# Acute Encephalopathy Associated With Influenza C Virus Infection

*To the Editors:*

Influenza C virus infection is considered to be milder than the infections caused by influenza viruses A and B; there are no reports of severe complications associated with influenza C virus infections.[1] Influenza C virus is distributed worldwide, and the seropositive rate in people aged above 10 years is approximately 100%. However, the clinical diagnosis of type C influenza is complicated by the rarity of specific symptoms and the dearth of facilities equipped with the resources for performing efficient viral isolation. Here we report the first case of acute encephalopathy associated with influenza C virus infection.

The patient (age, 2 years and 4 months) presented with hyperpyrexia. Several hours after the onset of hyperpyrexia, the patient had a generalized convulsion that lasted for approximately 10 minutes, after which the patient exhibited severely disturbed consciousness and symptoms of compensatory shock. His body temperature was 42.0°C.

The pharyngeal and nasal swabs collected on admission tested positive for the influenza C virus but negative for the other viruses. The serum hemagglutination-inhibition titer of antibodies against the isolated virus increased from less than 8-fold at the onset of hyperpyrexia to 128-fold on day 24.

CSF analysis revealed a normal cell count. The cytokine profile on admission revealed markedly elevated serum and CSF concentrations of interleukin (IL)-6 (1527.2 pg/mL and 951.3 pg/mL, respectively) and IL-10 (582.3 pg/mL and 49.3 pg/mL, respectively).

Diffusion-weighted imaging of the brain, performed on day 7, revealed diffuse high-intensity signals over the subcortical white matter. Diffusion-weighted imaging performed on day 24 revealed that the high-intensity signals indicating dendritic forms had disappeared; however, mild diffuse brain atrophy persisted.

Acute encephalopathy with prolonged febrile seizure and late reduced diffusion (AESD) has been suggested to be associated with infection by some viruses (eg, influenza A, influenza B, and human herpes virus type 6).[2] AESD is considered the primary form of excitotoxicity-induced acute encephalopathies. The MRI findings obtained in the present case are compatible with those noted in patients with AESD. AESD usually exhibits a biphasic clinical course, with status epilepticus at the onset. However, our patient underwent a monophasic clinical course, and status epilepticus was not noted. This could be attributable to the immediate intensive care that the patient received during the early phase. We considered that the diagnostic criteria for AESD were satisfied in the present case.

The invasion, uncoating, and proliferation mechanisms of the influenza C virus are fundamentally identical to those of the influenza A virus,[3] and we can assume that the influenza C encephalopathy is not associated with any unique pathophysiology. A unique feature of our case is the concomitant elevation in the CSF levels of IL-6 and IL-10. The marked elevation in the patient's IL-10, which is not observed in common AESD,[4] indicated CNS inflammation.

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## REFERENCES

1. Matsuzaki Y, Katsushima N, Nagai Y, et al. Clinical features of influenza C virus infection in children. *J Infect Dis*. 2006;193:1229–1235.
2. Takanashi J, Oba H, Barkovich AJ, et al. Diffusion MRI abnormalities after prolonged febrile seizures with encephalopathy. *Neurology*. 2006; 66:1304–1309; commentary 1291.
3. Palese P, Shaw ML. Orthomyxoviridae: the viruses and their replication. In: Knipe DM, Howly PM, et al, eds. *Fields Virology*. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2006: 1647–1689.
4. Ichiyama T, Suenaga N, Kajimoto M, et al. Serum and CSF levels of cytokines in acute encephalopathy following prolonged febrile seizures. *Brain Dev*. 2008;30:47–52.

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# Different effects of novel mtDNA G3242A and G3244A base changes adjacent to a common A3243G mutation in patients with mitochondrial disorders

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ABSTRACT

Two novel mitochondrial DNA base changes were identified at both sides of the 3243A > G mutation, the most common mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). One was a 3244G > A transition in a girl with MELAS. The other was a 3242G > A transition in a girl with a mitochondrial disorder without a MELAS phenotype. Although the two base changes were adjacent to the 3243A > G mutation, they had different effects on the clinical phenotype, muscle pathology, and respiratory chain enzyme activity. Investigations of the different effects of the 3244G > A and 3242G > A base changes may provide a better understanding of tRNA dysfunction in mitochondrial disorders.



## 1. Introduction

Mutations in mitochondrial tRNA genes are the most common molecular causes of mitochondrial encephalomyopathies. In particular, many mutations have been reported in the mitochondrial $tRNA^{Leu(UUR)}$ gene, indicating that the region is a hot spot for mutations (MITOMAP: a Human Mitochondrial Genome Database, http://www.mitomap.org/). Among them, an A-to-G mutation at nucleotide position (np) 3243 was the first reported in the mitochondrial $tRNA^{Leu(UUR)}$ gene (Goto et al., 1990) and is the most prevalent mutation in all ethnicities. This mutation demonstrates defects at several levels. At the molecular level, it causes decreased protein synthesis (Chomyn et al., 1992), transcription termination impairment (Hess et al., 1991), and an anticodon modification abnormality (Yasukawa et al., 2000; Suzuki et al., 2002). At the cellular level, it is associated with heteroplasmy and typical mitochondrial morphological findings, such as ragged-red fibers and strong SDH-reactive blood vessels (Goto et al., 1990; Sakuta and Nonaka, 1989). At the organ level, the mutation is strongly associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (Goto et al., 1990, 1992).

Mutations in the mitochondrial $tRNA^{Leu(UUR)}$ gene demonstrate marked phenotypic variability, ranging from pure myopathy (Campos et al., 2001; Hadjigeorgiou et al., 1999) to MELAS; however, the pathogenicity of only a few mutations including 3243A > G has been confirmed. As new cases of tRNA mutations accumulate and are analyzed, we will develop an understanding of their pathogenesis and the genotype–phenotype relationship.

Here, we report two new cases harboring novel base changes at both sides of the 3243A > G mutation. One was a 3244G > A transition and the other was a 3242G > A transition. We studied these patients clinically, pathologically, biochemically, and genetically to determine the different effects of these novel base changes that are adjacent to the common 3243A > G mutation.

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## 2. Materials and methods

### 2.1. Clinical investigations

#### 2.1.1. Patient 1

Patient 1 was a 6-year-old girl born to nonconsanguinous parents after an uncomplicated pregnancy and birth. Her family history was unremarkable, including that of her mother and sister. At 4 years of age, she had an attack with vomiting followed by loss of consciousness, and clonic convulsions of the right arm. After the first attack, she had recurrent seizures with episodic vomiting and gradually developed psychomotor deterioration. She was unable to run by the age of 6. At that time, she could converse, but often counted incorrectly. Her limbs were atrophic, and her muscle tone and power were reduced. The patient had no cerebellar signs, myoclonus, or abnormalities of the cranial nerves. A laboratory examination revealed elevated lactate and pyruvate levels in the blood (lactate, 4.81 mmol/l; pyruvate, 0.117 mmol/l; normal, 0.44–1.33 and 0.045–0.113, respectively) and in the cerebrospinal fluid (lactate, 13.3 mmol/l; pyruvate, 0.451 mmol/l). Computed tomography (CT) of the brain revealed calcifications of the basal ganglia and diffuse cerebral atrophy (Fig. 1A). Magnetic resonance imaging (MRI) images of the brain showed diffuse abnormal high T2-weighted signals in the cerebral white matter, especially around the lateral ventricles, and multiple patchy T2-weighted signals in the cerebral cortex.

#### 2.1.2. Patient 2

Patient 2 was a female born to nonconsanguinous parents. Her mother was healthy without any neurological symptoms. Her elder sister had a short stature due to Turner's syndrome with a typical 45X karyotype, but had no other symptoms of mitochondrial disorders. A birth weight of 1985 g at 37 weeks of gestation indicated intrauterine growth retardation. At birth, she had difficulty in sucking and became tachypneic and anemic, but she gradually improved and was discharged from the hospital. At 5 months of age, she was admitted to a hospital due to muscle floppiness and failure to thrive. She showed generalized hypotonia with absence of head control. She developed respiratory failure, heart failure, renal failure due to tubular dysfunction, and lactic acidosis, and therefore, she needed artificial ventilation. An echocardiogram indicated hypertrophic and dilatated cardiomyopathy. A laboratory examination revealed elevated lactate and pyruvate levels in the blood (lactate, 11.0 mmol/l; pyruvate, 0.31 mmol/l) and in the cerebrospinal fluid (lactate, 13.0 mmol/l; pyruvate, 0.44 mmol/l). She also had a significantly elevated creatine kinase (616 IU/l; normal, 43–170). CT and MRI images of the brain revealed nonspecific diffuse cerebral atrophy (Fig. 1A). After discharge from the hospital, she received artificial ventilation four times because of severe acidosis due to infection. However, after the age of 2 years, manifestations including acidosis, renal function, cardiac function, and muscle tonus slowly improved and she showed gradual psychomotor development without any deterioration.

Written informed consent was obtained from the parents of these patients to perform a muscle biopsy, molecular analysis, and biochemical studies.

### 2.2. Histopathological study

A biopsy from the *biceps brachii* muscle was frozen in isopentane chilled with liquid nitrogen, and serial frozen sections were stained with hematoxylin–eosin, modified Gomori trichrome (mGT), succinate dehydrogenase (SDH), cytochrome *c* oxidase (COX) by several histochemical methods.

### 2.3. Molecular genetic studies

DNA extraction, polymerase chain reaction (PCR), and total mitochondrial DNA (mtDNA) sequencing were performed, as described elsewhere (Akanuma et al., 2000). We applied the long

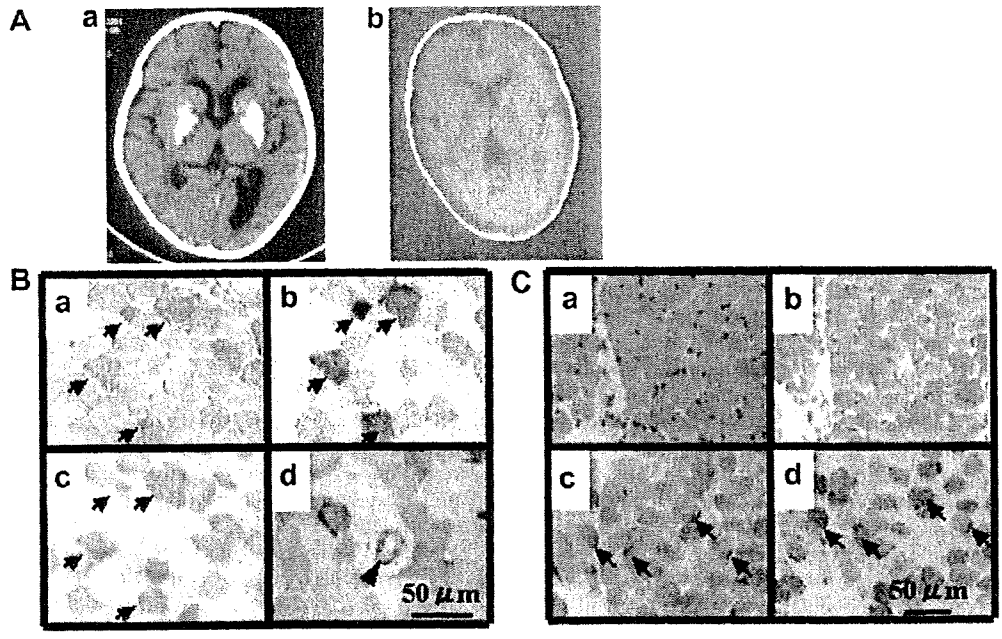


Fig. 1. Computed tomography and histochemical analysis. (A) Computed tomography of the brain of patient 1 (a) revealed calcifications of the basal ganglia and diffuse cerebral atrophy, and of patient 2 (b) revealed mild cerebral atrophy. Morphological analysis of the skeletal muscle of patient 1 (B) and patient 2 (C). In patient 1, ragged-red fibers (RRFs) (arrows) depicted by modified Gomori trichrome (a) and succinate dehydrogenase (SDH) stains. (b) Cytochrome c oxidase (COX) stain (c) revealed a focal COX deficiency, but most of the RRFs reflected intact COX activity (arrows). A strongly SDH-reactive blood vessel (SSV) was detected on the SDH stain (arrowhead). (d) In patient 2, hematoxylin–eosin (a) and modified Gomori trichrome (b) stain revealed moderate fiber size variation without RRFs, SDH, (c) and COX statin (d) showed subsarcolemmal accumulation of mitochondria (arrows) without COX-negative fibers.

