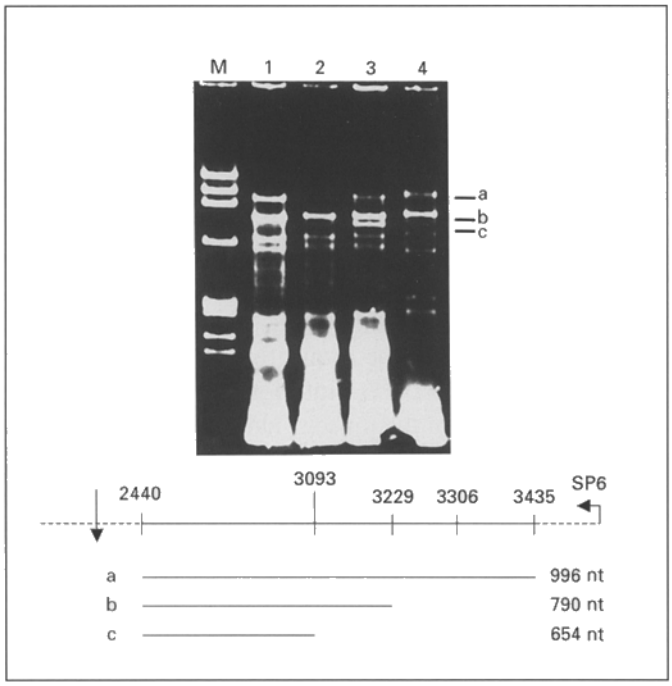

Fig. 2. RNase protection analysis of the 3′-end of the 16S rRNA transcripts. Upper panel: hybridization was performed with a riboprobe for the RNA of 143B cells, cybrid of the proband's mother, cybrid of the proband and cybrid of a MERRF patient (lanes 1–4, respectively). M represents the Φx174/*Hae* III DNA size marker. The protected RNA species were then subjected to electrophoresis on 1.5% agarose gel. Three protected fragments are indicated as (a) the 3′-end 16S rRNA + $tRNA^{Leu(UUR)}$ + the 5′-end of the ND1 transcript (996 nt), (b) the 3′-end of 16S rRNA (790 nt) and (c) the 3′-end of 16S rRNA to np 3093 (654 nt). Lower panel: a scheme indicating the protected region revealed by the RNase protection assay. The expected RNase-protected region of the 16S rRNA is indicated above the map.

