as acute necrotizing encephalopathy [3], acute encephalopathy with biphasic seizures and late reduced diffusion [4], and hemorrhagic and shock encephalopathy [5,6]. Although the influenza virus and HHV-6 (human herpes virus-6) are the main causative agents of these acute encephalopathies, many other viruses are also considered to be responsible for the disease [3,4,7].

It is estimated that more than 100 children die of IAE every year in Japan [8,9]. According to the first nationwide clinical survey of IAE in Japan, in many patients with IAE, multiple-organ failure developed, and rates of mortality (31.8%) and disability (27.7%) were high [2]. Although clinical and neuropathological studies suggested that blood–brain barrier destruction and hypercytokinemia in cerebrospinal fluid were closely related to the pathogenesis of IAE, the pathophysiology and mechanisms of disease onset are still unclear [3,7,10,11].

Recently, Chen et al. [12] reported that the thermolabile phenotypes of carnitine palmitoyltransferase II (CPT II) variations, [1055T > G/F352C] alone, and [1055T > G/F352C] + [1102G > A/V368I] were closely related to the pathomechanisms of IAE. The CPT system is a pivotal component of ATP generation through mitochondrial fatty acid oxidation in mammals [13]. Yao et al. [14] further characterized the enzyme properties of the CPT II variants as follows: (1) dominant-negative effect, (2) reduced activities, (3) thermal instability, and (4) short half-lives compared with the wild-type. They demonstrated that the thermolabile CPT II variants might cause mitochondrial fuel utilization failure in various organs and endothelial cells during periods of high fever, and, thus, might play an important role in the pathogenesis of brain edema in IAE. In the present study, we analyzed the CPT II polymorphism and peripheral blood ATP levels as a signal of "energy crisis" in patients with acute encephalopathy with and without influenza virus infection, septic encephalopathy, and febrile delirium during influenza virus infection, and analyzed the relationships among these data, age, and clinical manifestations.

2. Patients and methods

2.1. Patient profile for the study of CPT II polymorphism

This investigation was approved by the Ethics Review Committee for human genome analysis of our institution. All participants' caregivers gave written informed consent. Fifteen patients were included in the study. The clinical details are summarized in Table 1. The diagnoses of the 15 patients were as follows: 12 patients with acute encephalopathy (7 IAE, one human herpes virus type 6 (HHV-6) associated, one varicella-associated, one septic encephalopathy associated with *Hemophilus influenzae* type b, two acute encephalopathy with an unknown pathogenesis, highly suspected of being of viral origin), and three febrile delirium associated with influenza virus infection. Two patients (Case 1, IAE, and Case 2, septic encephalopathy) died 30 and 3 days after admission, respectively, despite intensive care. All 12 patients with acute encephalopathy were diagnosed based on prolonged seizures with high fever and/or consciousness disturbance lasting longer than 12 h associated with brain CT or MRI abnormalities.

3. Representative case presentations

3.1. Case 1

This 4-year-old girl was admitted to our hospital because of feeding difficulty, a lethargic state, and high fever lasting longer than 12 h. A rapid test for influenza A virus antigen in the nasal discharge was positive. She has been followed at our outpatient clinic with a diagnosis of severe psychomotor delay and epilepsy due to chromosome abnormality (46, XX, dup(2)(q21.1q24.2)) since the age of 3 years. Her seizure disorder was well-controlled with phenobarbital. On admission, except for a lethargic tendency, she showed no neck stiffness, involuntary movement, or convulsion, and her respiratory and circulatory conditions were stable. She was also able to follow an object. Neurological examination revealed normal light and corneal reflexes and normal deep tendon reflexes. Pathological reflexes were not induced. Her consciousness level, however, deteriorated 12 h after admission. On laboratory tests, blood glucose, ammonia, the white blood cell count (WBC), hemoglobin (Hb), and platelet count (Plt) were within normal ranges, and cereblospinal fluid (CSF) findings were unremarkable. Blood and CSF cultures were negative. Because she also showed sudden respiratory insufficiency and reduced blood pressure, she was immediately resuscitated and intubated. After that, she could not move and all brainstem reflexes disappeared. On brain CT the next day, as shown in Fig. 1a, cisterns surrounding the brainstem and cerebellum were not identified and auditory brainstem responses (ABR) showed only bilateral wave I. Rapid consciousness deterioration as well as brain CT and ABR findings suggested cerebral herniation due to influenza-associated brainstem encephalopathy. On the second CT 3 weeks later, severe brain edema and subarachnoid hemorrhage were observed. Despite intensive care, she died on the 31st day of hospitalization. She had a thermolabile F352C CPT II variant.

3.2. Case 2

This previously healthy 2-year-old boy was admitted to our hospital because of consciousness disturbance, a brief seizure cluster, and high fever lasting 24 h. On admission, neurological examination revealed coma, the absence of light and corneal reflexes, dilated and anisocoric pupils, and flaccid extremities. Neck stiffness was

Table 1
Clinical summary of patients and CPT II polymorphism.

