MELAS syndrome with mitochondrial tRNA^{Leu(UUR)} gene mutation in a Chinese family

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Abstract

The clinical features of a patient in a Chinese family with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS syndrome) are reported. The study revealed that hearing and visual impairments and miscarriages may be early clinical presentations in MELAS. A heteroplasmic A to G transition in the tRNA^{Leu(UUR)} gene was noted at the nucleotide pair 3243 in the mitochondrial DNA of muscle, blood, and hair follicles of the proband and his maternal relatives. Quantitative analysis of the mutated mitochondrial DNA revealed variable proportions in different tissues and subjects of maternal lineage in the family. Muscle tissue contained a higher proportion of the mutant mitochondria than other tissues examined. The function of the reproductive system of the proband seems to be impaired. In one clinically healthy sibling, the 3243rd point mutation was found in sperm mitochondrial DNA, although sperm motility was not affected. It seems that biochemical defects in mitochondrial respiration and oxidative phosphorylation are tissue specific expressions of the 3243rd point mutation in the mitochondrial DNA of the affected target tissues.

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Mitochondrial encephalomyopathies have been recognised as a clinically distinct class of maternally inherited disorders associated with mutations in mitochondrial DNA (mtDNA). Many different syndromes have now been associated with either point mutation or deletions of mtDNA. Among them, two closely related syndromes have specific molecular defects: (a) myoclonus epilepsy and ragged-red fibres with a point mutation in the mitochondrial tRNA^{Leu} gene at the 8344th nucleotide pair¹⁻³ and (b) mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) with other point mutations in the mitochondrial tRNA^{Leu(UUR)} gene at the 3243rd or 3271st position of the mtDNA.⁴⁻⁷

Clinical diagnosis of mitochondrial disease is difficult due to the diversity of clinical manifestations either in probands or in the unaffected relatives. Although some family studies have been reported, detailed survey of family

members with MELAS is still rare.^{5,8} Histopathological studies of muscle biopsies sometimes fail to reveal ragged-red fibres.^{9,10} In the past decade, however, molecular analysis of mtDNA has provided a clear identification for many of the mitochondrial diseases. Furthermore, patients with MELAS are usually thin and short and exhibit a delayed puberty, infertility, and hypogonadism. The aetiologies for infertility and delayed puberty remain poorly understood. In this communication, we report a detailed survey of a family with MELAS to find the early changes in the family members and to correlate clinical findings with mtDNA mutation. The characteristics of sperm motility were also investigated to assess the function of the reproductive system in patients with MELAS syndrome.

Case report

A 14 year old adolescent was admitted because of sudden onset of right limb weakness and incoherent speech for about one week. His birth history was unremarkable. On evaluation, he had a short stature (119 cm in body height) and weighed 21 kg. He had hypertelorism and high arched palate. On 17 March 1990, he developed headache, nausea, and incoherent speech. One week later, focal seizure over the right limb occurred. Mental retardation with a poor school performance, right hemiparesis, and right hemiparesthesia were found. There were no visual field defects, cataract, limitation of eyeball movements, retinitis pigmentosa, or optic nerve atrophy except for a poor vision with amblyopia. Neither myoclonus nor ataxia was noted. Laboratory investigations revealed a normal haemogram, normal values for creatine phosphokinase, lactate dehydrogenase and pyruvate (0.25 mg/dl), but a high serum lactate concentration (25.8 mg/dl). A chest radiogram, electrocardiogram, and echocardiogram were normal. During admission, electroencephalography showed a focal slow wave background associated with intermittent epileptiform discharges over the left temporoparietal area. A brain CT scan disclosed a contrast enhanced hypodense lesion in the left temporoparietal area. The initial diagnosis was *herpes simplex* encephalitis. Studies on CSF were normal, however, except for a high lactate concentration (33.8 mg/dl). The antibody titres to *herpes simplex* virus types I and II were high in serum but negative in CSF. He was treated with an intravenous injection of acyclovir (200 mg) every eight hours for 10 days and phenobarbitone (60 mg) daily. The

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seizure was controlled and his hemiparesis and hemiparesthesia improved gradually in the following months. The low density lesion had disappeared in a follow up CT scan on 3 April and he was discharged in late April.

On 21 August 1990, another episode of focal seizure of the left limbs occurred with left hemiparesis. Brain CT scan showed an additional hypodense lesion in the right temporal area. A cerebral perfusion study with ^{99m}Tc-hexamethyl propylene amine oxime (^{99m}Tc HMPAO) radionuclide single photon emission CT scan showed a decreased deposition of radioactivity in the left frontotemporooccipital and the right temporal areas. Although muscle biopsy revealed no ragged-red fibres and paracrystalline inclusion bodies in light and electron microscopic studies, repetitive stroke-like episodes in adolescence indicated a MELAS syndrome. The mitochondrial DNA isolated from the muscles showed a point mutation with an A to G substitution at the 3243rd nucleotide pair.