PCR-based sequencing method to avoid any adverse results associated with similar sequences in the nuclear DNA. The sequence data were compared with the Human DNA Revised Cambridge Reference Sequence (MITOMAP: a Human Mitochondrial Genome Database; http://www.mitomap.org/mitomap/mitoseq.html).

We devised a real-time PCR amplification method based on a previously described approach (Komaki et al., 2003) to accurately quantify the frequency of the 3244G > A mutation. The target sequence (np 3211–3322) was amplified using a pair of primers and two fluorogenic TaqMan™ probes (PE Applied Biosystems; Foster City, CA, USA) designed for the wild-type and mutant sequences (Table 1). The copy number of mtDNA containing each mutant or wild-type sequence was determined based on a standard curve created by the reaction of a known amount of plasmid containing the mtDNA fragment (np 3171–3350) with each wild-type or mutant sequence.

To identify the 3242G > A transition, we amplified the 126-bp PCR fragment with the forward and mismatched primers and digested the fragment by SacI. If the fragment did not have the 3242G > A base change, then 108- and 18-bp cleaved fragments would be obtained. Each fragment was detected in a 4% agarose gel (Nusieve 3:1 agarose; Bio-Whittaker Molecular Applications; Rockland, ME, USA) stained with ethidium bromide.

### 2.4. Biochemical studies of primary cultures and transmitochondrial cell lines

Primary skin fibroblasts were obtained from patients 1 and 2, and myoblasts could be obtained only from patient 2. The fibroblasts and myoblasts were grown in DMEM/F-12 medium with 20% fetal bovine serum (Invitrogen Corp. Carlsbad, CA, USA).

Transmitochondrial cell lines (cybrids) were obtained by polyethylene glycol fusing of enucleated fibroblasts from both patients with human osteosarcoma 143B/TK- cells lacking mtDNA (King and Attardi, 1989). Twenty clones derived from patient 2 were selected from a uridine-lacking medium and employed to measure ATP synthesis and enzymatic activity of individual mitochondrial respiratory complexes. The DNA was extracted from each clone for the quantification of the proportions of the 3244G > A mutation. Cybrid cells derived from patient 2 were used to measure enzymatic activity of individual mitochondrial respiratory complexes and for blue native polyacrylamide gel electrophoresis (BN-PAGE).

The methods to measure ATP synthesis in digitonin-permeabilized fibroblasts, myoblasts, or cybrids is described elsewhere (Komaki et al., 2003), along with several modifications of a method reported by Robinson (Robinson, 1996).

Enzymatic activity of individual mitochondrial respiratory complexes was performed on isolated mitochondria obtained from cultured $5 \times 10^5$ cybrid and 143B/TK-cells according to Trounce et al. with modifications (Trounce et al., 1996). The activities of complexes I, IV, and citrate synthase were measured by spectrophotometric assays as described. All samples were measured at least in duplicate and averaged.

**Table 1**
Fluoroeenie probes mid amplification primers for real-time PCR.

| Fluorogenic probes sequence | | Primer sequence |
|---|---|---|
| Wild | 5′ (FAM)-TGGCAGA GCCCGGT-(MGB) p3′ | Forward 5′-CCACCCAAGAACAGGGTTTG-3′ |
| Mutant | 5′ (VIC)-TGGCAGAACCCGGT-(MGB) p3′ | Reverse 5′-GGTTGGCCATGGGTATGTTG-3′ |

The underlined positions corresponded to np 3244. MGB: minor groove binder.

### 2.5. BN-PAGE and Western blot for immunodetection

Mitochondrial proteins were isolated from cultured 143B/TK-cells and cybrids derived from patient 2 ($3–6 \times 10^6$ cells) (Nijtmans et al., 2002). The mitochondrial proteins (100 μg) were solubilized in sample buffer (Invitrogen) containing 0.5% (w/v) *n*-dodecyl-β-d-maltoside (DDM). Electrophoresis was performed on 3–12% polyacrylamide gels (Invitrogen) (Nijtmans et al., 2002; D'Aurelio et al., 2006). Following BN-PAGE, the gels were soaked in a transfer buffer (Invitrogen) and blotted onto polyvinylidene fluoride (PVDF) membranes using the iBlot transfer system (Invitrogen) according to the manufacturer's instructions (20 V, 7 min). Subunit-specific mouse monoclonal antibodies (Molecular Probes) were used to immunodetect protein complexes. The cocktail of primary antibodies included the 39 kDa (complex I, 0.5 μg/mL), 70 kDa (complex II, 0.5 μg/mL), core II (complex III, 0.5 μg/mL), subunit I (complex IV, 2.5 μg/mL), and subunit β (complex V, 0.5 μg/mL). After removing the cocktail of primary antibodies, the alkaline phosphatase-conjugated anti-mouse secondary antibody was reacted, and nitroblue tetrazolium chloride (NBT)-derived chromogenic detection was performed. We determined the appropriate conditions to solubilize the mitochondrial membranes while preserving the intact respiratory chain complexes.

## 3. Results

### 3.1. Histopathological study

The histopathological study of the skeletal muscle from patient 1, at the age of 6 years, revealed the presence of numerous ragged-red fibers (RRFs), i.e., ⩾15% of the total fibers and some strongly succinate dehydrogenase-reactive blood vessels (SSVs). The COX stain revealed diffuse COX-negative fibers, whereas most of the RRFs were reactive (Fig. 1B). In patient 2, at the age of 9 months, there was increased subsarcolemmal accumulation of mitochondria in many fibers, which suggested mitochondrial abnormalities, but typical RRF, SSV, and COX-negative fibers were not detected (Fig. 1C).

### 3.2. Molecular genetic studies

No large-scale mtDNA rearrangements were detected by the long PCR method in either patient. Total mtDNA sequencing of the muscle from patient 1 revealed 38 base changes compared with the revised standard sequence (Table 2). According to the MITOMAP database, 37 changes have been previously reported as normal polymorphisms. One of the observed changes was a G-to-A mutation at np 3244 in the mitochondrial $tRNA^{Leu(UUR)}$ gene, and this change appeared to be heteroplasmic (i.e., both the mutant and the wild-type genome were present) on electropherograms (Fig. 2A). Real-time PCR amplification confirmed that the percentage of mutant mtDNA in the patient's muscle and fibroblasts was 94% and 90%, respectively. In patient 2, total mtDNA sequencing of muscle revealed 41 base changes compared with the standard sequence (Table 3). According to the MITOMAP database, 39 changes have been previously reported as normal polymorphisms. We identified a 6481T > C base change that had not been reported previously to the MITOMAP database; however, we detected this base change in her healthy mother. We also detected a G-to-A base change at np 3242 (Fig. 2B). This change was confirmed by restriction fragment polymorphism and was revealed to be homoplasmic (i.e., only the mutant genome was present) in the patient's tissues, including blood, but was absent in the blood of her healthy mother (Fig. 2C). Total mtDNA sequencing of blood from the proband's mother revealed the

**Table 2**
MtDNA sequence variants in patient 1.

| Gene product | np | Base-change | Amino acid change | MitoMap database |
|---|---|---|---|---|
| D-loop | 73 | A to G | | Reported polymorphism |
| D-loop | 152 | T to C | | Reported polymorphism |
| D-loop | 263 | A to G | | Reported polymorphism |
| D-loop | 311 | Insertion C | | Reported polymorphism |
| D-loop | 489 | T to C | | Reported polymorphism |
| 12S rRNA | 750 | A to G | | Reported polymorphism |
| 16S rRNA | 2706 | A to G | | Reported polymorphism |
| 16S rRNA | 3010 | G to A | | Reported polymorphism |
| 16S rRNA | 3206 | C to T | | Reported polymorphism |
| tRNA-Leu(UUR) | 3244 | G to A | | Unreported |
| NADH dehydrogenase 2 | 4763 | A to G | Synonymous | Reported polymorphism |
| NADH dehydrogenase 2 | 4883 | C to T | Synonymous | Reported polymorphism |
| NADH dehydrogenase 2 | 5178 | C to A | L to M | Reported polymorphism |
| Cytochrome c oxydase I | 7028 | C to T | Synonymous | Reported polymorphism |
| ATP synthase a | 8414 | C to T | L to F | Reported polymorphism |
| ATP synthase a | 8473 | T to C | Synonymous | Reported polymorphism |
| ATP synthase 0 | 8701 | A to G | T to A | Reported polymorphism |
| ATP synthase 0 | 8860 | A to G | T to A | Reported polymorphism |
| Cytochrome c oxydase 3 | 9540 | T to C | Synonymous | Reported polymorphism |
| Cytochrome c oxydase 3 | 9524 | T to C | Synonymous | Reported polymorphism |
| NADH dehydrogenase 3 | 10308 | A to G | T to A | Reported polymorphism |
| NADH dehydrogenase 3 | 10400 | C to T | Synonymous | Reported polymorphism |
| tRNA-Arg | 10410 | T to C | | Reported polymorphism |
| NADH dehydrogenase 4 | 10873 | T to C | Synonymous | Reported polymorphism |
| NADH dehydrogenase 4 | 11710 | G to A | Synonymous | Reported polymorphism |
| NADH dehydrogenase 5 | 12706 | C to T | Synonymous | Reported polymorphism |
| NADH dehydrogenase 6 | 14068 | C to T | Synonymous | Reported polymorphism |
| Cytochrome b | 14766 | C to T | I to T | Reported polymorphism |
| Cytochrome b | 14783 | T to C | Synonymous | Reported polymorphism |
| Cytochrome b | 14070 | T to C | I to T | Reported polymorphism |
| Cytochrome b | 15043 | G to A | Synonymous | Reported polymorphism |
| Cytochrome b | 15301 | G to A | Synonymous | Reported polymorphism |
| Cytochrome b | 15314 | G to A | A to T | Reported polymorphism |
| Cytochrome b | 15326 | A to G | T to A | Reported polymorphism |
| D-loop | 16085 | C to T | | Reported polymorphism |
| D-loop | 16120 | G to A | | Reported polymorphism |
| D-loop | 10223 | C to T | | Reported polymorphism |
| D-loop | 10223 | T to C | | Reported polymorphism |
| D-loop | 10500 | T to C | | Reported polymorphism |

same polymorphisms except for the 3242G > A base change (data not shown).

### 3.3. *Biochemical studies*

In patient 1, fibroblast ATP synthesis was significantly low when pyruvate/malate or glutamate/malate were used as the substrate (Fig. 2D), but the rate of synthesis was normal when succinate or TMPD/ascorbate were added to the cells. These findings revealed that fibroblasts derived from this patient had a complex I deficiency. To confirm that the 3244 mutation was pathogenic, we performed functional analysis of cybrids. We obtained 20 clones with a different percentage of the heteroplasmic np 3244 mutation and performed ATP synthesis assays using each clone. ATP synthesis was within the normal range when the percentage of mutant DNA remained under 90%. Clones carrying mutant mtDNA in high proportions, i.e., those exceeding the threshold level of approximately 95%, lost their ability to synthesize ATP when glutamate/malate or TMPD/ascorbate were used as the substrate (Fig. 2E,F). Moreover, analysis of enzymatic activity for individual mitochondrial respiratory complexes revealed that the activities of complexes I and II were apparently low in the cybrid cells carrying a high proportion of mutant mtDNA, although they were normal in cybrid cells carrying a low proportion of mutant mtDNA (Fig. 2G). These findings indicated that extremely high levels of the mutation led not only to a complex I deficiency, but also to complex IV and/or V deficiencies.

In contrast, the fibroblasts and myoblasts of patient 2 showed normal ATP synthesis (data not shown); however, her cybrid cells had low levels of complex IV activity (Fig. 2G).

### 3.4. *Studies on assembly of respiratory chain enzymes*

To understand the consequences of the 3242G > A mutation on the composition of the respiratory chain, BN-PAGE analysis was performed using the subunit-specific monoclonal antibodies on equal amounts of mitochondrial proteins extracted from the same number of mutant cybrids and human osteosarcoma 143B/TK- cells. Also, the amount of assembled respiratory chain complexes in cybrid clones carrying the 3242G > A mutation was estimated. Compared with 143B/TK-, cybrids showed a reduced level of the complex I-III-IV supercomplex and an increase in the amount of the complex I-III supercomplex. The levels of the complex III homodimer and complex II were assessed as a loading control (Fig. 2H).