Case no.	Age at onset	Pathogen	Diagnosis	CPT II polymorphism	Duration of high fever	Duration of seizure (min)	Therapy	Outcome
1[c]	4 years 10 months	Flu A	IAE	F352C	24 h	(−)	Gly, IVIG, m-PSL, Venti	Died
2	2 years 2 months	*H. influenza*	Hib septic AE	F352C, V368I	2 days	3	Venti, Epin, DOA, CTX	Died
3[c]	1 year	Unknown	AEU	F352C, V368I	2 days	30	Mann, MDZ	Severe MR, MD, Epi
4[c]	1 year 7 months	Flu A	IAE	(−)	30 h	40	Gly, m-PSL, Venti	Moderate MR, MD, Epi
5[c]	4 years 5 months	Flu A	IAE	F352C, V368I	5 days	90	Gly, MDZ, Pen, IVIG, m-PSL	Moderate MR
6[c]	2 years 1 months	Varicella	Varicella AE	F352C, V368I	24 h	90	Mann, MDZ, m-PSL	Mild MR
7[c]	6 years	Unknown	AEU	F352C, V368I	2 days	30	Mann, MDZ, m-PSL, HT	Mild MR
8[a]	1 years 4 months	Flu A	IAE	V368I	5 days	60	Gly, MDZ, Pen, PB, m-PSL	Mild MR
9[a]	2 years	Flu A	IAE	V368I	5 days	60	Gly, MDZ, Pen, IVIG, m-PSL	Mild MR
10[c]	11 months	HHV-6	HHV-6 AE	V368I	36 h	100	Gly, MDZ, Pen, m-PSL	Good
11[b]	2 years 5 months	Flu A	IAE	V368I, M647 V	24 h	40	MDZ, PB, m-PSL, HT	Good
12[a]	3 years 11 months	Flu A	IAE	V368I	2 days	40	MDZ, PB, m-PSL, HT, Venti	Good
13	4 years 9 months	Flu A	FD	F352C, V368I	4 days	2	(−)	Good
14	9 years 5 months	Flu A	FD	(−)	3 days	(−)	(−)	Good
15	11 years	Flu A	FD	V368I, M647V	3 days	(−)	(−)	Good

IAE: Influenza-associated encephalopathy, AEU: acute encephalopathy of unknown pathogen, FD: febrile delirium, Flu A: influenza A, HHV-6: human herpes virus-6, MR; mental retardation, MD: motor delay, Epi: epilepsy, Mann: mannitol, MDZ: midazolam, m-PSL: methylpredonisolone, HT: hypothermia, Venti: artificial ventilator, Epin: epinephrine, DOA: dopamine, CTX: cefotaxim, PB: Phenobarbital, Pen: pentobarbital, IVIG: intravenous infusion of gamma-globlin, Gly: glycerole.

[a] AESD (acute encephalopathy with biphasic seizures and late reduced diffusion).

[b] This case partially resembles ANE (acute necrotizing encephalopathy).

[c] Unclassified acute encephalopathy.

not observed. A rapid test for influenza virus antigen in the nasal discharge was negative. His head CT demonstrated diffuse brain edema, as shown in Fig. 1b. On laboratory investigation, blood glucose and ammonia, as well as liver and renal functions were within normal limits. WBC was 18,000/μL, Hb 11.6 g/dL, Plt 3,60,000/μL, and prothrombin time 68.7 s. Blood culture identified *H. influenzae* type b. Spinal tap was not performed because of the risk of cerebral herniation. The blood ATP level was 0.58 mM on admission. The acylcarnitine ratio ((C16 + C18:1)/C2) was high, at 0.203, on admission, compared with the upper cutoff value of 0.048 [12]. We diagnosed him with septic encephalopathy. Despite intensive care including antibiotics, ventilator support, and catecholamine infusion, he died 2 days later. He had compound thermolabile CPT II variants [F352C + V368I].

3.3. Case 12

This previously healthy 3-year-old boy was admitted to our hospital because of a febrile seizure status and high fever lasting longer than 24 h. His generalized tonic clonic seizure was suppressed with pentobarbital infusion 40 min after the onset. A rapid test for influenza virus antigen in the nasal discharge was positive for flu A. Brain CT revealed mild brain edema. So, he was sedated and intubated. Methylpredonisolone (m-PSL) pulse and hypothermia therapies were immediately started based on the diagnosis of IAE. The blood ATP value was 0.77 mM on admission, and it increased to 1.35 mM 2 weeks later. On the 6th day of hospitalization, he developed brief right-sided clonic seizure. Brain MRI (diffusion-weighted images) showed an abnormal high intensity in the left hemisphere (Fig. 1e). The clinical course and MRI findings were compatible with acute encephalopathy with biphasic seizures and late reduced diffusion [4]. Additional m-PSL therapy was given and the hypothermia therapy gradually discontinued. His neurological condition subsequently showed a full recovery. No apparent mental, motor, and social skill impairment was noted during follow-up 1 year later. He had a V368I CPT II variant.

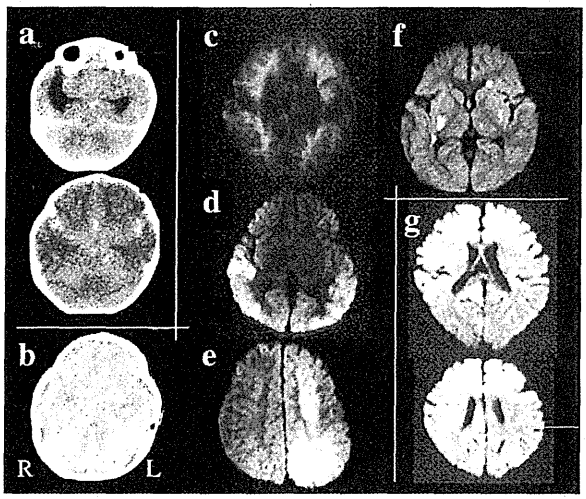

Fig. 1. (a) Brain CTs on 2nd and 21st day of hospitalization in Case 1 showing disappearance of cistern between brainstem and cerebellum (upper image) and severe brain edema (lower image). (b) Brain CT on admission showing severe brain edema in Case 2. (c–g) Brain MRIs showing abnormal high intensities in diffusion-weighted images in Cases 6,7,12,11 and 10, respectively.