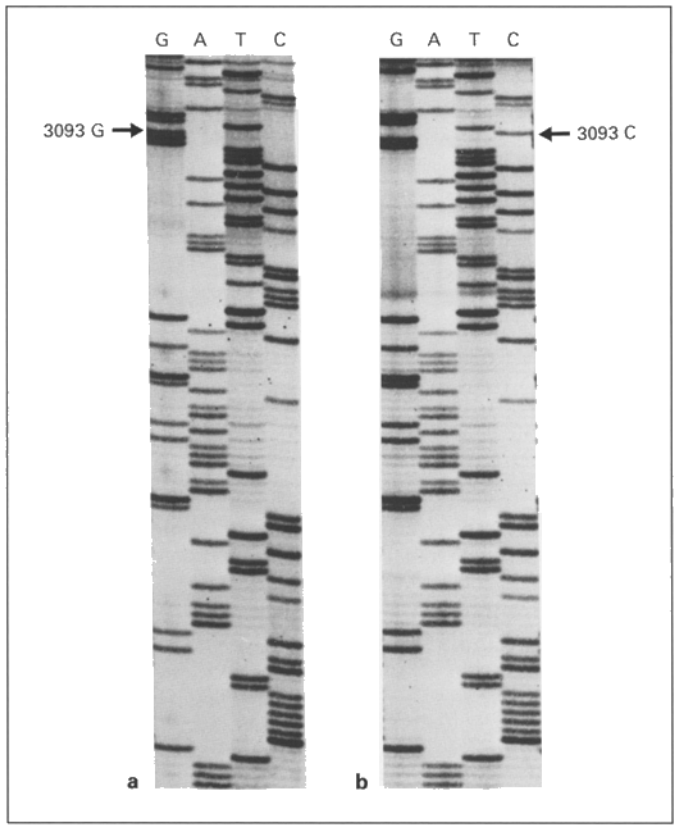

Fig. 3. Nucleotide sequence of the 3′-end of the mitochondrial 16S rRNA gene of the proband. The sequence is shown in the direction of 5′ to 3′ from top to bottom. **a** One C to G transversion was found at np 3093 of a cloned DNA fragment (np 3058 to 3343) from the mtDNA of the cybrid of the proband. **b** The nucleotide sequence of the same DNA fragment of wild-type mtDNA from a healthy subject.

143B cells and the three different cybrids. The PCR products were cloned and sequenced by cycle sequencing using internal labeling with [α-^{35}S]-dATP. In the cybrids from the proband, we detected a novel C to G transversion at np 3093 of mtDNA, which was not seen in the other two cybrids or the 143B cells (fig. 3). This point mutation was found to be located at the 16S rRNA gene of mtDNA. The nucleotide sequences flanking np 3093 of mtDNA from different species show that this nucleotide is highly conserved (table 1) and no mutations have ever been reported before. To examine the association between the C3093G mtDNA mutation and the disease, a 286-bp mtDNA fragment encompassing this and the A3243G mutation sites was amplified by PCR using specifically designed oligonucleotide primers. With this pair of primers, the PCR products amplified from a healthy individual would have the sequence ^{3090}GATC^{3093}, which could be recognized by *Dpn* II, which would digest the amplified DNA segments containing the wild-type sequence into two fragments of 250 and 36 bp. A C to G transversion at np 3093 of mtDNA would change this sequence to ^{3090}GATG^{3093}, which cannot be digested by *Dpn* II, and the 286-bp PCR product would remain intact. We added [α-^{33}P]-dATP to the PCR reaction mixture in the last cycle to determine the proportion of mtDNA with the C3093G mutation (fig. 4).

The proportions of mtDNA with the C3093G mutation were 9 and 4% in the blood cells and 22 and 10% in the hair follicles of the proband and her mother, respectively. The proportion of mtDNA with the C3093G mutation in muscle biopsies of the proband was 51%. The C3093G point mutation of mtDNA was not detected in

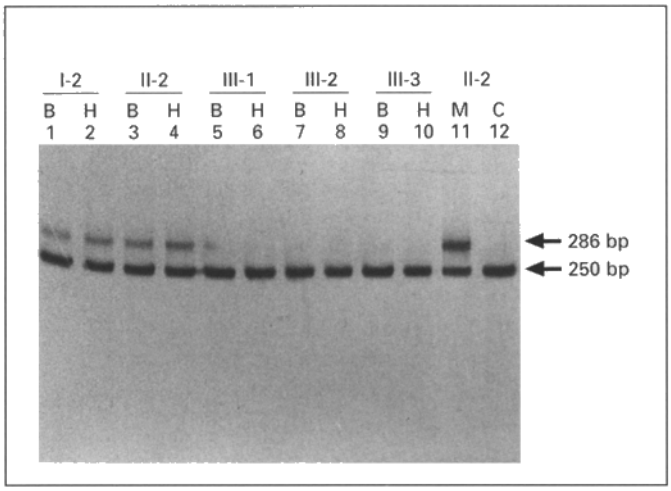

Fig. 4. *Dpn* II restriction analysis of the PCR-amplified DNA fragments for the C3093G mutation in the mtDNA of the proband and her relatives. Lanes 1–12 represent the *Dpn* II-digested PCR-amplified mtDNA fragments. B, H and M indicate the DNA samples of blood cells, hair follicles and muscle biopsy, respectively, of the indicated subjects. C (lane 12) indicates the DNA of blood cells from a healthy subject.