## 4. Discussion

MELAS is a maternally inherited disorder typically characterized by onset before the age 15 years, lactic acidosis, episodic vomiting, seizures, migraine-like headaches, and recurrent cerebral insults resembling strokes (Goto, 1995; Hirano and Pravlakis, 1994). The symptoms of patient 1, including the onset age, recurrent episodic vomiting, headache, hemiconvulsions, and severe lactic acidosis, were consistent with the clinical spectrum associated

**Table 3**
MtDNA sequence variants in patient 2.

| Gene product | np | Base change | Amino acid change | MitoMap database |
|---|---|---|---|---|
| D-loop | 73 | A to G | | Reported polymorphism |
| D-loop | 152 | T to C | | Reported polymorphism |
| D-loop | 263 | A to G | | Reported polymorphism |
| D-loop | 303 | Insertion C | | Reported polymorphism |
| D-loop | 311 | Insertion C | | Reported polymorphism |
| D-loop | 480 | T to C | | Reported polymorphism |
| 12S rRNA | 750 | A to G | | Reported polymorphism |
| 12S rRNA | 1438 | A to G | | Reported polymorphism |
| 16S rRNA | 2706 | A to G | | Reported polymorphism |
| 16S rRNA | 3010 | G to A | | Reported polymorphism |
| 16S rRNA | 3206 | C to T | | Reported polymorphism |
| tRNA-Leu(UUR) | 3242 | G to A | | Unreported |
| NADH dehydrogenase 2 | 4760 | A to G | Synonymous | Reported polymorphism |
| NADH dehydrogenase 2 | 4883 | C to T | Synonymous | Reported polymorphism |
| NADH dehydrogenase 2 | 5173 | C to A | L to M | Reported polymorphism |
| NADH dehydrogenase 2 | 5201 | G to A | A to T | Reported polymorphism |
| Cytochrome c oxydase I | 6481 | T to C | V to A | Unreported |
| Cytochrome c oxydase I | 7028 | C to T | Synonymous | Reported polymorphism |
| ATP synthase 8 | 8414 | C to T | L to F | Reported polymorphism |
| ATP synthase 8 | 8473 | T to C | Synonymous | Reported polymorphism |
| ATP synthase 6 | 3701 | A to G | T to A | Reported polymorphism |
| ATP synthase 6 | 8860 | A to G | T to A | Reported polymorphism |
| Cytochrome c oxydase 3 | 8540 | T to C | Synonymous | Reported polymorphism |
| NADH dehydrogenase 3 | 10,308 | A to G | T to A | Reported polymorphism |
| NADH dehydrogenase 3 | 10,400 | C to T | Synonymous | Reported polymorphism |
| tRNA-Arg | 10,410 | T to C | | Reported polymorphism |
| NADH dehydrogenase 4 | 10,873 | T to C | Synonymous | Reported polymorphism |
| NADH dehydrogenase 4 | 11,710 | G to A | Synonymous | Reported polymorphism |
| NADH dehydrogenase 5 | 12,706 | C to T | Synonymous | Reported polymorphism |
| NADH dehydrogenase 6 | 14,068 | C to T | Synonymous | Reported polymorphism |
| Cytochrome b | 14,766 | C to T | I to T | Reported polymorphism |
| Cytochrome b | 14,783 | T to C | Synonymous | Reported polymorphism |
| Cytochrome b | 14,870 | T to C | I to T | Reported polymorphism |
| Cytochrome b | 15,043 | G to A | Synonymous | Reported polymorphism |
| Cytochrome b | 15,301 | G to A | Synonymous | Reported polymorphism |
| Cytochrome b | 15,314 | G to A | A to T | Reported polymorphism |
| Cytochrome b | 15,320 | A to G | T to A | Reported polymorphism |
| D-loop | 16,120 | G to A | | Reported polymorphism |
| D-loop | 16,223 | C to T | | Reported polymorphism |

The T64S1C base change was observed in a healthy mother of patient 2.

mutations of mtDNA were found, but we did identify base changes adjacent to the 3243A > G mutation, which is the most common mutation in MELAS patients, including a 3244G > A transition in patient 1 and a 3242G > A transition in patient 2 (Fig. 3A). The 3244G > A and 3242G > A base changes have not been reported clinically, but as a somatic mutation in gastric carcinoma (Habano et al., 2000) and in the bone marrow of a patient with myelodysplastic syndrome (Gattermann et al., 2004), respectively.

Several lines of evidence support a causal association between the 3244G > A base change mutation and MELAS. First, this base change was not observed in over 200 normal individuals. Second, the 3244G > A transition disrupted a highly conserved nucleotide in the tRNA structure (Fig. 3B). Third, most mutations in typical cases of MELAS were located in the same tRNA gene, including the 3271T > C (Goto et al., 1991), 3291T > C (Goto et al., 1994), 3252A > G (Morten et al., 1993), and 3260A > G mutations (Nishino et al., 1996). Moreover, the 3244G > A base change was located adjacent to the most common mutation in MELAS patients, namely, the 3243A > G mutation (Fig. 3A). Fourth, this mutation existed under heteroplasmic conditions, which is a common feature of pathogenic mtDNA mutations. Finally, a functional analysis of cybrids revealed a significant decrease in the respiratory chain function, which was observed in cells with a relatively high percentage of mutant mtDNA. The results of these assays indicated deficiencies of complexes I and IV and a threshold effect of mutant load on respiratory chain enzyme activity, which has often been observed in MELAS patients carrying the 3243A > G mutation (Goto, 1995; Koga et al., 1995).

In patient 2, 6481T > C and 3242G > A base changes were detected; these polymorphisms were not reported previously. The 6481T > C change resulted in the replacement of valine with alanine; however, we detected this base change in her healthy mother. Therefore, it is reasonable to conclude that this base change is not pathogenic. In patient 2, the 3242G > A base change existed in all tissues in a homoplasmic condition, which is different than the 3244G > A or the more common 3243A > G mutation (Fig. 2C), thus making its pathogenicity difficult to confirm. However, there is evidence, including the biochemical defects in cybrid cells, to support the pathogenicity of this base change (Chinnery et al., 1999; McFarland et al., 2004). First, this transition was segregated with the disease; it was not detected in more than 200 normal individuals. Moreover, it was not detected in the blood of her healthy mother, although the base change was present in blood of the proband (Fig. 2C). Second, the 3242G > A transition affected a highly conserved position in the $tRNA^{Leu(UUR)}$ gene (Fig. 3B). Third, pathogenic mutations of several mitochondrial diseases have involved the dihydrouridine (DHU) stem of this tRNA (Kawarai et al., 1997; Nishigaki et al., 2003; Hao and Moraes, 1996). It is important to note that the DHU stem appears to be a rather weak structure and might thus be more prone to alterations leading to structural disturbances. The 3242G > A transition can alter the secondary and possibly the tertiary structure of the DHU-stem due to

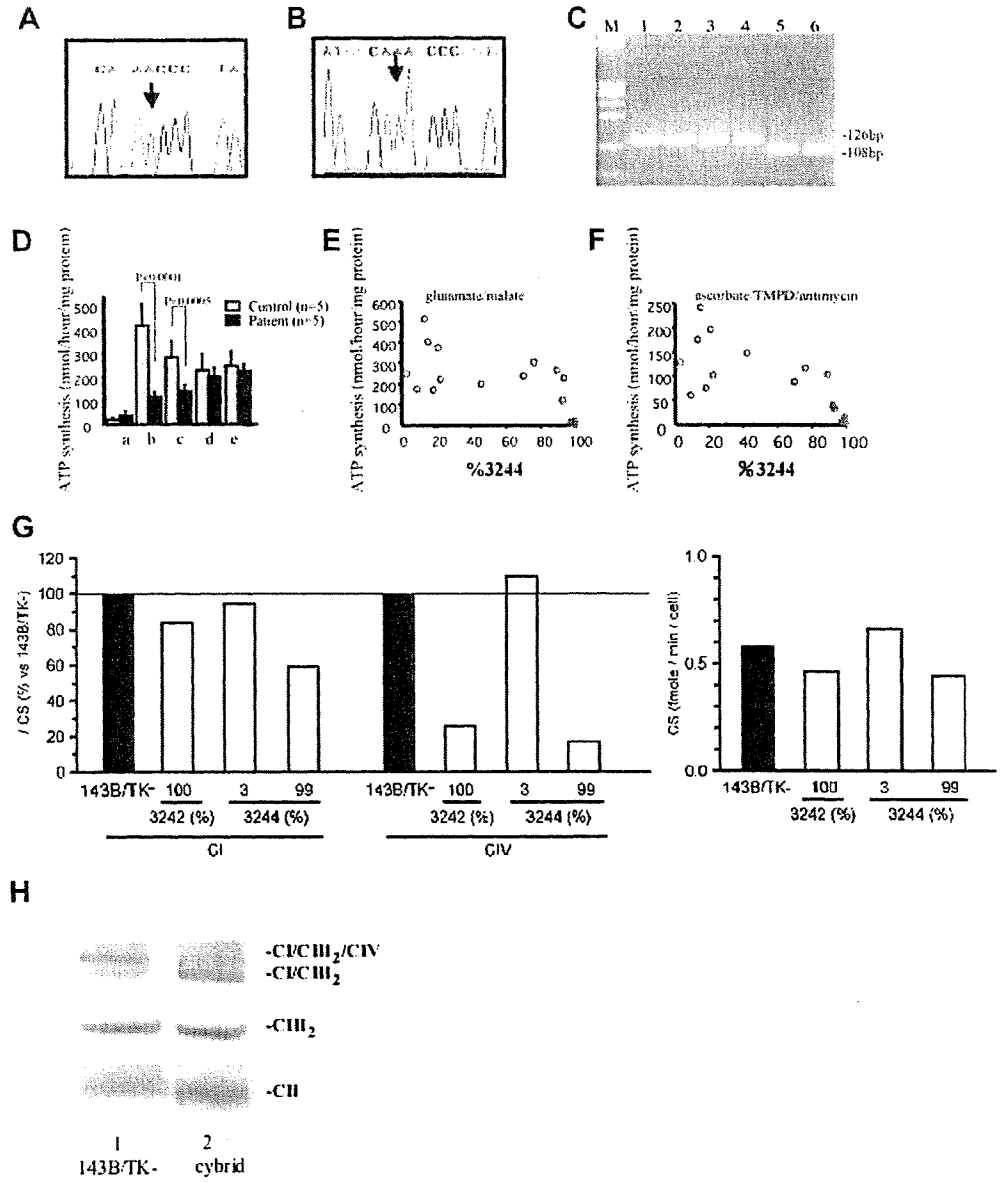


**Fig. 2.** Molecular and biochemical analysis. (A) An electropherogram based on mtDNA sequencing from muscle specimens revealed a heteroplasmic G-to-A substitution at nucleotide position 3244 (arrow) in the $tRNA^{Leu(UUR)}$ gene of patient 1, and a (B) G-to-A substitution at nucleotide position 3242 (arrow) in patient 2. (C) SacI digestion of the mutant 3242G > A mtDNA is indicated by a 126-bp band. Wild-type mtDNA is indicated by the presence of a 108-bp band. M, molecular weight markers; lane 1, patient's muscle; lane 2, myoblast; lane 3, fibroblast; lane 4, blood; lane 5, mother's blood; lane 6, wild-type control. (D) ATP synthesis in a digitonin-treated primary culture of fibroblasts with 90% mutants. ATP synthesis was measured using the following combinations of substrates and specific inhibitors: a, none; b, pyruvate/malate; c, glutamate/malate; d, succinate/rotenone; e, ascorbate/TMPD/antimycin. The results of this assay are expressed as nanomoles ATP per hour per milligram of cell protein. The control values are presented as the mean ±1 SD. ATP synthesis in 143B/TK- derived cybrid clones with various percentages of the 3244G > A mutation (open circles) and 143B/TK- cells (closed circles). (E) Glutamate + malate was used as the substrate. (F) Ascorbate and TMPD were used as the substrates, and antimycin was used as an inhibitor. (G) Activities of the respiratory chain complexes relative to citrate synthase in 143B/TK- cells and cybrid clones carrying the 3242G > A mutation (100% mutant) and the 3244G > A mutation (3% and 99% mutant). The figure to the right shows the activity of citrate synthase in these clones. CI, complex I; CIV, complex IV; CS, citrate synthase. (H) $CI/CIII_2/CIV$ supercomplex level decreases and $CI/CIII_2$ supercomplex level increases in cybrid clones carrying the 3242G > A mutation (lane 2), compared to 143B/TK-cells (lane 1). $CI/CIII_2/CIV$, complex I-III-IV supercomplex; $CI/CIII_2$, complex I-III supercomplex; $CIII_2$, complex III homodimer; CII, monomeric complex II.

with MELAS. Morphological analysis of the muscle biopsy also showed typical findings of MELAS. We detected diffuse COX-negative fibers and numerous RRFs, some of which stained positive for COX activity, as has previously been reported in MELAS patients but not in those with other mitochondrial myopathies (Goto et al., 1992). We also observed SSVs, which is an important finding in MELAS patients (Hasegawa et al., 1991). Patient 2 showed multiple tissue involvement including severe lactic acidosis, cardiomyopathy, renal tubular dysfunction, cerebral atrophy, generalized hypotonia in infancy, and increased subsarcolemmal accumulation of mitochondria in the muscle biopsy, which strongly suggested that she had a mitochondrial disease. However, the clinical picture and pathological findings were apparently different from MELAS.