3.4. Patient profile for the study of the blood ATP level

Twenty-five patients were included in this study. The diagnoses of the 25 patients were as follows: 10 patients with acute encephalopathy (mean age: 3 years and 11 months, age range: 7 months–10 years and 8 months, one IAE, one *Salmonella*-associated, one HHV-6-associated, three unknown virus-associated, one methylmalonic aciduria, one hepatic encephalopathy, one hemolytic uremic syndrome, and one septic encephalopathy (Case 2 in Table 1)), nine febrile seizure status (mean age: 1 year and 5 months, age range: 4 months–4 years 9 months), and six mitochondrial disease (mean age: 9 years and 8 months, age range: 2–25 years, two partial cytochrome c oxidase deficiency, three Leigh syndrome, and one chronic progressive external ophthalmoplegia). All 10 patients with acute encephalopathy were analyzed regarding the blood ATP levels in the acute phase (within 24 h of disease onset), and five of the 10 patients were also analyzed in the convalescent phase. Among the 15 patients who were analyzed for CPT II polymorphism, only Cases 2 and 12 were included in this study.

4. Methods

4.1. Analysis of CPT II polymorphism

Genomic DNA from whole blood was purified as previously described [15]. PCR of five exons of the CPT II gene was carried out with intron-based primers in genomic DNA. For haplotype analysis, the CPT II exon four region was cloned into the pCR® 2.1 vector (Invitrogen). The sequences of the PCR products and cloned CPT II gene were analyzed employing the ABI DyeDeoxy Terminator Cycle Sequencing Kit with an ABI-PRISM 3100 Genetic Analyzer (PE-Applied Biosystems). Each PCR product was sequenced at least twice independently.

4.2. Preparation of patients' lymphoblasts and culture

Blood samples (2 mL) were obtained from patients by venipuncture into a sterile EDTA blood collection tube. Lymphocytes were separated from peripheral blood, diluted (1:1, v/v) with sterile saline, by centrifugation ($800\times g$, 20 min) over 2 mL of Lymphoprep (Nycomed). The lymphocyte layer was recovered and washed twice with PBS by centrifugation at $250\times g$ for 10 min each, and then maintained in PRMI-1640 (GIBCO) supplemented with 12.5% FCS. Cells were incubated with 5% CO_2 at 37 °C for 7 days. Lymphoblastic cell lines were established by infecting peripheral blood lymphocytes with the Epstein Barr virus. Cells were grown in suspension in an SC flask (Greiner 658190) in an upright position, in 10 ml of PRMI-1640 medium that contained 12.5% FCS, maintained at 37 °C. Fluid was routinely changed every 2 days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

4.3. Analysis of CPT II activity

CPT II activities of patients' lymphoblasts were analyzed as previously described [14]. To prepare whole cell extracts, cells were harvested and washed twice with PBS (−) at $250\times g$ for 10 min and then lysed with 0.5 mL of ice-cold lysis buffer (5 mM Tris–HCl buffer, pH 7.4, containing 1% Tween-20 and 0.5 M KCl), then centrifuged at $147{,}600\times g$ for 1 h at 4 °C. To analyze the heat stability of CPT II, cell lysates were pre-incubated at 30, 37 and 41 °C for 0–120 min. Protein concentrations in the cell lysates were measured using the BCATM Protein Assay Kit (Thermo SCIENTIFIC).

4.4. Measurement of blood ATP levels

ATP concentrations in whole blood lysate were measured by an ENLITEN® ATP assay system bioluminescence detection kit (Promega) according to the instructions provided by the manufacturer and the values were expressed as ATP levels in whole blood.

5. Results

5.1. CPT II polymorphism in the patients

As shown in Table 1, among the 15 patients studied, seven had a thermolabile F352C CPT II variant (1 F352C only and six [F352C + V368I]), four V368I only,

two [V368I + M647 V], and two no polymorphisms. In 12 patients with acute encephalopathy (Cases 1–12), six (Cases 1–3 and 5–7) had a thermolabile F352C CPT II variant (1 F352C only and five [F352C + V368I]), and five (Cases 8–12) had the V368I CPT II variant (4 V368I only and one [V368I + M647 V]) and one (Case 4) showed no CPT II variant. Two patients with acute encephalopathy who died (Cases 1 and 2) had a thermolabile F352C CPT II variant (1 F352C only and the other [F352C + V368I]). In three patients with febrile delirium associated with influenza infection (cases 13–15), only case 13 (brief febrile seizure and unusually long febrile delirium) had the [F352C + V368I] CPT II variant. No other reported CPT II mutations or polymorphisms were detected.

There was no significant difference in the age at onset (41.0 ± 23.3 vs. 24.3 ± 12.7 months of age, $p = 0.18$), duration of high fever (52.0 ± 35.3 vs. 63.0 ± 44.9 h, $p = 0.28$), and duration of seizures (40.5 ± 40.1 vs. 56.7 ± 23.4 h, $p = 0.12$) between the six patients with acute encephalopathy with a thermolabile F352C CPT II variant (Cases 1–3,5–7) and six patients with acute encephalopathy without this thermolabile variant (Cases 4,8–12) (Mann–Whitney U-test).

5.2. Lymphocyte CPT II activity in the patients

As shown in Fig. 2(b), CPT II activity using peripheral lymphocytes of a patient with a thermolabile F352C CPT II variant was significantly reduced to about 50% during incubation for 120 min at 41 °C as compared to those at 30 and 37 °C. All patients with a thermolabile F352C CPT II variant showed a significant reduction of CPT II activity at 41 °C.