the blood cells and hair follicles of three children of the proband (III-1, III-2 and III-3, fig. 1). We also screened extensively for this mutation in healthy subjects and several patients with other types of mitochondrial disease. The results showed that the C3093G mutation was absent in 8 patients with MELAS syndrome who harbored the A3243G mutation, 5 patients with chronic progressive external ophthalmoplegia (CPEO) harboring mtDNA deletions of different sizes and 23 healthy subjects. When the above-mentioned 286-bp PCR product was subjected to digestion by *Apa* I, we found that the proportions of mtDNA with the A3243G mutation were 82, 55, 35 and 58%, respectively, in the muscle, hair follicles, blood cells and fibroblasts of the proband. The blood cells and hair follicles from the proband's relatives all contained much lower levels of mtDNA with the A3243G mutation.

We then determined the mitochondrial respiratory function of the skin fibroblasts from the proband, 45% of which harbored the C3093G mutation and 58% the A3243G mutation of mtDNA. The activities of complex I in the skin fibroblasts of the proband, the proband's

Table 1. Comparison of the nucleotide sequences of the 33-bp DNA segment encompassing np 3093 in the mtDNA of different species

Species	Nucleotide sequence (5′→3′)			Reference
np	3073	3093	3105	
Patient	CAGACCGGAGTAATCCAGGT	**G**	GGTTTCTATCTA	this study
Human	CAGACCGGAGTAATCCAGGT	**C**	GGTTTCTATCTA	[2]
Mouse	CAGACCGGAGTAATCCAGGT	**C**	GGTTTCTATCTA	[26]
Bovine	CAGACCGGAGTAATCCAGGT	**C**	GGTTTCTATCTA	[3]
Gorilla	CAGACCGGAGTAATCCAGGT	**C**	GGTTTCTATCTA	[16]
Chicken	CAGACCGGAGTAATCCAGGT	**C**	GGTTTCTATCTA	[10]
Chimpanzee	CAGACCGGAGTAATCCAGGT	**C**	GGTTTCTATCTA	[16]

Table 2. Mitochondrial respiratory enzyme activities in the fibroblasts from five members of the family of a woman with MELAS syndrome

Subject	Respiratory enzyme activities, nmol/min/mg				
	complex I	complex II	complex II-III	complex III	complex IV
I-2	22.3±3.0	62.9±5.2	64.8±6.9	401.7±21.5	49.6±2.7
II-2	13.8±4.0*	83.9±11.0*	80.3±8.8*	386.0±16.1	49.0±1.5
III-1	26.4±2.2	62.0±8.9	67.0±5.9	396.4±13.1	50.5±2.0
III-2	25.9±2.8	64.9±7.9	73.5±4.7	423.0±22.3	51.7±4.4
III-3	25.2±4.7	66.4±5.8	72.1±6.6	416.2±22.9	52.1±2.7
Control	29.5±2.7	65.3±4.4	72.7±3.0	411.0±11.3	52.3±2.6

The skin fibroblasts from six subjects without any of the known mitochondrial diseases were used as control. The values of the activities of the five respiratory enzymes in each subject are the mean ± SD of the data obtained from three determinations. Mann-Whitney nonparametric independent analysis was used to determine the statistical significance of the difference in the indicated enzyme activity between each of the members of the MELAS family and the control. * Significant difference as compared with the control ($p < 0.05$).

mother and three sons of the proband were 13.8 ± 4.0, 22.3 ± 3.0, 26.4 ± 2.2, 25.9 ± 2.8 and 25.2 ± 4.7 nmol/min/mg, respectively (table 2). The results indicate that complex I activity in the proband's skin fibroblasts was severely decreased compared with that in the fibroblasts of the proband's mother, three sons of the proband and the healthy subjects. The skin fibroblasts of the proband had slightly higher activities of complex II and complex II-III compared with the fibroblasts from her relatives and control subjects (table 2).