The underlying molecular defects involved in these cases were distinct from the common causes of mitochondrial disorders including MELAS. Here, none of the previously reported pathogenic

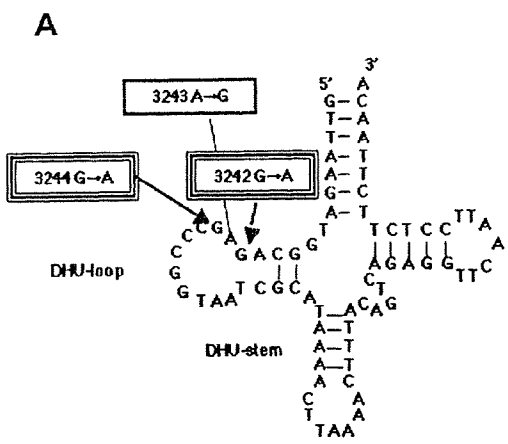


Fig. 3. (A) Secondary structure of the human mitochondrial $tRNA^{Leu(UUR)}$ gene and positions of the 3242G > A, 3243A > G, and 3244G > A mutations. (B) Comparison of mitochondrial $tRNA^{Leu(UUR)}$ among several species. 3242G > A and 3244G > A mutations are boxed.

"strengthening" of the stem by the formation of an additional A–U pairing instead of the well-conserved C–U pair (Helm et al., 2000) (Fig. 3A). Fourth, a somatic mutation of the 3242G > A was recently detected in CD34+ bone marrow cells, but not in peripheral blood cells (Gattermann et al., 2004). The authors supported the pathogenicity of this mutation, which they thought was associated with a maturation defect, and that the dysfunction of the mitochondria carrying the mutation contributed to ineffective hematopoiesis in their patient. Finally, we found that the cybrid cells carrying the 3242G > A mutation derived from patient 2, which excluded any influence of nuclear genes, revealed the functional defect of the mutation; the enzymatic activity of complex IV was apparently low in cybrid cells and the BN-PAGE analysis of the cybrid cells showed a reduced level of the complex I–III–IV supercomplex and an increase in the complex I–III supercomplex. Recently, an abnormal respiratory supercomplex was reported in a human disease (McKenzie et al., 2006). They proposed that unstable respiratory chain supercomplexes affect respiratory activities and subsequent pathology. In our case, the above findings suggest that the destabilization of the supercomplex due to the dissociation of complex IV is related to the activity of complex IV.

Point mutations at many of the 75 nucleotides in the $tRNA^{Leu(UUR)}$ gene have been associated with several distinct mitochondrial diseases with variable phenotypes ranging from pure myopathy to multisystemic disorders such as MELAS (Campos et al., 2001; Hadjigeorgiou et al., 1999; Goto et al., 1990; Sevidei, 2002; Moraes et al., 1992; Seneca et al., 2001; Jaksch et al., 2001). However, the reasons for the differences between the genotypes and phenotypes are not clear. The novel 3244G > A mutation emphasizes the crucial role of $tRNA^{Leu(UUR)}$ dysfunction in the pathogenesis of MELAS. Because its biochemical effects were similar to those of the 3243A > G mutation, this portion of the tRNA gene is likely to be very important for the maintenance of tRNA function. However, the effects of the 3242G > A base change in the DHU stem on the clinical phenotype, pathological findings, and biochemical functions are different from those of the 3243A > G mutation. Because both novel base changes are adjacent to the common 3243A > G mutation, these different effects may be a key to clarify the molecular pathogenesis of mitochondrial disorders including MELAS.

Regarding the pathogenesis of the $tRNA^{Leu(UUR)}$ gene mutations including the 3243A > G mutation, several groups have pointed out the possibility of an abnormality at the transcription level, because these mutations occur in a control region responsible for the termination of transcription at the end of rRNA genes (Hess et al., 1991; King et al., 1992). Other groups have demonstrated that this type of mutant tRNA may be functionally deficient (Yasukawa et al., 2000; Chomyn et al., 2000). One of the groups revealed a specific correlation between the modification deficiency in mutant tRNA and the clinical features of mitochondrial disorders (Kirino et al., 2005). They reported that mitochondrial $tRNA^{Leu(UUR)}$ harboring mutations, such as the 3244G > A transition, detected in tissues from patients with symptoms of MELAS lacked the normal taurine-containing modification at the anticodon wobble position. In contrast, mitochondrial $tRNA^{Leu(UUR)}$ with mutations, including the 3242G > A transition, detected in patients that have mitochondrial diseases but do not show the MELAS symptoms had the normal modifications. Further investigations of the different effects of the np 3244 and np 3242 base changes on the phenotypes and the similarities shared by the np 3243 and 3244 base changes may provide clues for elucidating the actual impact of $tRNA^{Leu(UUR)}$ gene mutations on the phenotypic expression of MELAS. The present results can contribute to a better understanding of tRNA function and provide insight into the complicated issues surrounding the association between genotype and phenotype in mitochondrial disorders.

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## References

Akanuma, J., Muraki, K., Komaki, H., Nonaka, I., Goto, Y., 2000. Two pathogenic point mutations exist in the authentic mitochondrial genome, not in the nuclear pseudogene. J. Hum. Genet. 45, 337–341.

Campos, Y., Gamez, J., Garcia, A., Andreu, A.L., Rubio, J.C., Martin, M.A., del Hoyo, P., Navarro, C., Cervera, C., Garesse, R., Arenas, J., 2001. A new mtDNA mutation in the $tRNA^{Leu(UUR)}$ gene associated with ocular myopathy. Neuromuscul. Disord. 1, 477–480.

Chinnery, P.F., Howell, N., Andrews, R.M., Turnbull, D.M., 1999. Mitochondria DNA analysis: polymorphisms and pathogenicity. J. Med. Genet. 36, 505–510.

Chomyn, A., Martinuzzi, A., Yoneda, M., Daga, A., Hurko, O., Johns, D., Lai, S.T., Nonaka, I., Angelini, C., Attardi, G., 1992. MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. Proc. Natl. Acad. Sci. USA 89, 4221–4225.

Chomyn, A., Enriquez, J.A., Micol, V., Fernandez-Silva, P., Attardi, G., 2000. The mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like

episode syndrome-associated human mitochondrial $tRNA^{Leu(UUR)}$ mutation causes aminoacylation deficiency and concomitant reduced association of mRNA with ribosomes. J. Biol. Chem. 275, 19198–19209.

D'Aurelio, M., Gajewski, C.D., Lenaz, G., Manfredi, G., 2006. Respiratory chain supercomplexes set the threshold for respiration defects in human mtDNA mutant cybrids. Hum. Mol. Genet. 15, 2157–2169.

Gattermann, N., Wulfert, M., Junge, B., Germing, U., Haas, R., Hofhaus, G., 2004. Ineffective hematopoiesis linked with a mitochondrial tRNA mutation (3242G > A) in a patient with myelodysplastic syndrome. Blood 103, 1499–1502.

Goto, Y., 1995. Clinical features of MELAS and mitochondrial DNA mutations. Muscle Nerve 3, S107–112.

Goto, Y., Nonaka, I., Horai, S., 1990. A mutation in the $tRNA^{Leu(UUR)}$ gene associated with the MELAS subgroup of mitochondrial encephalopathies. Nature 348, 651–653.

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

Goto, Y., Horai, S., Matsuoka, T., Koga, Y., Nihei, K., Kobayashi, M., Nonaka, I., 1992. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS): a correlative study of the clinical features and mitochondrial DNA mutation. Neurology 42, 545–550.

Goto, Y., Tsugane, K., Tanabe, Y., Nonaka, I., Horai, S., 1994. A new point mutation at nucleotide pair 3291 of the mitochondrial $tRNA^{Leu(UUR)}$ gene in a patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Biochem. Biophys. Res. Commun. 202, 1624–1630.

Habano, W., Sugai, T., Nakamura, S.I., Uesugi, N., Yoshida, T., Sasou, S., 2000. Microsatellite instability and mutation of mitochondrial and nuclear DNA in gastric carcinoma. Gastroenterology 118, 835–841.

Hadjigeorgiou, G.M., Kim, S.H., Fischbeck, K.H., Andreu, A.L., Berry, G.T., Bingham, P., Shanske, S., Bonilla, E., DiMauro, S., 1999. A new mitochondrial DNA mutation (A3288G) in the $tRNA^{Leu(UUR)}$ gene associated with familial myopathy. J. Neurol. Sci. 164, 153–157.

Hao, H., Moraes, C.T., 1996. Functional and molecular mitochondrial abnormalities associated with C->T transition at position 3256 of the human mitochondrial genome. J. Biol. Chem. 271, 2347–2352.

Hasegawa, H., Matsuoka, T., Goto, Y., Nonaka, I., 1991. Strongly succinate dehydrogenase-reactive blood vessels in muscles from patients with mitochondrial myopathy, encephalopathy lactic acidosis, and stroke-like episodes. Ann. Neurol. 29, 601–605.

Helm, M., Brule, H., Friede, D., Giege, R., Putz, D., Florentz, C., 2000. Search for characteristic structural features or mammalian mitochondrial tRNAs. RNA 6, 1356–1379.

Hess, J.F., Parisi, M.A., Bennett, J.L., Clayton, D.A., 1991. Impairment of mitochondrial transcription termination by a point mutation associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 351, 236–239.

Hirano, M., Pravlakis, S., 1994. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS): current concepts. J. Child Neurol. 9, 4–13.

Jaksch, M., Lochmuller, H., Schmitt, F., Volpel, B., Obermaier-Kusser, B., Horvath, R., 2001. A mutation in mt $tRNA^{Leu(UUR)}$ causing a neuropsychiatric syndrome with depression and cataract. Neurology 57, 1930–1931.

Kawarai, T., Kawakami, H., Kozuka, K., Izumi, Y., Matsuyama, Z., Watanabe, C., Kohriyama, T., Nakamura, S., 1997. A new mitochondrial DNA mutation associated with mitochondrial myopathy: $tRNA^{Leu(UUR)}$ 3254C-to-G. Neurology 49, 598–600.

King, M.P., Attardi, G., 1989. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science 246, 500–503.

King, M.P., Koga, Y., Davidson, M., Schon, E.A., 1992. Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the $tRNA^{Leu(UUR)}$ mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. Mol. Cell Biol. 12, 480–490.

Kirino, Y., Goto, Y., Campos, Y., Arenas, J., Suzuki, T., 2005. Specific correlation between the wobble modification deficiency in mutant tRNAs and the clinical features of a human mitochondrial disease. Proc. Natl. Acad. Sci. USA 102, 7127–7132.

Koga, Y., Davidson, M., Schon, E.A., King, M.P., 1995. Analysis of cybrids harboring MELAS mutations in the mitochondrial $tRNAs^{Leu(UUR)}$ gene. Muscle Nerve 3, S119–123.

Komaki, H., Akauma, J., Iwata, H., Takahashi, T., Mashima, Y., Nonaka, I., Goto, Y., 2003. A novel mtDNA C11777A mutation in Leigh syndrome. Mitochondrion 2, 293–304.

McFarland, R., Elson, J.L., Taylor, R.W., Howell, N., Turnbull, D.M., 2004. Assigning pathogenicity to mitochondrial tRNA mutations: when 'definitely maybe' is not good enough. Trends Genet. 20, 591–596.

McKenzie, M., Lazarou, M., Thorborn, D.R., Ryan, M.T., 2006. Mitochondrial respiratory chain supercomplexes are destabilized in Barth syndrome patients. J. Mol. Biol. 361, 462–469.