Fig. 2(a) shows CPT II activity in a patient with the V368I CPT II variant without reduction even at 41 °C.

5.3. Blood ATP levels in patients with acute encephalopathy

As shown in Fig. 3, ATP levels in the extracts of whole blood in the acute phase of encephalopathy during high fever were significantly low (0.58 ± 0.16 mM, $n = 10$) compared with those in the convalescent phase (1.08 ± 0.27 mM, $n = 5$) and with those of patients with febrile seizure status (1.01 ± 0.36 mM, $n = 9$). The blood ATP levels in the acute phase of encephalopathy revealed no significant difference when compared to those of patients with mitochondrial disease exhibiting several symptoms (0.79 ± 0.39 mM, $n = 6$).

6. Discussion

Although the precise pathomechanisms of acute encephalopathy have yet to be clarified, it is postulated that some genetically-determined factors might be involved, because some types of acute encephalopathy are more frequent in Japanese than in Caucasians. Chen et al. [12] demonstrated that the thermolabile phenotype of CPT II variations such as the F352C CPT II variant or complex [F352C + V368I] CPT II variant might be a principal genetic background of IAE in Japanese. On the basis of the analysis of fatty acid oxidation and cellular ATP production in COS-7 cells transfected with wild-type and variant *CPT2* cDNAs at 37 and 41 °C, Yao et al. [14] suggested that the compound *CPT2* variants with thermolabile phenotypes are the main cause of multiple-organ failure, particularly in high ATP-consuming organs as well as endothelial cells and play a major role in the etiology of IAE.

In the 12 patients with acute encephalopathy studied, six patients (Cases 1–3 and 5–7) had thermolabile F352C CPT II variants (F352C CPT II variant alone in one case and complex [F352C + V368I] CPT II variants in five cases), which were reported to be frequently noted in severe IAE patients [12,14]. Of the six patients, two patients (Case 1, IAE and Case 2, *Hemophilus influenzae*-associated septic encephalopathy) died despite intensive care. Case 2, who died of fatal septic encephalopathy [10], showed a high acylcarnitine ratio ((C16 + C18:1)/C2:0.203) on admission. This value corresponded to the ratio (>0.09) of the high-risk group of patients with IAE showing a fatal outcome, thus reflecting the disorder of mitochondrial β-oxidation. [12]. The remaining six patients (Cases 4 and 8–12) with acute encephalopathy without a thermolabile F352C CPT II variant followed a relatively mild clinical course (Table 1). Out of the six patients, five had a V368I CPT II variant.

As shown in Fig. 2, the CPT II activities of lymphocyte in patients with the F352C CPT II variant showed thermal instability, that is, a marked activity reduction at 41 °C, while those in patients with the V368I CPT II variant did not. There was no significant difference in the age at onset, duration of high fever, and duration of seizures between the six patients with the F352C CPT II variant (Cases 1–3 and 5–7) and six patients without this variant (Cases 4 and 8–12). Therefore, taken together, it seems likely that a thermolabile F352C CPT II variant might be related to the severity of disease, that is, the rapidity of progression of brain edema. In Caucasians, two polymorphisms of CPT II, p.V368I and p.M647 V, occur with a frequency of 0.5 and 0.25, respectively, exhibiting a Hardy–Weinberg equilibrium. A third polymorphism, p.F352C, occurs with a frequency of 0.21 exclusively in the Japanese population [17]. Therefore, this thermolabile F352C CPT II variant might be one of the predisposing factors to trigger the pathomechanism of acute encephalopathy in Japanese.

The CPT system regulates the entry of long-chain fatty acids into the mitochondrial matrix for β-oxidation. Fatty acid oxidation is an important source of acetyl-CoA for maintaining the tricarboxylic acid cycle.

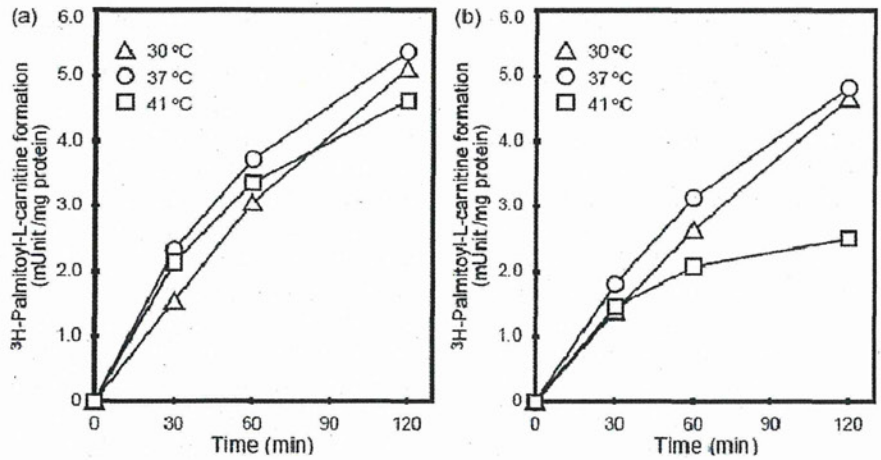

Fig. 2. (a) Lymphocyte CPT II activity in case 12 (influenza-associated encephalopathy) with V368I CPT II variant at 30, 37 and 41 °C. No definite reduction of CPT II activity was observed at 41 °C. (b) Lymphocyte CPT II activity in Case 1 (influenza-associated encephalopathy) with a thermolabile F352C CPT II variant at 30, 37 and 41 °C. At 41 °C, the CPT II activity decreased to about 50% of that at 37 °C after 2-h-incubation.