Discussion

The MELAS patient examined in this study has been investigated and reported previously [25]. The proband was first diagnosed to have non-insulin-dependent diabetes mellitus and hyperthyroidism. Molecular analysis of mtDNA showed an A3243G point mutation in the $tRNA^{Leu(UUR)}$ gene and a 260-bp tandem duplication in the D-loop region of mtDNA of the proband and her relatives [25]. In this study, a novel C to G point mutation at np 3093 in the 16S rRNA gene of mtDNA was further identified in muscle, blood cells and hair follicles of this MELAS patient. No point mutations in the rRNA genes of human mtDNA have ever been found to occur in patients with MELAS syndrome. A homoplasmic A to G mutation at np 1555 in a highly conserved region of the 12S rRNA gene of mtDNA was found in families with aminoglycoside-induced deafness as well as in an Arab-Israeli family with maternally inherited deafness [5]. The A1555G mtDNA mutation was also found to associate with nonsyndromic hearing loss [12, 36]. However, some researchers have reported that the A1555G mutation was not associated with nonsyndromic hearing impairment [1, 8]. This portion of the RNA molecule is part of the aminoacylation site in which the mRNAs are decoded, and it lies at the interface between the two rRNA subunits of the ribosome. In a series of mitochondrially inherited chloramphenicol-resistant mutants isolated from human and mouse cells, several point mutations were found in the 16S rRNA gene of mtDNA [17, 33]. Two point mutations of T2991C and C2939A of mtDNA in human cells [33] and four point mutations of G2161A, G2375, A2379 and T2433C of mtDNA in mouse cells have been identified to be associated with the chloramphenicol-resistant phenotype [17].

The C3093G mutation of mtDNA lies near the chloramphenicol binding site, but not in this region. We have compared the nucleotide sequence flanking the np 3093 of mtDNA of different species. The results showed that this nucleotide is highly conserved and no polymorphisms have ever been reported at this site (table 1). The C3093G point mutation was absent in the mtDNA of 8 MELAS patients, 5 CPEO patients, 23 healthy subjects and the three asymptomatic sons of the proband harboring the A3243G mutation of mtDNA. The finding that the novel C3093G mutation exists in multiple tissues of the proband but was not detectable in the unaffected family members and healthy subjects suggests that the association of this mtDNA mutation with this patient with atypical MELAS syndrome is causative. Moreover, compared with the Cambridge sequence of human mtDNA, the sequences of the 3′-end of the 16S rRNA gene of this MELAS patient and a healthy Chinese subject showed a missing C at np 3106/3107, which is an mtDNA polymorphism previously found in some Asians [29].

In Northern hybridization, a novel RNA species corresponding to the unprocessed transcript of 16S rRNA + $tRNA^{Leu(UUR)}$ + ND1 genes was identified [23]. This species was designated RNA19, which is a partially processed full-length polycistronic H-strand precursor RNA. A distinct band corresponding to RNA19 was identified in the total RNA isolated from both wild-type and mutant cybrids. However, there was a significant increase in the steady-state levels of RNA19 in the mutant cybrids as compared to the cybrids harboring wild-type mtDNA [9, 28]. Further support for this finding comes from an experiment on S1 nuclease protection, in which Koga et al. [24] demonstrated that the intensity of the RNA19 in mutant cybrids was much stronger than that in wild-type cybrids or in 143B cells. However, we observed that unprocessed RNA coexists with mature RNAs in 143B cells and in cybrids of the proband (fig. 2). Presumably, the MELAS-specific A3243G mutation alters normal processing of the primary transcript, resulting in an elevated level of RNA19. RNA19 contains 16S rRNA as one of its three components. Thus, it is possible that the RNA19 containing the C3093G mutation within 16S rRNA could be incorporated into the ribosome, which might not be able to function efficiently. Recently, many laboratories have reported that decreased efficiency in aminoacylation of $tRNA^{Leu(UUR)}$ may lead to mistranslation of leucine during protein synthesis [6, 38]. It is plausible that the C3093G mutation of the 16S rRNA gene is another pathogenic factor causing the multisystem disorders in the proband examined in this study.