Moraes, C.T., Ricci, E., Bonilla, E., DiMauro, S., Schon, E.A., 1992. The mitochondrial $tRNA^{Leu(UUR)}$ mutation in mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS): genetic, biochemical, and morphological correlation in skeletal muscle. Am. J. Hum. Genet. 50, 934–949.

Morten, K.J., Cooper, J.M., Brown, G.K., Lake, B.D., Pike, D., Poulton, J., 1993. A new point mutation associated with mitochondrial encephalomyopathy. Hum. Mol. Genet. 2, 2081–2087.

Nijtmans, L.G., Henderson, N.S., Holt, I.J., 2002. Blue native electrophoresis to study mitochondrial and other protein complexes. Methods 26, 327–334.

Nishigaki, Y., Tadesse, S., Bonilla, E., Shungu, D., Hersh, S., Keats, B.J., Berlin, C.I., Goldverg, M.F., Vockley, J., DiMauro, S., Hirano, M., 2003. A novel mitochondrial $tRNA^{Leu(UUR)}$ mutation in a patient with features of MERRF and Kearns-Sayre syndrome. Neuromuscul. Disord. 13, 334–340.

Nishino, I., Komatsu, M., Kodama, S., Horai, S., Nonaka, I., Goto, Y., 1996. The 3260 mutation in mitochondrial DNA can cause mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Muscle Nerve 19, 1603–1604.

Robinson, B.H., 1996. Use of fibroblast and lymphoblast cultures for detection of respiratory chain defects. In: Attardi, G.M., Chomyin, A. (Eds.), Methods in Enzymology, No. 264. Academic Press USA, San Diego, CA, pp. 454–463.

Sakuta, R., Nonaka, I., 1989. Vascular involvement in mitochondrial myopathy. Ann. Neurol. 25, 594–601.

Seneca, S., Verhelst, H., De Meirleir, L., Meire, F., Ceuterick-De Goote, C., Lissens, W., Van Coster, R., 2001. A new mitochondrial point mutation in the transfer $RNA^{Leu(UUR)}$ gene in a patient with a clinical phenotype resembling Kearn-Sayre syndrome. Arch. Neurol. 58, 1113–1118.

Sevidei, S., 2002. Mitochondrial encephalomyopathies: gene mutation. Neuromuscul. Disord. 12, 524–529.

Suzuki, T., Suzuki, T., Wada, T., Saigo, K., Watanabe, K., 2002. Taurine as a constituent of mitochondrial tRNAs: new insights into the functions of taurine and human mitochondrial diseases. EMBO J. 21, 6581–6589.

Trounce, I.A., Kim, Y.L., Jun, A.S., Wallace, D.C., 1996. Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. In: Attardi, G.M., Chomyin, A. (Eds.), Methods in Enzymology, No. 264. Academic Press USA, San Diego, CA, pp. 484–509.

Yasukawa, T., Suzuki, T., Ueda, T., Ohta, S., Watanabe, K., 2000. Modification defect at anticodon wobble nucleotide of mitochondrial $tRNAs^{Leu(UUR)}$ with pathogenic mutations of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. J. Biol. Chem. 275, 4251–4257.

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Original article

# Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in perinatal asphyxia

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**Abstract**

Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) play important roles in the function of the blood–brain-barrier (BBB). We investigated the roles of MMP-9 and TIMP-1 in the pathogenesis of hypoxic–ischemic encephalopathy following perinatal asphyxia. Serum concentrations of MMP-9 and TIMP-1 were determined by ELISA in 12 neonates with perinatal asphyxia and 15 controls on the birth day and the next day. Serum MMP-9 concentrations in asphyxiated neonates with neurological sequelae ($n = 5$) were significantly higher than concentration in asphyxiated neonates without sequelae ($n = 7$) and controls on birth day ($p = 0.003$ and $p < 0.001$, respectively). The ratios of serum MMP-9/TIMP-1 on birth day in asphyxiated neonates with neurological sequelae were significantly higher than those in asphyxiated neonates without sequelae ($p = 0.048$). There were no significant differences in the serum MMP-9 concentrations or the ratios of MMP-9/TIMP-1 between asphyxiated neonates with and without neurological sequelae on the day after birth. Our preliminary study suggests that serum MMP-9 levels on birth day are important for predicting neurological prognosis of neonates with asphyxia.


*Keywords:* Asphyxia; Neonates; Matrix metalloproteinase-9; Tissue inhibitor of metalloproteinase-1

## 1. Introduction

Perinatal asphyxia remains a major cause of pediatric mortality and morbidity, with possible long-term neurologic sequelae, such as cerebral palsy, mental retardation, or epilepsy [1,2]. Hypoxic–ischemic encephalopathy (HIE) is a neurological syndrome that frequently accompanies perinatal asphyxia [3]. Evaluation of the severity of HIE at an early phase is important for tailoring medical treatment. It would be useful for clinicians to decide whether or not to perform potentially neuroprotective therapies, which include hypothermia, free radical scavengers, and magnesium sulfate [3], if the severity of HIE could be predicted within the first day of life in the neonatal intensive care unit (NICU) [4]. Multiple clinical parameters have been suggested, but many have not been available [5].

Matrix metalloproteinases (MMPs) constitute a family of enzymes that mediate the degradation of extracellular matrix proteins [6]. MMPs play important roles in normal and pathological processes including embryogenesis, wound healing, inflammation, arthritis, cardiovascular diseases, pulmonary diseases and cancer [7]. MMP-9, a member of this family which is capable of degrading collagen IV, is a major component of the basement membrane of the cerebral endothelium, and promotes the migration of cells through tissue or across the blood–brain-barrier (BBB) [8]. The activity of MMPs is further controlled by specific tissue inhibitors

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of metalloproteinases (TIMPs) [9]. TIMP-1 exhibits a high affinity for MMP-9 [10].

To elucidate the utility of MMP-9 and TIMP-1 as early predicting parameters of the prognosis of neonates with HIE, we determined the serum concentrations of MMP-9 and TIMP-1 on birth day and the next day in neonates with perinatal asphyxia.

## 2. Patients and methods

Informed consent was obtained from the parents of the neonates. The protocol was approved by the Institutional Review Board of Yamaguchi University Hospital (H19-83).

### 2.1. Perinatal asphyxia

Twelve neonates (4 boys and 8 girls; mean gestational age, 38.3 weeks; mean birth weight, 2851 g) with perinatal asphyxia (Apgar score at 1 min: lower than 6 points) admitted to our NICU between February 2003 and December 2007 were enrolled in this study (Tables 1 and 2). We divided neonates with the disease into two groups, those who had cerebral palsy as neurological sequelae (Group 1, $n = 5$), and those who survived (Group 2, $n = 7$) (Table 2). Cerebral palsy was diagnosed by pediatric neurologists at patient ages of 4–12 months. The follow-up periods for the patients were 9 months to 5 years (mean, 1.6 years). Three patients (Patients 1–3) had severe spastic tetraplegia with mechanical ventilation and could not control their necks or roll over, and two patients (Patients 4 and 5) had spastic diplegia and could control their necks, roll over, and sit alone.

Table 1
Clinical data of asphyxiated neonates and controls

| | Asphyxiated neonates $N = 12$ | Controls $N = 13$ |
|---|---|---|
| Sex (female:male) | 4:8 | 7:6 |
| Gestational weeks (mean, range) | 38.3, 34W5D–40W4D | 37.6, 35W2D–40W2D |
| Birth weight (g, mean, range) | 2851, 1632–4268 | 2797, 2006–3714 |
| Comorbid conditions | | LBWI: 5<br>TTN: 2<br>Caesarean-section: 2<br>Melena: 1<br>ACC: 1<br>Maternal Graves' disease: 1<br>Back-knee: 1 |

LBWI, low-birth weight infant; TTN, transient tachypnea of the newborn; ACC, agenesis of corpus callosum.

### 2.2. Controls

Thirteen newborns (6 boys and 7 girls; mean gestational age, 37.6 weeks; mean birth weight, 2797 g) without perinatal asphyxia (Apgar score at 1 min: higher than 7 points) admitted to our NICU were enrolled in this study (Table 1). The comorbid conditions of the controls included low-birth weight ($n = 5$), transient tachypnea of the newborn ($n = 2$), Caesarean-section ($n = 2$), melena ($n = 1$), agenesis of corpus callosum ($n = 1$), maternal Graves' disease ($n = 1$) and back-knee ($n = 1$). None had neurologic sequelae.

### 2.3. Serum sampling and determination of MMP-9 and TIMP-1 concentrations

All serum samples were collected during routine blood examinations which were performed as part of the normal medical care on birth day and the next day. The specimens were stored frozen at −80 °C until assay.

Table 2
Clinical characteristics of asphyxiated neonates

| Patient No./sex | Gestational age | Birth weight (g) | Apgar score at 1 min/5 min | Outcome |
|---|---|---|---|---|
| *Group 1, patients with neurological sequelae* | | | | |
| 1/Female | 39W3D | 3046 | Unknown | Severe spastic tetraplegia |
| 2/Male | 40W0D | 2700 | 1/1 | Severe spastic tetraplegia |
| 3/Female | 40W4D | 4268 | 1/6 | Severe spastic tetraplegia |
| 4/Female | 39W6D | 3296 | 3/5 | Spastic diplegia |
| 5/Female | 34W5D | 1632 | 1/3 | Spastic diplegia |
| *Group 2, patients without neurological sequelae* | | | | |
| 6/Male | 35W1D | 2230 | 1/8 | Normal |
| 7/Female | 38W5D | 3180 | 3/6 | Normal |
| 8/Female | 37W6D | 2642 | 6/10 | Normal |
| 9/Female | 38W4D | 2262 | 2/4 | Normal |
| 10/Female | 39W1D | 3256 | 3/8 | Normal |
| 11/Male | 40W2D | 3090 | 3/6 | Normal |
| 12/Female | 35W6D | 2608 | 1/4 | Normal |

The serum concentrations of MMP-9 and TIMP-1 were determined with sandwich-type ELISA kits (Amersham, Buckinghamshire, UK). A monoclonal coating antibody was adsorbed onto polystyrene microwells to bind to MMP-9 or TIMP-1 in the samples or the standard. A horse radish peroxidase-conjugated monoclonal antibody with neutralizing activity toward MMP-9 or TIMP-1 was added to bind to MMP-9 or TIMP-1 captured by the first antibody. A substrate solution, reactive with horse radish peroxidase, was then added to the wells to produce a color reaction in proportion to the amount of MMP-9 or TIMP-1, and the absorbance was measured. The detection limits were 2.5 ng/ml for MMP-9 and 2.4 ng/ml for TIMP-1. The assay for MMP-9 recognizes the pro- and active forms of MMP-9.

### 2.4. Statistical analysis

The differences between groups were analyzed using the Mann–Whitney $U$ test. The differences in the results between birth and the next day were analyzed by the Wilcoxon matched paired test. $p$-Values less than 0.05 were considered significant. Analyses and calculations were performed using SPSS-12.0 (SPSS, Inc., Chicago, IL, USA).

## 3. Results

There were no significant differences in serum MMP-9 or TIMP-1 concentrations, or the ratio of MMP-9/TIMP-1, on birth day between asphyxiated neonates and controls (data not shown). Serum MMP-9 concentrations in asphyxiated neonates with neurological sequelae ($n = 5$) on birth day were significantly higher than those in asphyxiated neonates without sequelae ($n = 7$) and controls ($p = 0.003$ and $p < 0.001$, respectively), as shown in Fig. 1. The serum MMP-9 concentrations of three patients with severe spastic tetraplegia (Patients 1–3) were 222.7, 365.3, and 151.5 ng/ml, respectively, and those of two patients with spastic diplegia (Patients 4 and 5) were 272.9 and 203.0 ng/ml, respectively. Serum TIMP-1 concentrations in asphyxiated neonates with neurological sequelae on birth day were significantly higher than those in controls ($p = 0.026$). The ratios of serum MMP-9/TIMP-1 in asphyxiated neonates with neurological sequelae on birth day were significantly higher than those in asphyxiated neonates without sequelae ($p = 0.048$).

There were no significant differences in serum MMP-9 concentrations on the next day among asphyxiated neonates with neurological sequelae, those without neurological sequelae, and controls (Fig. 2). Serum TIMP-1 concentration in asphyxiated neonates with and without neurological sequelae on the next day were significantly higher than those in controls ($p = 0.001$ and $p = 0.006$, respectively). Serum MMP-9/TIMP-1 ratios in asphyxiated neonates with neurological sequelae on the next day were significantly lower than those in controls ($p = 0.046$).