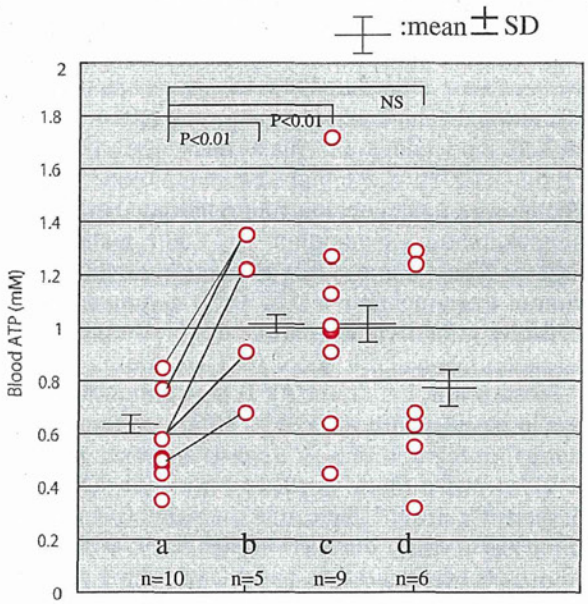

Fig. 3. ATP levels in whole blood in patients with acute encephalopathy (acute (a) and convalescent phase (b)), febrile seizure status (c) and mitochondrial disease (d). In five patients with acute encephalopathy, blood ATP level recovered at convalescent phase.

The CPT II is ubiquitously expressed in all tissues that require fatty acid oxidation as an energy-producing pathway [18]. CPT II deficiency is a disorder of long-chain fatty acid oxidation. It is classified into three clinical types based on the age at onset and disease severity: lethal neonatal form, severe infantile hepatocardiomuscular form, and myopathic form. It is clear that our patients' clinical manifestations did not correspond to any of these three types. The thermolabile instability of the F352C CPT II variant in our cases explains the situation whereby impaired energy metabolism could occur during high fever due to a secondary CPT II deficiency in spite of the absence of symptomatic manifestations of CPT II disorder in daily life at a normal temperature [12,14].

Olpin et al. [19] reported based on mutation analysis that when CPT II activities are above 20% of controls, fatty acid oxidation in fibroblasts is usually within the normal range (>70% of controls). However, under heat stress, fasting, acidosis, and seizures, moderately lowered CPT II activity due to the thermolabile F352C CPT II variant may accelerate the disease process of acute encephalopathy.

Blood ATP levels in the acute phase of encephalopathy during high fever were significantly lower than those in the convalescent phase and also with those of patients with febrile seizure status. This suggests that mitochondrial energetic failure may be more severe in patients with acute encephalopathy, and the pathological process of acute encephalopathy should differ from the febrile seizure status. The low levels of ATP in the acute phase of encephalopathy were normalized in the convalescent phase in line with clinical recovery. Interestingly, blood ATP levels in the acute phase of encephalopathy corresponded to those of mitochondrial disease with several symptoms. Yao et al. [14] showed that COS-7 cells transfected with thermolabile [F352C + V368I] CPT II variants exhibited significantly decreased fatty acid oxidation and subsequent intracellular ATP reduction at 41 °C. The decreased ATP levels seemed to reflect systemic mitochondrial dysfunction including the blood brain barrier (BBB) at the acute phase of encephalopathy in our cases. The ATP demand per body weight is so high in infants that a thermolabile CPT II variant induced-ATP reduction might lead to a greater susceptibility to the pathophysiology of encephalopathy in children than in adults.

The brain capillary endothelium is characterized by a greater density of mitochondria than that of peripheral capillaries [20]. This greater mitochondrial density is required to maintain the significant active transport mechanisms, electrochemical gradients, autoregulatory adjustments, and regulation of tight junctional complexes. As such, the requirement of a constant ATP supply may make the BBB particularly susceptible to acute hypoxic insult [21]. From a similar perspective, BBB breakdown may occur at an initial stage of encephalopathy under the condition of ATP reduction, thus leading to subsequent brain edema due to complex cascade of hypercytokinemia, excitotoxicity, and oxidative stress. Although there is one hypothesis that cytokine storm due to virus–glial cell interaction might cause endothelial cell damage (BBB breakdown) leading to brain edema and neuronal injury [11], we consider that endothelial cell damage might induce in turn cytokine production resulting in neuronal damage in patients with thermolabile F352C CPT II variant irrespective of encephalopathy type .

In three patients with febrile delirium associated with influenza virus infection (Cases 13–15), Case 13 with a thermolabile F352C CPT II variant developed a short seizure and an intermittent confused state with visual hallucinations and agitation lasting 6 h. Cases 14 and 15 without F352C CPT II variant showed short-term consciousness alteration and abnormal behavior without seizures. All patients' brain MRIs were normal, and they fully recovered. Although more extensive study is needed, the grade of febrile delirium associated with influenza virus was more severe in a case with a thermolabile F352C CPT II variant when compared with that in cases without F352C CPT II variant.

Given that a thermolabile CPT II variant might be one of the predisposing factors for acute encephalopathy, we should revise the therapeutic strategy from the acute phase. Considering the rapid progression of encephalopathy and associated low CPT II activity during high fever, immediate hypothermia, sufficient glucose infusion, and L-carnitine supplementation should be adopted as treatment options. We speculate that the immediate hypothermia led to the recovery of the lowered CPT II activity and, thus, mitochondrial energy failure became minimal in many tissues including the brain capillary endothelium, leading to less severe damage to the central nervous system.