In 1993, an endocrinological study showed normal concentrations of triiodothyronine (93.5 ng/dl), tetraiodothyronine (6.23 µg/dl), thyrotropin (1.55 µIU/ml), adrenocorticotropin (27.0 pg/ml), cortisol (10.5 µg/dl), blood sugar (94.0 mg%), human growth hormone (< 0.1 ng/dl), prolactin (23.0 ng/ml), luteinising hormone (1.9 mIU/ml), and follicle stimulating hormone (5.2 mIU/ml). Testosterone concentration was 0.5 ng/ml (reference 2.8–8.8 ng/ml). To understand the clinical manifestations and their correlations with the heteroplasmy of the mutant mtDNA in the family members, we have conducted a detailed survey of the MELAS family.

Materials and methods

Nine members in three generations of the family were included in our study. They were asked to answer a questionnaire enquiring about age, sex, body weight, body height, histories of birth and development, headache, nausea, vomiting, hearing acuity, vision, cataract, unsteady gait, seizure, myoclonus, and stroke-like episodes. Indices of sexual function including impotence, sexual desire, morning erection, spontaneous ejaculation in men, and age of menarche, menstruation (amount and duration), miscarriages, and gynecological history in women were stressed. A physical examination including assessment for hypertelorism, low set ear, saddle nose, high arched palate, simian crease, and pes cavus was performed. Special attention was given to secondary characteristics of pubic and axillary hair, breast development, testicles, and bulbocavernosal reflex. Examination of the testicles focused on the consistency and length of the longest diameter. We also carried out a detailed neurological examination including visual field, eye ball movement, visual acuity, retinitis pigmentosa, optic nerve atrophy, muscle power, and tendon reflexes.

Biopsy specimens were obtained from the right vastus lateralis muscles in five subjects. Sections were stained with haematoxylin and

eosin, modified Gomori-trichrome, succinate dehydrogenase (SDH), and nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR). Blood, hair follicles, and semen (if available) were also obtained. The semen was evaluated for sperm count and motility was measured by a computer assisted semen analyser (CASA). The mtDNAs were isolated from the muscle, blood, hair follicles, and sperm for detection of the mitochondrial genetic defect.

MOLECULAR ANALYSIS OF mtDNA

Total DNA was extracted from various tissues of each subject in the MELAS family according to the standard method described previously.¹¹

For restriction analysis of the 3243-mutation, a 1159 base pair fragment ranging from nt 2678 to nt 3836 was amplified by the polymerase chain reaction technique.^{12,13} After amplification, the DNA fragment was digested with the restriction enzyme *Apa* I which recognises the nt 3243 mutation and gave rise to two additional fragments (591 and 568 bp). The *Apa* I-digested polymerase chain reaction products were subjected to electrophoresis on a 1.5% agarose gel at 100 V for two hours. After electrophoresis, the gel was soaked in double distilled water containing 0.5 µg/ml ethidium bromide for 20 minutes and was subsequently destained in double distilled water with slow agitation. The gel was then photographed under transillumination with short wavelength UV radiation. Ethidium bromide is intercalated into DNA molecules and the complex thus formed emits fluorescence on UV radiation. The intensity of fluorescence is proportional to the total amount of DNA in each band in the agarose gel. Thus after photography the negative film was scanned with a densitometer (Molecular Dynamics, Sunnyvale, CA), and the relative quantity of the mutant DNA (*Apa* I-digested fragments) and wild-type DNA (uncut fragment) were estimated by the ratio of their intensities on the negative film.¹⁴

For restriction analysis of the 3271-mutation, a 223 base pair fragment was amplified from the mtDNA of each subject by the polymerase chain reaction technique using the following pair of primers: 5'3079GGAG-TAATCCAGGTCGGT30963' and 3'3272-AATtcCAGTCTCCAAGTTAAGGAGAAG AAT33015'.⁷ The mismatches were specially designed to create a *Bfr* I (*Afl* II) recognition site after amplification of the DNA segment encompassing the putative 3271 point mutation in the mtDNA of the patients with MELAS. The polymerase chain reaction products of the 3271-mutant mtDNA were then cleaved by *Bfr* I into a 197 base pair and a 26 base pair fragments, and the digested DNA mixture was analysed by agarose gel electrophoresis as described.