Serum TIMP-1 concentrations on the next day were significantly higher than those on birth day in asphyxiated neonates ($p = 0.021$), as shown in Fig. 3. There were no significant differences in serum MMP-9 concentrations and MMP-9/TIMP-1 ratios between birth and the next day in asphyxiated neonates. There were no significant differences in serum MMP-9 or TIMP-1 concentrations or MMP-9/TIMP-1 ratios between birth and the next day in controls.

## 4. Discussion

To determine predictive parameters, several biochemical markers such as proinflammatory cytokines, chemo-

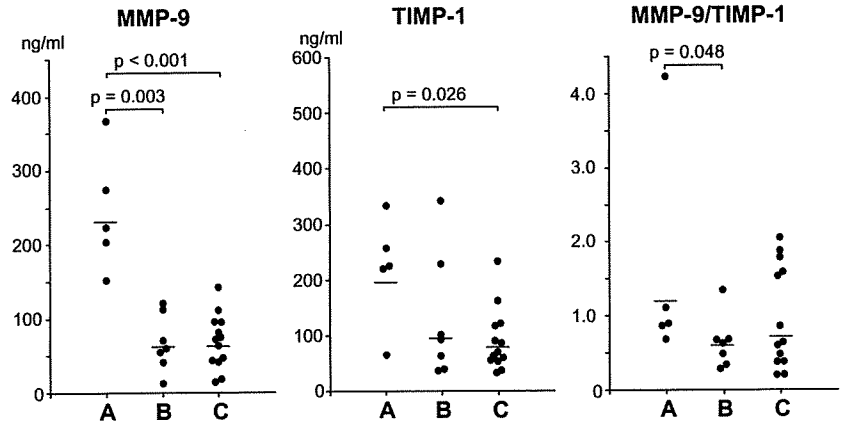


Fig. 1. Serum concentrations of MMP-9 and TIMP-1 and the ratios of MMP-9/TIMP-1 in asphyxiated neonates with/without neurological sequelae and controls on birth day. (A) Asphyxiated neonates with neurological sequelae ($n = 5$); (B) asphyxiated neonates without neurological sequelae ($n = 7$); (C) controls ($n = 13$). Horizontal lines indicate geometric mean values.

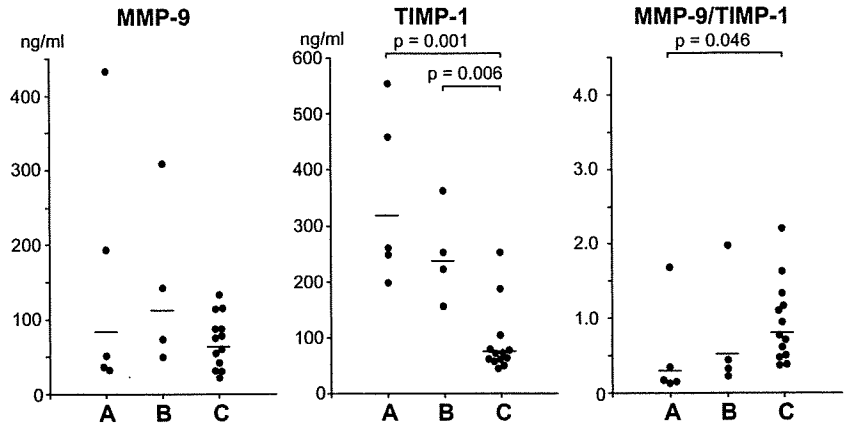


Fig. 2. Serum concentrations of MMP-9 and TIMP-1 and the ratios of MMP-9/TIMP-1 in asphyxiated neonates with/without neurological sequelae and controls on the day after birth. (A) Asphyxiated neonates with neurological sequelae ($n = 5$); (B) asphyxiated neonates without neurological sequelae ($n = 4$); (C) controls ($n = 13$). Horizontal lines indicate geometric mean values.

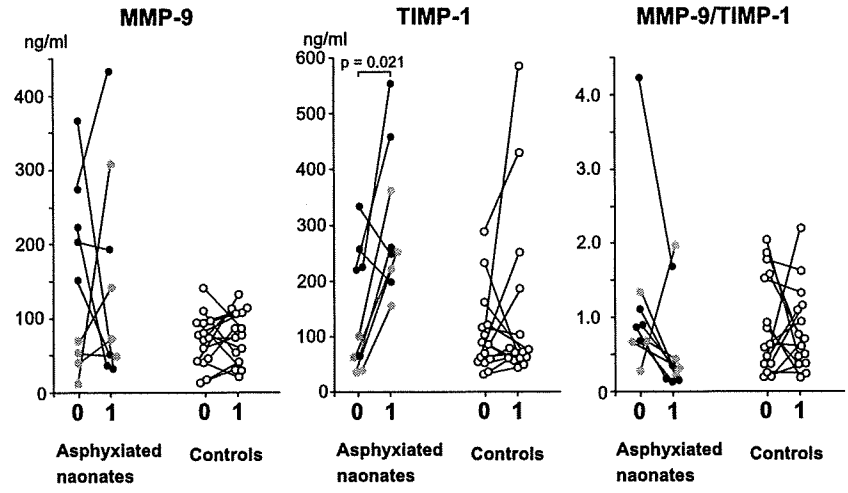


Fig. 3. The relationship between serum concentrations of MMP-9 and TIMP-1 and the ratios of MMP-9/TIMP-1 at birth day and the next day in asphyxiated neonates and controls. "0" indicates birth day and "1" indicates the day after birth. Black circles in asphyxiated neonates are those with neurological sequelae and gray are those without neurological sequelae. Lines indicate samples from the same infants.

kines, neuron-specific enolase, and uric acid in the blood or cerebrospinal fluid have been investigated in asphyxiated neonates [4,11–15]. Moreover, there have been reports on magnetic resonance imaging in asphyxiated neonates [2,16,17]. We measured serum MMP-9 and TIMP-1 concentrations in asphyxiated neonates on birth day and the next day to determine whether or not this could predict a neurological prognosis at an early time. Previous studies suggested that MMP-9 induced brain edema and injury after ischemia due to BBB leakage [18–20]. Inhibition of MMP-9 and knock-out of the MMP-9 gene reduced brain injury after ischemia [18,20]. It has been reported that TIMP-1 protects the function of BBB by inhibiting MMP-9 activity which reduces brain damage after ischemia [21–24]. These reports suggest that the balance of MMP-9 and TIMP-1 is important for BBB function.

We have previously investigated serum MMP-9 and TIMP-1 levels in neurologic diseases. In patients with acute disseminated encephalomyelitis with gadolinium-enhanced lesions observed via brain MRI, serum MMP-9 levels and the MMP-9/TIMP-1 ratio in the acute stage were higher than those in the convalescent stage, suggesting that MMP-9 and TIMP-1 were related to the activity of the lesions [25]. In addition, serum MMP-9 levels and MMP-9/TIMP-1 ratios in patients with subacute sclerosing panencephalitis at Jabbour stage III were significantly higher than in those at Jabbour stage II, suggesting that MMP-9 and MMP-9/TIMP-1 ratios were related to the stage of the disease [26]. Furthermore, we have reported that serum MMP-9 and TIMP-1 levels and MMP-9/TIMP-1 ratios were related to unfavorable prognosis in influenza-associated encephalopathy, acute encephalopathy following pro-

longed febrile seizures, and hemolytic uremic syndrome with encephalopathy [27–29]. These data indicate that MMP-9 and TIMP-1 are related to the severity and outcome of the neurological diseases. In the present study, our results showed that serum levels of MMP-9 on birth day were high in asphyxiated neonates with neurological sequelae. We suggest that high levels of serum MMP-9 on birth day could injure the BBB and result in irreversible brain damage. Recent studies reported that MRI findings could be markers of outcome in asphyxiated neonates [2,16,17]. To obtain MR imaging of asphyxiated neonates, they should be transported to the MR scanner from NICU. It may be risky for them. Several previous studies demonstrated that proinflammatory cytokines were good markers of prognosis in asphyxiated neonates, and the CSF levels were better indexes than the serum or plasma ones in most of these biochemical markers [4,15,16]. To obtain CSF by lumbar puncture imposes on asphyxiated neonates, compared with obtaining peripheral blood. Therefore, we believe that serum MMP-9 levels are superior to previous reported ones.

Immunohistopathological studies revealed that activated microglia, infiltrated neutrophils and vascular endothelial cells produced MMP-9 in the ischemic brain [30,31]. We suggest that MMP-9 produced in the brain enters the blood. Another hypothesis is that MMP-9 is produced by peripheral blood leukocytes and vascular endothelial cells. We have reported that peripheral blood monocytes/macrophages were activated in asphyxiated neonates [32]. In addition, we revealed that serum TIMP-1 levels on the day after birth were significantly higher than those on birth day in asphyxiated neonates, suggesting that TIMP-1 production on the day after birth corresponded to MMP-9 production on birth day. As a result, their serum MMP-9 levels seemed to be quickly decreased on the next day after birth. It is likely that high serum TIMP-1 levels in asphyxiated neonates with neurological sequelae on the next day after birth responded to a massive MMP-9 release in the serum. The serum TIMP-1 levels on the next day were increased in 10 of 12 asphyxiated neonates but were decreased in two patients with neurological sequelae (Patients 1 and 2), compared with birth day. One hypothesis is that the two patients could not produce sufficient TIMP-1, even though MMP-9 was abundant in their blood. Alternatively, it may be that TIMP-1 was consumed quickly in the two patients, with a consequent drop in the serum TIMP-1 level. Our results further suggest that measurement of serum MMP-9 and TIMP-1 levels should be performed on birth day, but not the next day. A further large-scale study is necessary to clarify this point.

Our preliminary study suggests that serum MMP-9 levels on birth day are important for predicting neurological prognosis of asphyxiated neonates. We believe that knowledge of serum MMP-9 levels on birth day, but not the next day, will be useful for clinicians to predict neurological prognosis.

## References

[1] Dilenge ME, Majnemer A, Shevell MI. Long-term developmental outcome of asphyxiated term neonates. J Child Neurol 2001;16:781–92.
[2] Boichot C, Walker PM, Durand C, Grimaldi M, Chapuis S, Gouyon JB, et al. Term neonate prognoses after perinatal asphyxia: contributions of MR imaging, MR spectroscopy, relaxation times, and apparent diffusion coefficients. Radiology 2006;239:839–48.
[3] Volpe JJ. Hypoxic–ischemic encephalopathy: clinical aspects. In: Volpe JJ, editor. Neurology of the Newborn. Philadelphia: WB Saunders; 2001. p. 331–94.
[4] Tekgul H, Yalaz M, Kutukculer N, Ozbek S, Kose T, Akisu M, et al. Value of biochemical markers for outcome in term infants with asphyxia. Pediatr Neurol 2004;31:326–32.
[5] Patel J, Edwards AD. Prediction of outcome after perinatal asphyxia. Curr Opin Pediatr 1997;9:128–32.
[6] Chandler S, Miller KM, Clements JM, Lury J, Corkill D, Anthony DC, et al. Matrix metalloproteinases, tumor necrosis factor and multiple sclerosis: an overview. J Neuroimmunol 1997;72:155–61.
[7] Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 2003;253:269–85.
[8] Lukes A, Mun-Bryce S, Lukes M, Rosenberg GA. Extracellular matrix degradation by metalloproteinases and central nervous system diseases. Mol Neurobiol 1999;19:267–84.
[9] Murphy G, Knäuper V. Relating matrix metalloproteinase structure to function: why the "hemopexin" domain? Matrix Biol 1997;15:511–8.
[10] Lacraz S, Nicod LP, Chicheportiche R, Welgus HG, Dayer JM. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J Clin Invest 1995;96:2304–10.
[11] Akisü M, Kültürsay N. Value of the urinary uric acid to creatinine ratio in term infants with perinatal asphyxia. Acta Pediatr Jpn 1998;40:78–81.
[12] Yoon BH, Park CW, Chaiworapongsa T. Intrauterine infection and the development of cerebral palsy. BJOG 2003;110:124–7.
[13] Fotopoulos S, Mouchtouri A, Xanthou G, Lipsou N, Petrakou E, Xanthou M. Inflammatory chemokine expression in the peripheral blood of neonates with perinatal asphyxia and perinatal or nosocomial infections. Acta Pediatr 2005;94:800–6.
[14] Aly H, Khashaba MT, El-Ayouty M, El-Sayed O, Hasanein BM. IL-1β, IL-6 and TNF-α and outcomes of neonatal hypoxic–ischemic encephalopathy. Brain Dev 2006;28:178–82.
[15] Hussein MH, Daoud GA, Kakita H, Hattori A, Murai H, Yasuda M, et al. The sex differences of cerebrospinal fluid levels of interleukin 8 and antioxidants in asphyxiated newborns. Shock 2007;28:154–9.
[16] Kaufman SA, Miller SP, Ferriero DM, Glidden DH, Barkovich AJ, Partridge JC. Encephalopathy as a predictor of magnetic resonance imaging abnormalities in asphyxiated newborns. Pediatr Neurol 2003;28:342–6.
[17] Hunt RW, Nei JJ, Coleman LT, Kean MJ, Inder TE. Apparent diffusion coefficient in the posterior limb of the internal capsule predicts outcome after perinatal asphyxia. Pediatrics 2004;114:999–1003.
[18] Romanic AM, White RF, Arleth AJ, Ohlstein EH, Barone FC. Matrix metalloproteinase expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infarct size. Stroke 1998;29:1020–30.