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ORIGINAL ARTICLE

The N-ERC index is a novel monitoring and prognostic marker for advanced malignant pleural mesothelioma

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ABSTRACT

Background: Although N-ERC/mesothelin (N-ERC) is an attractive diagnostic and treatment monitoring biomarker for malignant pleural mesothelioma (MPM), its clinical utility for predicting the prognosis has not yet been clarified. The aim of this study is to investigate whether the serum N-ERC level can accurately predict the outcome in patients with MPM.

Methods: Twenty-six patients with MPM were enrolled. Serum N-ERC level was measured before and after chemotherapy. The N-ERC index was determined by the logarithm of the division of the N-ERC level after two courses of chemotherapy by the prior level.

Results: The median N-ERC index in the partial response (PR) group was significantly lower than that in patients with the stable disease (SD) plus the progressive disease (PD) group. The overall survival in the group whose median N-ERC index was lower than its median value was significantly longer than the group whose median N-ERC index was higher than its median value.

Conclusions: The N-ERC index is therefore considered to be a useful biomarker for predicting not only the chemotherapeutic response, but also the prognosis in patients with advanced MPM.

KEY WORDS Mesothelioma; biomarker; N-ERC index; response; prognosis

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Introduction

Malignant mesothelioma is a rare and highly aggressive disease arising from the serosal surfaces of the pleura and peritoneum. Asbestos exposure is the most common risk factor for malignant pleural mesothelioma (MPM). The incidence of MPM is increasing worldwide due to widespread asbestos exposure. Pemetrexed plus cisplatin chemotherapy has been demonstrated to improve the overall median survival in patients with advanced stage disease (1). The chemotherapeutic response is evaluated by Modified RECIST (2) on computed tomography. However, the determination of the tumor response is not always easy because MPM usually does not form tumors and spread to the pleura. In addition, it tends to be difficult to predict the prognosis after chemotherapy, even though several prognostic biomarkers have been reported. Therefore, new biomarkers are needed that can predict the chemotherapeutic response and prognosis at the time of evaluation of chemotherapeutic response. Although serum mesothelin, osteopontin and soluble mesothelin-related protein (SMRP) have been identified as candidates for diagnostic markers of mesothelioma (3-5), it remains unclear as to which marker is clinically superior (6). In addition, although mesothelin and SMRP have been reported as prognostic markers, no biomarkers that can predict the chemotherapeutic response as well as the prognosis have yet been identified.

We previously reported the renal carcinoma *ERC* gene to be expressed in renal carcinoma of the Eker rat (7). We also identified that ERC is a homolog of human megakaryocyte potentiating factor (MPF)/mesothelin gene (8,9). Rat Erc and the human MPF/mesothelin are functional orthologues.

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We designated this protein as ERC/mesothelin. The ERC/mesothelin gene encodes a 71-kDa precursor protein and the protein is cleaved by a furin-like protease into the 31 kDa N-terminal fragment (N-ERC/mesothelin) and 40 kDa C-terminal fragment (C-ERC/mesothelin) (10,11). We established a novel ELISA assay for the detection of human ERC/mesothelin as previously reported (3,4). The serum N-ERC level is a sensitive marker for early diagnosis of MPM especially in the epithelioid-type of the disease and tends to increase according to the stage of the disease (4). We also reported that since N-ERC values decreased following chemotherapy among PR-responsive patients with MPM, thus N-ERC was a reliable monitoring marker for MPM (12).

In this study, we assessed whether N-ERC is a reliable biomarker, which can not only evaluate the chemotherapeutic response, but also predict the prognosis at the time of the second course of chemotherapy in patients with advanced MPM.

Patients and methods

Between June 2005 and June 2010, twenty-six inoperable patients with histologically confirmed MPM were recruited for treatment with chemotherapy at Juntendo University Hospital. The serum N-ERC levels were measured before (on the same day and just before administering chemotherapy) and following two courses of chemotherapy. All blood samples after two courses of chemotherapy were collected from the patients who were completely relieved from chemotherapeutic adverse effects. Serum specimens were immediately obtained from blood samples and stored in aliquots at –80 °C until analysis. The serum level of N-ERC was measured using the sandwich ELISA kit (Immuno-Biological Laboratories, Ltd., Gunma, Japan) as previously reported (3). The chemotherapeutic assessment was performed using a CT scan with Modified RECIST criteria (2) before and after the two courses of chemotherapy. This study was approved by the Juntendo University Research Ethics Committee. Written informed consent was obtained from all patients enrolled in this study.

Statistical analyses

The N-ERC index was defined as Log_2 (N-ERC value after 2 courses of chemotherapy/N-ERC value prior chemotherapy). In order to analyze the overall survival (OS), survival curves were generated using the Kaplan-Meier method. The OS was calculated from the date of initiation of chemotherapy to the date of death. The statistical analysis was performed with Wilcoxon signed-rank test to compare the N-ERC index between the PR and SD/PD groups. The OS rates were compared using the log-rank test according to the N-ERC index (a group whose N-ERC index is above the median value *vs.* a group whose N-ERC index is below the median value). The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software program version19.0F (SPSS Inc.). Differences between the levels were considered to be statistically significant at $P<0.05$.

Results

The characteristics of the participants enrolled in this study are shown in Table 1. Briefly, 26 patients who were diagnosed with MPM were included, 21 men and 5 women. The median age was 63.8 years (range, 51-78 years). Of 26 patients, 21 were of epithelial type, 4 were sarcomatoid type and 1 was biphasic type. The clinical stage of all patients was as follow: one patient in stage I, 5 in stage II, 8 in stage III and 12 in stage IV. The patient in stage I was inoperable due to an advanced age and a low respiratory function. The chemotherapy regimen is also shown in Table 1. The most frequently used regimen was pemetrexed plus cisplatin. The overall response rate was 19.2% with 5 partial responses (PR), 10 patients with stable disease (SD) and 11 patients with progressive disease (PD).