Deficiency in mitochondrial enzyme activities and a decrease in ATP synthesis have also been reported in mitochondrial multisystem disorders [11, 15, 19, 35].

Cells containing mtDNA mutations are quite sensitive to increased energy demand and respiratory enzyme dysfunction. This is one of the important factors contributing to the pathophysiology of mitochondrial diseases. The cells harboring the C3093G mutation of mtDNA exhibited decreased complex I activity. High levels of mtDNA with the A3243G point mutation were found in skin fibroblasts of the proband and all of the proband's relatives. However, only the skin fibroblasts of the proband harbored both A3243G and C3093G mutations. Additionally, no mtDNA with the 260-bp duplication was found in the proband's fibroblasts. This implies that the two mutations of mtDNA might play a synergistic role in the pathogenesis of the multisystem disorders of this patient. We conjecture that the ribosomes might not be able to assemble efficiently on the 16S rRNA molecule with this mutation, or that they might not be able to incorporate charged tRNAs as rapidly as seen in the ribosomes comprising wild-type 16S rRNA. It is possible that the two mtDNA mutations in this patient with atypical MELAS syndrome elicit synergistic impairment of mitochondrial respiration and oxidative phosphorylation and thereby cause the activity of energy metabolism to fall below a threshold required by the target tissues of the patient. We found that the activity of the respiratory enzyme complex I in the skin fibroblasts of the proband was much more severely decreased compared with that of the asymptomatic relatives of the proband. These findings may explain, at least in part, the clinical observation that the MELAS patient examined in this study exhibited much more complicated clinical features compared with patients with typical MELAS syndrome.

Acknowledgments

This work was supported by a grant (NHRI-GT-EX89S505L) from the National Health Research Institutes and partly by a grant (NSC89-2320-B-010-146) from the National Science Council, Republic of China. Y.-H.W. wishes to express his sincere appreciation to the National Science Council and the Medical Foundation in Memory of Dr. Chi-Shuen Tsou for the support of his research on mitochondrial DNA mutations in human diseases.

References

1 Abe S, Usami S, Shinkawa H, Weston MD, Overbeck LD, Hoover DM, Kenyon JB, Horai S, Kimberling WJ. Phylogenetic analysis of mitochondrial DNA in Japanese pedigrees of sensorineural hearing loss associated with the A1555G mutation. Eur J Hum Genet 6:563–569;1998.

2 Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. Nature 290:457–465;1981.

3 Anderson S, de Bruijn MHL, Coulson AR, Eperon IC, Sanger F, Young IG. Complete sequence of bovine mitochondrial DNA. Conserved features of the mammalian mitochondrial genome. J Mol Biol 156:683–717;1982.

4 Ballinger SW, Shoffner JM, Hedaya EV, Trounce I, Polak MA, Koontz DA, Wallace DC. Maternally transmitted diabetes and deafness associated with a 10.4 kb mitochondrial DNA deletion. Nat Genet 1:11–15;1992.

5 Blanc H, Adams CW, Wallace DC. Different nucleotide changes in the large rRNA gene of the mitochondrial DNA confer chloramphenicol resistance on two human cell lines. Nucleic Acids Res 9:5785–5795;1981.

6 Borner GV, Zeviani M, Tiranti V, Carrara F, Hoffmann S, Gerbitz KD, Lochmuller H, Pongratz D, Klopstock T, Melberg A, Holme E, Paabo S. Decreased aminoacylation of mutant tRNAs in MELAS but not in MERRF patients. Hum Mol Genet 9:467–475;2000.

7 Brockington M, Sweeney MG, Hammans SR, Morgan-Hughes JA, Harding AE. A tandem duplication in the D-loop of human mitochondrial DNA is associated with deletions in mitochondrial myopathies. Nat Genet 4:67–71; 1993.

8 Casano RA, Bykhovskaya Y, Johnson DF, Hamon M, Torricelli F, Bigozzi M, Fischel-Ghodsian N. Hearing loss due to the mitochondrial A1555G mutation in Italian families. Am J Med Genet 79:388–391;1998.