[19] Gasche Y, Fujimura M, Morita-Fujimura Y, Copin JC, Kawase M, Massengale J, et al. Early appearance of activated matrix metalloproteinase-9 after focal cerebral ischemia in mice: a possible role in blood–brain barrier dysfunction. J Cereb Blood Flow Metab 1999;19:1020–8.
[20] Svedin P, Hagberg H, Sävman K, Zhu C, Mallard C. Matrix metalloproteinase-9 gene knock-out protects the immature brain after cerebral hypoxia–ischemia. J Neurosci 2007;27:1511–8.
[21] Krizanac-Bengez L, Hossain M, Fazio V, Mayberg M, Janigro D. Loss of flow induces leukocyte-mediated MMP/TIMP imbalance in dynamic in vitro blood–brain barrier model: role of pro-inflammatory cytokines. Am J Physiol Cell Physiol 2006;291:C740–9.
[22] Sellner J, Leib SL. In bacterial meningitis cortical brain damage is associated with changes in parenchymal MMP-9/TIMP-1 ratio and increased collagen type IV degradation. Neurobiol Dis 2006;21:647–56.
[23] Yamamoto S, Nguyen JH. TIMP-1/MMP-9 imbalance in brain edema in rats with fulminant hepatic failure. J Surg Res 2006;134:307–14.
[24] Förster C, Kahles T, Kietz S, Drenckhahn D. Dexamethasone induces the expression of metalloproteinase inhibitor TIMP-1 in the murine cerebral vascular endothelial cell line cEND. J Physiol 2007;580:937–49.
[25] Ichiyama T, Kajimoto M, Suenaga N, Maeba S, Matsubara T, Furukawa S. Serum levels of matrix metalloproteinase-9 and its tissue inhibitor (TIMP-1) in acute disseminated encephalomyelitis. J Neuroimmunol 2006;172:182–6.
[26] Ichiyama T, Siba P, Suarkia D, Takasu T, Miki K, Kira R, et al. Serum levels of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases 1 in subacute sclerosing panencephalitis. J Neurol Sci 2007;252:45–8.
[27] Ichiyama T, Morishima T, Kajimoto M, Matsushige T, Matsubara T, Furukawa S. Serum levels of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases 1 in influenza-associated encephalopathy. Pediatr Infect Dis J 2007;26:542–4.
[28] Suenaga N, Ichiyama T, Kubota M, Isumi H, Tohyama J, Furukawa S. Roles of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases 1 in acute encephalopathy following prolonged febrile seizures. J Neurol Sci 2008;266:126–30.
[29] Shiraishi M, Ichiyama T, Matsushige T, Iwaki T, Iyoda K, Fukuda K, et al. Soluble tumor necrosis factor receptor 1 and tissue inhibitors of metalloproteinases-1 in hemolytic uremic syndrome with encephalopathy. J Neuroimmunol 2008;196:147–52.
[30] Kolev K, Skopál J, Simon L, Csonka E, Machovich R, Nagy Z. Matrix metalloproteinase-9 expression in post-hypoxic human brain capillary endothelial cells: $H_2O_2$ as a trigger and NF-κB as a signal transducer. Thromb Haemost 2003;90:528–37.
[31] Rosell A, Ortega-Aznar A, Alvarez-Sabín J, Fernández-Cadenas I, Ribó M, Molina CA, et al. Increased brain expression of matrix metalloproteinase-9 after ischemic and hemorrhagic human stroke. Stroke 2006;37:1399–406.
[32] Hasegawa K, Ichiyama T, Isumi H, Nakata M, Sase M, Furukawa S. NF-κB activation in peripheral blood mononuclear cells in neonatal asphyxia. Clin Exp Immunol 2003;132:261–4.

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Original Article

# Clinical characteristics of respiratory syncytial virus infection-associated acute otitis media

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**Abstract** ***Background***: It is known that children with respiratory syncytial virus (RSV) infection frequently have complications of acute otitis media (AOM).
***Methods***: The hospital records of 148 inpatients aged 6–35 months who had RSV infection between January 2004 and December 2007, were retrospectively investigated.
***Results***: Forty-six out of 148 children (31%) had AOM. There was a significantly greater number of children with fever who had AOM ($P = 0.005$). The percentage of children with β-lactamase-non-producing ampicillin-resistant (BLNAR) *Haemophilus influenzae* in nasopharyngeal culture who had AOM showed a tendency to be greater than that of those who did not have AOM, but this was not statistically significant ($P = 0.068$). Moreover, BLNAR *H. influenzae* was positive in middle ear fluid specimens from four of five children with AOM who underwent tympanocentesis. There were no significant differences in the incidence of lower airway infection, leukocytes counts, or serum C-reactive protein levels between children with and without AOM.
***Conclusions***: Children who had RSV infection with AOM had a higher incidence of fever than those without AOM.

**Key words** acute otitis media, β-lactamase-non-producing ampicillin-resistant, *Haemophilus influenzae*, respiratory syncytial virus.

Respiratory syncytial virus (RSV) is the most important respiratory pathogen of infancy and early childhood.[1] Epidemics tend to recur annually at regular, predictable intervals. In temperate climates, RSV activity usually peaks in the winter and extends into the spring. This virus produces its most severe disease in the first few weeks or months of life. An infant's first encounter with RSV is almost always apparent, but the symptoms may range from a mild cold to severe bronchiolitis or pneumonia. In a welfare nursery of 90 infants with and without previous infection, an outbreak of RSV resulted in pneumonia in 40% and febrile upper respiratory tract illness in 53%.[1] In addition, acute otitis media (AOM) is frequently associated with RSV infections in young children.[1–6] RSV has been detected in middle ear aspirates, alone or, more commonly, simultaneously with a bacterial pathogen, suggesting that RSV may play both a primary and a secondary role in the pathogenesis of AOM.[1]

Usually, because of a preceding viral upper respiratory tract infection, the mucosa in the area becomes hyperemic and swollen, resulting in obstruction and providing an opportunity for bacterial multiplication in the middle ear. The most common bacterial pathogens in AOM of childhood are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*.[7] Of these pathogens, β-lactamase-resistant strains of *H. influenzae* isolates are increasing in frequency.[8] The peak incidence of AOM occurs in children between 6 and 24 months.[9] By the age of 3 years, 83% of children have experienced at least one episode of AOM.[9]

There have been few reports of bacteriological studies in Japanese children with RSV infection who also had AOM. Therefore, we designed a retrospective case–control study to investigate the clinical profile of 148 children with RSV infection aged 6–35 months during the past 4 years, particularly regarding the clinical characteristics of AOM.

## Methods

### Patients with RSV infection

One hundred and forty-eight children aged 6–35 months who were admitted to Yamaguchi University Hospital between January 2004 and December 2007 were enrolled in the present study (Table 1). The diagnosis of RSV infection was based on virus antigen detection in nasopharyngeal swab specimens using the immunochromatography test (SA Scientific, San Antonio, TX, USA).[10] All inpatients underwent otoscopy by a pediatrician, and definite diagnosis was then made by an otolaryngologist. The diagnosis of AOM was based on signs of inflammation (red or yellow color or bulging) of the tympanic membrane, and the presence of middle ear fluid.[6,7]

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**Table 1** Clinical data of patients with RSV infection (mean ± SD)

| | Patients with AOM ($n = 46$) | Patients without AOM ($n = 102$) |
|---|---|---|
| Age (months) | 15.8 ± 7.5 | 14.1 ± 7.7 |
| Male : Female | 27:19 | 51:51 |
| Day | 4.8 ± 2.9 | 3.9 ± 1.7 |
| Bodyweight (kg) | 9.7 ± 2.9 | 9.2 ± 2.2 |
| Gestational age (weeks) | 38.3 ± 3.0 | 37.6 ± 3.5 |
| Birthweight (g) | 2886 ± 702 | 2763 ± 673 |
| Comorbid conditions | Trisomy 21 ($n = 1$) | Trisomy 21 ($n = 2$)<br>VSD ($n = 2$)<br>CLD ($n = 1$)<br>Bronchomalacia ($n = 1$)<br>Congenital intestinal atresia ($n = 1$) |

AOM, acute otitis media; CLD, chronic lung disease; RSV, respiratory syncytial virus; VSD, ventricular septal defects.

To evaluate the clinical characteristics of children with RSV infection regardless of the presence of AOM, clinical data including the differential white blood cell (WBC) counts, platelet counts, hemoglobin, serum C-reactive protein (CRP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) levels, body temperature at admission, existence of lower airway infection, and nasopharyngeal culture, were investigated retrospectively from the hospital records. The onset day of fever and/or respiratory symptoms, such as cough and rhinorrhea, was considered as the first day of illness. Fever was defined as temperature ≥37.5°C.[2] A child was defined as having lower airway infection when auscultation by pediatrician detected moist or dry rale, and/or chest X-ray indicated bronchiolitis or pneumonia.

Five children with RSV infection who had AOM underwent tympanocentesis on admission or on the next day after admission. All children with AOM were treated with antibiotics, which were effective for bacteria in nasopharyngeal and/or middle ear fluid specimens.

### *Statistical analysis*

All data were log transformed to obtain an approximately normal distribution. The differences in the results between groups were analyzed on *t*-test and $\chi^2$ test, and those with $P < 0.05$ were considered significant. Analyses and calculations were performed using SPSS 12.0 (SPSS, Chicago, IL, USA).

## Results

Table 1 presents the clinical data of children with RSV infection in the presence or absence of AOM. Forty-six (31.1%) of 148 patients with RSV infection had AOM. There were no significant differences in the age, sex, day of admission, bodyweight on admission, gestational age or birthweight between patients with and without AOM. The comorbid conditions of the patients with AOM included trisomy 21 ($n = 1$), and those without AOM included trisomy 21 ($n = 2$), ventricular septal defects ($n = 2$), chronic lung disease ($n = 1$), bronchomalacia ($n = 1$), and congenital intestinal atresia ($n = 1$). With regard to children with RSV infection who had definite prior RSV infection, three had AOM and three did not. Nine of 46 children (19.6%) with AOM had low birthweight, whereas low birthweight was found in 15 of 102 children (14.7%) without AOM, and there was no significant difference between these groups. Children who had fever on and/or before admission comprised 96% of those with RSV infection and AOM, which was significantly higher than in the non-AOM group (odds ratio [OR], 2.26; 95% confidence interval [CI]: 1.53–30.01; $P = 0.005$; Table 2). There were no significant differences in the incidence of lower airway infection and laboratory findings, including WBC, platelet counts, hemoglobin, serum CRP, ALT, AST or LDH levels between patients with and without AOM.

Nasopharyngeal culture for bacteria was performed in 105 of 148 children with RSV infection. Table 3 presents the data on nasopharyngeal cultures in children with RSV infection in the presence or absence of AOM. The percentage of children with β-lactamase-non-producing ampicillin-resistant (BLNAR) *H. influenzae* in nasopharyngeal culture among those with AOM had a tendency to be higher than that of those without AOM, but not significant (OR, 2.26; 95%CI: 1.00–5.09; $P = 0.068$). Moreover, BLNAR *H. influenzae* was positive in middle ear fluid specimens from four of five children with AOM who underwent

**Table 2** Clinical and laboratory findings of children with RSV infection (mean ± SD)

| | Patients with AOM ($n = 46$) | Patients without AOM ($n = 102$) |
|---|---|---|
| Fever, *n* (%) | 44 (96)* | 78 (76) |
| Presence of lower airway infection, *n* (%) | 37 (80) | 84 (82) |
| WBC (/μL) | 13 032 ± 5675 | 12 257 ± 5150 |
| Platelet (×104/μL) | 35.3 ± 1.9 | 33.6 ± 11.5 |
| Hemoglobin (g/dL) | 11.6 ± 0.9 | 11.9 ± 1.1 |
| CRP (mg/dL) | 2.99 ± 3.69 | 2.11 ± 2.66 |
| ALT (U/L) | 20 ± 17 | 23 ± 25 |
| AST (U/L) | 40 ± 17 | 42 ± 20 |
| LDH (U/L) | 332 ± 67 | 346 ± 90 |

*$P = 0.005$ vs patients without AOM.