The average N-ERC level was 21.19 ng/mL (range: 1.58-97.54 ng/mL) before chemotherapy. The median value of the N-ERC index in patients with PR was significantly lower than that in patients with SD/PD (Wilcoxon signed-rank test, P=0.015, Figure 1). The overall survival analyses were performed by stratification at a high level (above median) and at a low level (below median) of the N-ERC index. The overall survival in a group whose N-ERC index was below the median level was significantly longer [26.6 months (95% CI, 15.9-37.2 months)] than a group whose N-ERC index was above the median level [10.3 months (95% CI, 5.8-14.1 months)] (P=0.027, Figure 2). The causes of mortality for all patients were the underlying disease. In addition, the low N-ERC level group included 4 PR patients, 4 SD patients and 5 PD patients, while the high N-ERC level group included 1PR patient, 6 SD patients and 6 PD patients.

Discussion

Many biomarkers for MPM have been investigated in patients with MPM to aid in making an early diagnosis. For example, Cytokeratin fragment 21-1, TPA, CA15-3, CA19-9 and CEA have been considered to be potential tumor markers for MPM. However, the findings of such studies still remain controversial (6) i.e., the specificity of these biomarkers is quite low. Therefore, many researchers have so far struggled to identify novel biomarkers whose sensitivity and specificity are higher than those of classical markers. Recently, several investigators reported that mesothelin is useful diagnostic biomarkers, with a high sensitivity and specificity, for MPM (5,13).

We previously reported N-ERC to be a sensitive diagnostic

Table 1. Participant characteristics.

Number	26
Age	
Average [range]	63.8 [51-78]
Gender	
Male/female	21/5
Histology	
Epi/Sar/Bi	21/4/1
Stage	
I/II/III/IV	1/5/8/12
Regimen	
CDDP + Pemetrexed	18
CDDP + Gemcitabin	2
CBDCA + Gemcitabin	5
Pemetrexed	1
Response	
PR	5
SD	10
PD	11
Response rate	19.2%
N-ERC [ng/mL]	
Average [range]	21.19 [1.58-97.54]

Abbreviation: CDDP, cisplatin; CBDCA, carboplatin; Epi, epitheloid; Sar, sarcomatoid; Bi, biphasic; PR, partial response; SD, stable disease; PD, progressive disease.

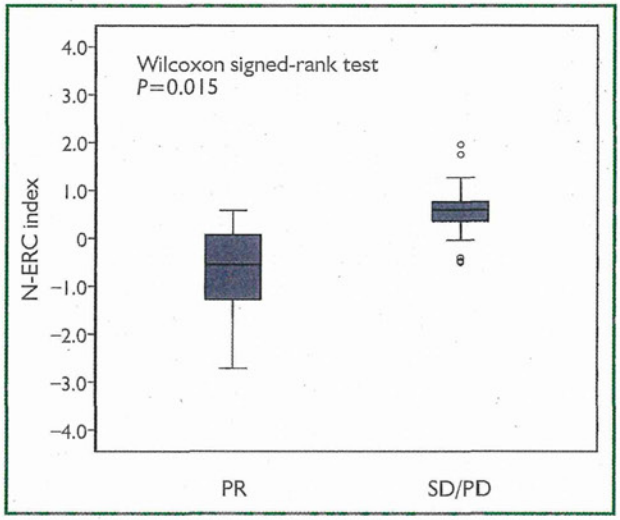

Figure 1. The comparison of the N-ERC index between PR patients and SD/PD patients. The N-ERC index was calculated by Log_2 (N-ERC level after 2 courses of chemotherapy level /N-ERC level prior to chemotherapy) P=0.015.

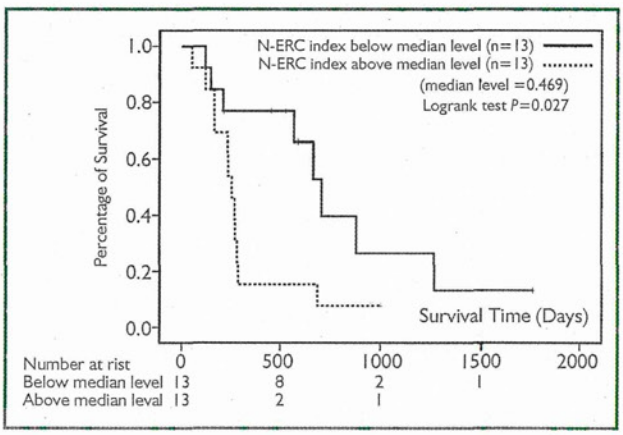

Figure 2. The comparison of the overall survival between the patients whose N-ERC index were below the median level and those whose N-ERC index were above the median level. The horizontal bar indicates the survival time (days), while the vertical bar indicates the percentage of survival. The median value of N-ERC index is 0.469. P=0.027.

marker (4) and useful monitoring marker for MPM (12). In this study, we employed a new index, "N-ERC index", which is calculated by Log_2 (N-ERC value after 2 courses of chemotherapy/N-ERC value prior chemotherapy). The reason why logarithmic transformation was applied in our study is due to the fact that a wide variance in the N-ERC baseline level can be adjusted and also the ratio of N-ERC change before and after chemotherapy can be more accurately evaluated by logarithmic transformation. This mathematical method was adopted from the previous report by Vollmer RT *et al.* (14). We demonstrated that the N-ERC index in patients with PR is significantly lower than that in patients with SD/PD. This result is consistent with our previous report (12). In addition, we also showed that patients whose N-ERC index is below 0.469 (median N-ERC value) survived significantly longer than those whose N-ERC index is over 0.469 (median N-ERC value). These results indicated that N-ERC could be a novel and useful marker for predicting not only the chemotherapeutic response, but also the survival at the time that chemotherapy is evaluated. Interestingly, the low N-ERC level group included 4 SD patients and 5 PD patients. One of the possible reasons for this could be due to difficulties in evaluating tumor reduction based on the Modified RECIST criteria. This finding may also suggest that there could be a deviation between the therapeutic response and prognosis. However, further validation of our findings by a large scale study is needed because our sample size is too small to make any definitive conclusions.