9 Chomyn A, Meola G, Bresolin N, Lai ST, Scarlato G, Attardi G. In vitro genetic transfer of protein synthesis and respiration defects to mitochondrial DNA-less cells with myopathy-patient mitochondria. Mol Cell Biol 11:2236–2244;1991.

10 Desjardins P, Morais R. Sequence and gene organization of the chicken mitochondrial genome. A novel gene order in higher vertebrates. J Mol Biol 212:599–634;1990.

11 Dubeau F, De Stefano N, Zifkin BG, Arnold DL, Shoubridge EA. Oxidative phosphorylation defect in the brains of carriers of the $tRNA^{Leu(UUR)}$ A3243G mutation in a MELAS pedigree. Ann Neurol 47:179–185;2000.

12 Friedman RA, Bykhovskaya Y, Sue CM, DiMauro S, Bradley R, Fallis-Cunningham R, Paradies N, Pensak ML, Smith RJ, Groden J, Li XC, Fischel-Ghodsian N. Maternally inherited nonsyndromic hearing loss. Am J Med Genet 84:369–372;1999.

13 Gerbitz KD, van den Ouweland JM, Maassen JA, Jaksch M. Mitochondrial diabetes mellitus: A review. Biochim Biophys Acta 1271:253–260;1995.

14 Goto Y, Nonaka I, Horai S. A new mtDNA mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS). Biochim Biophys Acta 1097:238–240;1991.

15 Heddi A, Stepien G, Benke PJ, Wallace DC. Coordinate induction of energy gene expression in tissues of mitochondrial disease patients. J Biol Chem 274:22968–22976;1999.

16 Horai S, Satta Y, Hayasaka K, Kondo R, Inoue T, Ishida T, Hayashi S, Takahata N. Man's place in Hominoidea revealed by mitochondrial DNA genealogy. J Mol Evol 35:32–43; 1992.

17 Howell N, Lee A. Sequence analysis of mouse mitochondrial chloramphenicol-resistant mutants. Somat Cell Mol Genet 15:237–244; 1989.

18 Hsieh RH, Hou JH, Hsu HS, Wei YH. Age-dependent respiratory function decline and DNA deletions in human muscle mitochondria. Biochem Mol Biol Int 32:1009–1022; 1994.