ALT, alanine aminotransferase; AOM, acute otitis media; AST, aspartate aminotransferase; CRP, C-reactive protein; LDH, lactate dehydrogenase; RSV, respiratory syncytial virus; WBC, white blood cells.

**Table 3** Nasopharyngeal culture of children with RSV infection

| | Patients with AOM ($n = 39$) $n$ (%) | Patients without AOM ($n = 66$) $n$ (%) |
|---|---|---|
| *Haemophilus influenzae* | 22 (56) | 30 (45) |
| BLNAS | 2 (5) | 9 (14) |
| BLNAR | 20 (52) | 21 (32) |
| *Streptococcus pneumoniae* | 23 (59) | 43 (65) |
| PSSP | 9 (23) | 12 (18) |
| PISP | 5 (13) | 18 (27) |
| PRSP | 9 (23) | 13 (20) |
| *Moraxella catarrhalis* | 14 (36) | 26 (39) |
| *Staphylococcus aureus* | 0 (0) | 3 (5) |

AOM, acute otitis media; BLNAR, β-lactamase-non-producing ampicillin-resistant; BLNAS, β-lactamase-non-producing ampicillin-sensitive; PISP, penicillin-intermediate *Streptococcus pneumoniae*; PRSP, penicillin-resistant *Streptococcus pneumoniae*; PSSP, penicillin-sensitive *Streptococcus pneumoniae*; RSV, respiratory syncytial virus.

tympanocentesis. There were no significant differences for other bacteria strains between the patients with and without AOM.

## Discussion

We investigated the clinical characteristics of children with RSV infection between 6 and 35 months of age who frequently had AOM. Specifically, patients with AOM were compared with those without AOM. In the present study the diagnosis of AOM was made at screening using otoscopy, and the patients were finally diagnosed as having AOM by otolaryngology specialists. Regarding the diagnosis of AOM using otoscopy by a pediatrician, pneumatic otoscopy and/or tympanometry might aid in the diagnosis of AOM. The clinical symptoms suggesting AOM are variable, such as fever, irritability, earache, ear tugging and so on.[7,9] We demonstrated that 96% of children with RSV infection and AOM had fever on and/or before admission, while 76% of children with RSV infection who did not have AOM exhibited fever. A previous study found that patients with RSV infection exhibited fever more often than those with rhinovirus infection or virus-negative children with AOM,[11] suggesting that fever is an important symptom in AOM patients with RSV infection. The present results support these previous findings. There were no significant differences, however, in the incidence of lower airway infection and upper airway infection alone between patients with and without AOM. A previous study reported that an abnormal WBC count <5000/μL or 15 000–30 000/μL was not associated with a serious concurrent bacterial infection in infants and young children with RSV infection.[12] In the present study there were no significant differences in laboratory findings between patients with and without AOM. Consistent with previous studies, the present results suggest that it is difficult to evaluate whether or not infants and young children with RSV infection have AOM based upon laboratory findings.

Nasopharyngeal culture from children with RSV infection and AOM detected *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in the present study. These results were consistent with those of previous reports.[7,13] Of these pathogens, the prevalence of antibiotic-resistant strains in isolates has recently increased.[8,14–17] We could not find, however, any reports about definite antibiotic-resistant strains in children with RSV infection and AOM. The present study has newly demonstrated that the percentage of BLNAR *H. influenzae* in nasopharyngeal culture in children with RSV infection who had AOM had a tendency to be higher than that in those who did not have AOM, but not significant. We did not investigate the incidence in children with other respiratory infections who had BLNAR *H. influenzae* in the community. It is necessary to investigate the aforementioned problem and the incidence of AOM patients with RSV infection and BLNAR *H. influenzae* among other communities. Moreover, BLNAR *H. influenzae* was positive in middle ear fluid specimens of four of five patients with AOM who underwent tympanocentesis. The tympanocentesis was performed for serious patients to alleviate the aural symptoms, but who did not have definite criteria indicating tympanocentesis due to the retrospective nature of the study. The BLNAR strain is resistant to oral antibiotics, compared with the BLNA-sensitive (BLNAS) strain.[18,19] One possibility is that the incidence of the positive BLNAR strain in children with AOM is higher than in those without AOM and RSV-infected children with AOM is higher than those without AOM.

In summary, we found that infants and young children with RSV infection and concurrent AOM had a high incidence of fever. We emphasize that children with RSV infection who have fever should be examined closely for AOM and that laboratory findings are not useful in distinguishing those with AOM from those without AOM.

## References


1 Hall CB. Respiratory syncytial virus. In: Feigin RD, Cherry JD (eds). *Textbook of Pediatric Infectious Diseases*, 4th edn. WB Saunders, Philadelphia, 1998; 2084–111.
2 Monobe H, Ishibashi T, Nomura Y, Shinogami M, Yano J. Role of respiratory viruses in children with acute otitis media. *Int. J. Pediatr. Otorhinolaryngol.* 2003; **67**: 801–6.
3 Kafetzis DA, Astra H, Tsolia M, Liapi G, Mathioudakis J, Kallergi K. Otitis and respiratory distress episodes following a respiratory syncytial virus infection. *Clin. Microbiol. Infect.* 2003; **9**: 1006–10.
4 Nokso-Koivisto J, Räty R, Blomqvist S *et al.* Presence of specific viruses in the middle ear fluids and respiratory secretions of young children with acute otitis media. *J. Med. Virol.* 2004; **72**: 241–8.
5 Sagai S, Suetake M, Yano H *et al.* Relationship between respiratory syncytial virus infection and acute otitis media in children. *Auris Nasus Larynx* 2004; **31**: 341–5.
6 Patel JA, Nguyen DT, Revai K, Chonmaitree T. Role of respiratory syncytial virus in acute otitis media: Implications for vaccine development. *Vaccine* 2007; **25**: 1683–9.
7 Kerschner JE. Otitis media. In: Kliegman RM, Behrman RE, Jenson HB, Stanton BF (eds). *Nelson Textbook of Pediatrics*, 18th edn. Saunders, Philadelphia, 2007; 2632–46.
8 Crawford SE, Daum RS. Haemophilus influenzae. In: Kliegman RM, Behrman RE, Jenson HB, Stanton BF (eds). *Nelson Textbook of Pediatrics*, 18th edn. Saunders, Philadelphia, 2007; 1173–7.
9 Teele DW, Klein JO, Rosner B. Epidemiology of otitis media during the first seven years of life in children in greater Boston: A prospective, cohort study. *J. Infect. Dis.* 1989; **160**: 83–94.
10 Kuroiwa Y, Nagai K, Okita L *et al.* Comparison of an immunochromatography test with multiplex reverse transcription-PCR for

rapid diagnosis of respiratory syncytial virus infections. *J. Clin. Microbiol.* 2004; **42**: 4812–14.
11 Arola M, Ruuskanen O, Ziegler T *et al.* Clinical role of respiratory virus infection in acute otitis media. *Pediatrics* 1990; **86**: 848–55.
12 Purcell K, Fergie J. Lack of usefulness of an abnormal white blood cell count for predicting a concurrent serious bacterial infection in infants and young children hospitalized with respiratory syncytial virus lower respiratory tract infection. *Pediatr. Infect. Dis. J.* 2007; **26**: 311–15.
13 Korppi M, Leinonen M, Koskela M, Mäkelä PH, Launiala K. Bacterial coinfection in children hospitalized with respiratory syncytial virus infections. *Pediatr. Infect. Dis. J.* 1989; **8**: 687–92.
14 Hasegawa K, Kobayashi R, Takada E *et al.* High prevalence of type b β-lactamase-non-producing ampicillin-resistant *Haemophilus influenzae* in meningitis: The situation in Japan where Hib vaccine has not been introduced. *J. Antimicrob. Chemother.* 2006; **57**: 1077–82.
15 Mokaddas EM, Rotimi VO, Albert MJ. Increasing prevalence of antimicrobial resistance in Streptococcus pneumoniae in Kuwait: Implications for therapy. *Microb. Drug Resist.* 2007; **13**: 227–33.
16 Mölstad S, Erntell M, Hanberger H *et al.* Sustained reduction of antibiotic use and low bacterial resistance: 10-year follow-up of the Swedish Strama programme. *Lancet Infect. Dis.* 2008; **8**: 125–32.
17 Yu S, Yao K, Shen X, Zhang W, Liu X, Yang Y. Serogroup distribution and antimicrobial resistance of nasopharyngeal isolates of Streptococcus pneumoniae among Beijing children with upper respiratory infections (2000–2005). *Eur. J. Clin. Microbiol. Infect. Dis.* **2008**; 27: 649–55.
18 Kim IS, Ki CS, Kim S *et al.* Diversity of ampicillin resistance genes and antimicrobial susceptibility patterns in Haemophilus influenzae strains isolated in Korea. Antimicrob. *Agents Chemother.* 2007; **51**: 453–60.
19 Sekiya Y, Eguchi M, Nakamura M, Ubukata K, Omura S, Matsui H. Comparative efficacies of different antibiotic treatments to eradicate nontypeable Haemophilus influenzae infection. *BMC Infect. Dis.* 2008; **8**: 15.

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Original article

# Serum and cerebrospinal fluid levels of cytokines in acute encephalopathy associated with human herpesvirus-6 infection

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**Abstract**

Human herpesvirus-6 (HHV-6) is a causative agent of exanthema subitum. The immunological pathogenesis of acute encephalopathy associated with HHV-6 infection is still unclear. We measured the concentrations of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-6, IL-10, and soluble TNF receptor 1 (sTNFR1) in serum and cerebrospinal fluid (CSF) during the acute stage in 15 infants with acute encephalopathy and 12 with febrile seizures associated with HHV-6 infection. The serum IL-6, IL-10, sTNFR1, CSF IL-6, and sTNFR1 levels of infants with encephalopathy who had neurological sequelae ($n = 9$) were significantly higher than those with febrile seizures ($p = 0.011$, 0.043, 0.002, 0.029, and 0.005, respectively). In acute encephalopathy, serum IL-6, sTNFR1, and CSF IL-6 levels in infants with neurological sequelae were significantly higher than those without ($n = 6$) neurological sequelae ($p = 0.043$, 0.026, and 0.029, respectively), and serum IFN-γ, IL-6, IL-10, and sTNFR1 levels were significantly higher than those in the CSF ($p = 0.037$, 0.037, 0.001, and 0.021, respectively). There were no significant differences in serum or CSF cytokine levels between infants who were positive for HHV-6 DNA in the CSF ($n = 6$) compared to those who were negative ($n = 9$). We suggest that cytokines mediate the pathogenesis of acute encephalopathy associated with HHV-6 infection, and that the elevated levels of serum IL-6, sTNFR1, and CSF IL-6 are important for predicting neurological sequelae.


*Keywords:* Cytokine; Encephalopathy; Human herpesvirus-6; Interleukin-6; Soluble tumor necrosis factor receptor

## 1. Introduction

Human herpesvirus-6 (HHV-6) is well-known as the causative agent of exanthema subitum, a common infectious disease in infants [1,2]. Exanthema subitum is characterized by an abrupt rise in temperature to as high as 40 °C, followed in 2–4 days by a rapid drop in temperature that coincides with the appearance of an erythematous maculopapular rash that persists for 1–3 days [3]. HHV-6 infection occasionally accompanies neurologic complications, including febrile seizures and acute encephalitis/encephalopathy [3]. These neurologic complications may be caused by direct invasion into the central nervous system (CNS) [3–5] and secondary immune-mediated CNS injury [6–10]. However, the immunological pathogenesis of the acute encephalopathy remains unclear. There has been very little research on the outcome of HHV-6 encephalitis/encephalopathy [4,11]. To determine the role of cytokines in the pathogenesis of acute encephalopathy associated with HHV-

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