Results

Table 1 summarises the clinical presentation of nine persons in the MELAS family. Only

Table 1 Clinical manifestations and mitochondrial DNA mutation in a Chinese family with MELAS syndrome

	I1	I2	II1	II3	II4	II5	III1	III2	III3
Age(y)/Sex	54/M	50/F	29/F	25/M	23/F	17/M	7/F	3/F	1/F
Body weight (kg)	68	44	45	45	42	21	25	17	11
Body height (cm)	168	157	146	163	157	119	120	111	86
Headache	—	—	+	—	—	+	—	—	—
Vomiting	—	—	—	—	—	+	—	—	—
Hearing impairment	—	—	—	—	+	+	—	—	—
Visual abnormality	—	—	—	—	+	+	—	—	—
Cataract	—	—	—	—	—	—	—	—	—
Myoclonus	—	—	—	—	—	—	—	—	—
Seizure	—	—	—	—	—	+	—	—	—
Gait disturbance	—	—	—	—	—	—	—	—	—
Hemiparesis	—	—	—	—	—	+	—	—	—
Ragged-red fibres in muscle	NA	+	—	+	—	—	NA	NA	NA
Point mutation at 3243rd position*:									
Blood (%)	—	+ (6)	+ (3)	+ (25)	+ (33)	NA	+ (18)	—	NA
Hair follicle (%)	NA	+ (20)	+ (9)	+ (30)	+ (26)	NA	+ (34)	—	NA
Muscle (%)	NA	+ (22)	+ (21)	+ (45)	+ (48)	+ (89)	NA	NA	NA
Sperm (%)	NA	—	—	+ (38)	—	NA	—	—	—

*Numbers in parentheses represent percentages of mutant mtDNA; NA = not available.

proband II5 had prominent features of MELAS including short stature, headache, vomiting, hearing impairment, seizure, lactic acidosis, and hemiparesis. Among the family members, one (II1) had headache and one (II4) had hearing impairment and visual

abnormality, which may be related to MELAS syndrome. Skeletal abnormality and facial dysmorphism including short stature, hyper-telorism, and high arched palate were only seen in the proband. Histochemical examination of muscle specimens obtained from I2, II1, II3, II4, and II5 showed ragged-red fibres only in two persons (I2 and II3) with the modified Gomori-trichrome stain. Increased SDH and NADH-TR activities in muscle fibres were also noted in these two subjects. There were no ragged-red fibres in proband II5. Table 2 shows the secondary sexual characteristics in the family. The secondary sexual features included loss of libido, impotency, failure of penile erection, absence of axillary and pubic hairs, and peanut size testes (1.5 cm in the longest diameter) in the proband. In the family, subject II3 also had small testes (2.5 cm, reference range: 4.0 (SD 0.3) cm) and subject II4 had frequent miscarriages, which usually occurred in the first trimester of pregnancy. A bulbocavernosal reflex was absent in I1 and II5. Semen samples were only available from subject II3. The sperm concentration was $59 \times 10^6/\text{ml}$ (reference $20 \times 10^6/\text{ml}$). Sperm motility was normal with 58% rapid moving (reference $> 25\%$), 13% of medium velocity (reference 10–25%), and 3% slow moving (reference $< 10\%$). The mean path velocity was $38 \mu\text{m/s}$ (reference $55.1(10.5) \mu\text{m/s}$), the mean progress velocity (MPRV) was $31 \mu\text{m/s}$ (reference $42.2(8.3) \mu\text{m/s}$), and the ratio of mean path velocity: mean progress velocity was 82% (reference 77.2(4.8)%).

Table 2 Secondary sexual features in a Chinese family with MELAS syndrome

	I1	I2	II1	II3	II4	II5
Age (y)/sex	54/M	50/F	29/F	25/M	23/F	17/M
Loss of libido	—	—	—	—	+	+
Infertility	—	—	—	—	+	—
Impotence	—	—	—	—	+	—
Morning erection	—	—	—	+	—	+
Bulbocavernosal reflex	—	—	—	+	—	—
Testes size (diameter)	N(4.5 cm)	—	—	S(2.5 cm)	—	S(1.5 cm)
Sperm motility	NA	—	—	N	—	NA
Menarche(y)	—	15	13	—	16	—
Menstruation	—	N	N	—	N	—
Breast development	—	N	N	—	N	—
Miscarriages	—	—	—	—	+	—
Axillary hair	N	N	N	N	N	Ab
Pubic hair	N	N	N	N	N	Ab

N = normal; S = small; Ab = absence; NA = not available.