19 James AM, Sheard PW, Wei YH, Murphy MP. Decreased ATP synthesis is phenotypically expressed during increased energy demand in fibroblasts containing mitochondrial tRNA mutations. Eur J Biochem 259:462–469;1999.
20 Kadowaki T, Kadowaki H, Mori Y, Tobe K, Sakuta R, Suzuki Y, Tanabe Y, Sakura H, Awata T, Goto YI, Hayakawa T, Matsuoka K, Kawamori R, Kamada T, Horai S, Nonaka I, Hagura R, Akanuma Y, Yazaki Y. A subtype of diabetes mellitus associated with a mutation of mitochondrial DNA. N Engl J Med 330:962–968;1994.
21 Kameoka K, Isotani H, Tanaka K, Azukari K, Fujimura Y, Shiota Y, Sasaki E, Majima M, Furukawa K, Haginomori S, Kitaoka H, Ohsawa N. Novel mitochondrial DNA mutation in $tRNA^{Lys}$ (8296A→G) associated with diabetes. Biochem Biophys Res Commun 245:523–527; 1998.
22 King MP, Attardi G. Human cells lacking mtDNA: Re-population with exogenous mitochondria by complementation. Science 246: 500–503;1989.
23 King MP, Koga Y, Davidson M, Schon EA. Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the $tRNA^{Leu(UUR)}$ mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes. Mol Cell Biol 12:480–490;1992.
24 Koga Y, Davidson M, Schon EA, King MP. Fine mapping of mitochondrial RNAs derived from the mtDNA region containing a point mutation associated with MELAS. Nucleic Acids Res 21:657–662;1993.
25 Li JY, Kong KW, Chang MH, Cheung SC, Lee HC, Pang CY, Wei YH. MELAS syndrome associated with a tandem duplication in the D-loop of mitochondrial DNA. Acta Neurol Scand 93:450–455;1996.
26 Loveland BE, Wang CR, Yonekawa H, Hermel E, Fischer-Lindahl K. Maternally transmitted histocompatibility antigen of mice: A hydrophobic peptide of a mitochondrially encoded protein. Cell 60:971–980;1990.
27 Manfredi G, Servidei S, Bonilla E, Shanske S, Schon EA, DiMauro S, Moraes CT. High levels of mitochondrial DNA with an unstable 260-bp duplication in a patient with a mitochondrial myopathy. Neurology 45:762–768;1995.
28 Morgan-Hughes JA, Hanna MG. Mitochondrial encephalomyopathies: The enigma of genotype versus phenotype. Biochim Biophys Acta 1410:125–145;1999.
29 Obayashi T, Hattori K, Sugiyama S, Tanaka M, Tanaka T, Itoyama S, Deguchi H, Kawamura K, Koga Y, Toshima H, Takeda N, Nagano M, Ito T, Ozawa T. Point mutations in mitochondrial DNA in patients with hypertrophic cardiomyopathy. Am Heart J 124:1263–1269; 1992.
30 Onishi H, Inoue K, Osaka H, Kimura S, Nagatomo H, Hanihara T, Kawamoto S, Okuda K, Yamada Y, Kosaka K. Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) and diabetes mellitus: Molecular genetic analysis and family study. J Neurol Sci 114:5–8;1993.
31 Otabe S, Yasuda K, Mori Y, Shimokawa K, Kadowaki H, Jimi A, Nonaka K, Akanuma Y, Yazaki Y, Kadowaki T. Molecular and histological evaluation of pancreata from patients with a mitochondrial gene mutation associated with impaired insulin secretion. Biochem Biophys Res Commun 259:149–156;1999.
32 Pavlakis SG, Phillips PC, DiMauro S, De Vivo DC, Rowland LP. Mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes: A distinctive clinical syndrome. Ann Neurol 16:481–488;1984.
33 Prezant TR, Agapian JV, Bohlman MC, Bu X, Oztas S, Qiu WQ, Arnos KS, Cortopassi GA, Jaber L, Rotter JI, Shohat M, Fischel-Ghodsian N. Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. Nat Genet 4:289–294; 1993.
34 Sakuta R, Goto Y, Horai S, Nonaka I. Mitochondrial DNA mutations at nucleotide positions 3243 and 3271 in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes: A comparative study. J Neurol Sci 115:158–160;1993.
35 Suzuki Y, Goto Y, Taniyama M, Nonaka I, Murakami N, Hosokawa K, Asahina T, Atsumi Y, Matsuoka K. Muscle histopathology in diabetes mellitus associated with mitochondrial $tRNA^{Leu(UUR)}$ mutation at position 3243. J Neurol Sci 145:49–53;1997.
36 Torroni A, Cruciani F, Rengo C, Sellitto D, Lopez-Bigas N, Rabionet R, Govea N, Lopez De Munain A, Sarduy M, Romero L, Villamar M, del Castillo I, Moreno F, Estivill X, Scozzari R. The A1555G mutation in the 12S rRNA gene of human mtDNA: Recurrent origins and founder events in families affected by sensorineural deafness. Am J Hum Genet 65:1349–1358;1999.
37 Van den Ouweland JM, Lemkes HH, Trembath RC, Ross R, Velho G, Cohen D, Froguel P, Maassen JA. Maternally inherited diabetes and deafness is a distinct subtype of diabetes and associates with a single point mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene. Diabetes 43:746–751;1994.
38 Yasukawa T, Suzuki T, Ueda T, Ohta S, Watanabe K. Modification defect at anticodon wobble nucleotide of mitochondrial $tRNA^{Leu(UUR)}$ with pathogenic mutations of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. J Biol Chem 275:4251–4257;2000.