Restriction analysis of mtDNAs from various tissues of family members in the MELAS pedigree. A 1159 base pair (bp) polymerase chain reaction product was obtained from mtDNA of specified tissues of proband and family members indicated with a pair of primers H2678–2696 and L3836–3817. After digestion with *Apa* I, the polymerase chain reaction product can be cleaved into two fragments (591 bp and 568 bp) in maternal lineage family members except for subject III2. B = blood; M = muscle; H = hair follicle; mk = marker.

MITOCHONDRIAL DNA ANALYSIS

Whole blood was obtained from the proband and all the family members, hair follicles from seven persons (I2, II1, II2, II4, III1, III2, and III3), muscle biopsies from five (I2, II1, II2, II4 and II5), and sperm cells from one (II3). Restriction analysis of the polymerase chain reaction products with *Apa* I revealed three fragments including the undigested 1159 base pair DNA fragment and two newly generated 591 and 568 base pair fragments in whole blood, hair follicles (I2, II1, II3, II4, and III1), muscle biopsies (I2, II1, II3, II4, and

II5), and sperm cells (II3) except for blood and hair follicles of subjects I1, III2, and III3 (figure). The proportion of mutant mtDNA in muscle was consistently higher than those in blood, hair follicles and sperm of either the proband or his maternal relatives (table 1). Restriction analysis of the 223 base pair polymerase chain reaction products with *Bfr* I revealed no point mutation with a T to C transition at the nucleotide pair 3271 in the mtDNAs from the patient and all the members of the family.

Discussion

The report describes the clinical features of a patient with MELAS, and a hearing impairment, visual disturbance, and miscarriages possibly related to the syndrome in two of the family members. Moreover, mtDNA analysis showed a point mutation of tRNA^{Leu(UUR)} gene at the 3243rd nucleotide pair in all the members of maternal lineage except for a child (III2). Ciafaloni *et al*⁵ reported that 11 out of 25 relatives had oligosymptoms in 18 families with MELAS. Only two maternal relatives had no tRNA^{Leu(UUR)} gene mutation in their mtDNAs. Furthermore, from our study, neither facial dysmorphism nor skeletal abnormality was found.

Muscle biopsy with ragged-red fibres has been widely used as a proof in the diagnosis of mitochondrial myopathy. Some patients, however, including ours may have no ragged-red fibres, particularly when a single biopsy from one site is examined.^{9,10} In this study, two unaffected family members (subjects I2 and II3) had ragged-red fibres. This suggests that a family survey may be helpful particularly when the proband has MELAS features but no ragged-red fibres in muscle biopsies.

A delayed sexual development in the proband and small testes and frequent miscarriages without mechanical causes of congenital abnormality of the uterus, cervical incompetence, and fibroids in the uterus and other systemic diseases of high fever, congestive heart failure, and chronic renal failure in his siblings indicated that the reproductive system may also be involved in the MELAS syndrome. Interestingly, the 3243 point mutation was also found in the mtDNA of the sperm from one of the family members, but sperm motility was not affected. Although the sperm motility was normal, the affected sperm, that carry a proportion of mutant mitochondrial DNA, may not be able to fertilise ova as effectively as normal sperm. Further study, with the hamster egg penetration assay, may confirm the hypothesis. For the same reason, although the ova were not studied, the affected ova may be not so healthy as unaffected ova and result in miscarriages. To the best of our knowledge, a sperm study has never been reported in patients with MELAS or their family members.

The proportion of mutant mtDNA in the muscle was higher than that in blood

($p < 0.05$, Student's *t* test) and hair follicles. The proportion of mutant mtDNAs in muscle biopsies was also higher in the proband than in his maternal relatives. These findings are similar to those of Shoffner *et al*¹ and Ciafaloni *et al*⁵ in patients with MELAS, which suggest that a small number of wild type mtDNAs may have a protective effect against the mutant mtDNAs. In our family, patients II3 and II4 had an almost identical percentage of mutant mtDNA in muscle, but only patient II3 had ragged-red fibres. Similar findings were obtained in patients I2 and II1 and the series of Martinuzzi *et al*.⁸ The data suggest no obvious correlation between the ragged-red fibres and proportion of mutated mtDNA. Interestingly, the point mutation at the 3243rd nucleotide position was not found in the family member III2 (3rd generation). The question whether she will develop this point mutation in the mtDNA in the future awaits further investigation. To understand the onset and progression of the MELAS syndrome, a longitudinal follow up study on the clinical manifestations, histochemistry, and mtDNA mutation of the unaffected family members is warranted.

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