In general, patients with MPM who are subjected to chemotherapy tend to be elderly and fragile because of age-related comorbidities. Therefore, predicting the patients' prognosis after 2 courses of chemotherapy is extremely important. Although the performance status after 2 courses

of chemotherapy deteriorates in certain patients, the patients whose N-ERC index is quite low were found to be able to survive longer than those with a high N-ERC index.

There are several limitations associated with our study. First, our study was a kind of pilot study comprising 26 patients. Secondly, it included patients with a variety of stages and chemotherapeutic regimens. Therefore our small study could not lead to any definitive conclusions, and further validation is therefore required in order to establish the N-ERC index as a valid biomarker for MPM.

In conclusion, we herein demonstrated the serum N-ERC level to correlate with the therapeutic effect of chemotherapy and that the N-ERC index could be associated with the overall survival. We designated the relative N-ERC change ratio as the "N-ERC index". Our novel biomarker could therefore be an innovative tool for determining disease management. Our results suggest that the "N-ERC index" may therefore accurately reflect the therapeutic effect. It may therefore serve as a useful guide for predicting the patient prognosis in MPM after treatment with chemotherapy.

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Disclosure: The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Hydration with magnesium and mannitol without furosemide prevents the nephrotoxicity induced by cisplatin and pemetrexed in patients with advanced non-small cell lung cancer

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ABSTRACT

Background: The aim of this study was to examine the effect of hydration with magnesium and mannitol without furosemide on the nephrotoxocity accompanying combination chemotherapy using cisplatin and pemetrexed in patients with advanced non-small cell lung cancer (NSCLC).

Methods: Fifty patients with NSCLC who received cisplatin plus pemetrexed, using either old hydration protocol including normal saline with mannitol and furosemide, or a new one including normal saline with magnesium and mannitol without furosemide were retrospectively analyzed. Nephrotoxicity was compared between patients treated using the old protocol and those treated with the new protocol. Univariate and multivariate analyses were performed to identify the independent factors associated with protection against nephrotoxicity in patients with NSCLC who received cisplatin plus pemetrexed.

Results: Thirty patients received the old hydration protocol, while 20 patients were treated using the new hydration protocol. The patients treated using the new hydration protocol showed a significantly greater increase in creatinine clearance (P=0.0004) and a decrease in the serum creatinine level (P=0.0148) after one course of chemotherapy compared with those treated using the old hydration protocol. There were no differences in the chemotherapeutic response or overall survival between the groups (P=0.572). The new hydration protocol with supplemented magnesium with mannitol without furosemide was an independent factor for the protection against nephrotoxicity induced by cisplatin and pemetrexed in patients with advanced NSCLC [HR 0.232 (95% CI: 0.055-0.986), P=0.039].

Conclusions: These results demonstrate that the new hydration protocol comprising supplementation with magnesium without furosemide could prevent the nephrotoxicity induced by cisplatin and pemetrexed without affecting the treatment outcome.

KEY WORDS Lung cancer; cisplatin; magnesium; nephrotoxicity; pemetrexed

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Introduction

Lung cancer is a leading cause of death in Japan and other developed countries (1). More than 60% of patients with lung cancer, especially those with non-small cell lung cancer (NSCLC), are inoperable at the time of diagnosis and need to receive chemotherapy containing cisplatin (2). Recently, the percentage of adenocarcinomas among cases of NSCLC has been increasing (3), therefore, regimens containing cisplatin and pemetrexed are expected to be more frequently used (4). Cisplatin is one of the most active and widely used drugs, and still remains a standard component of combination chemotherapy for lung cancer (5). However, nephrotoxicity is a well-known side effect of cisplatin treatment (6). The logistic regression analyses have shown that the risk factors for the development of nephrotoxicity include older age, female gender, current smoking, and hypoalbuminemia (7). The nephrotoxic damage appears to be a clinical problem in 28-42% of patients who receive cisplatin (8). Therefore, many researchers have sought less toxic methods for administering cisplatin. It has been reported that hydration with magnesium supplementation can reduce the nephrotoxocity induced by cisplatin (8,9). In contrast,

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Old regimen			time	New regimen		time
Pre hydration	①	Normal saline 500 mL		①	ST1® 500 mL	2 hr
		metoclopramide 10 mg	2 hr	②	ST3® 500 mL	
	②	ST3®* 500 mL	2 hr		$MgSO_4$ 8 mEq	2 hr
	③	Normal saline 100 mL		③	Normal saline 50 mL	
		5HT3 receptor antagonist			5HT3 receptor antagonist	
			30 min		dexamethasone 12 mg	
					H_2 blocker	15 min
PEM	④	Normal saline 100 mL		④	Normal saline 100 mL	
		Pemetrexed	10 min		Pemetrexed	10 min
	⑤	Normal saline 100 mL	30 min	⑤	Mannitol 200 mL	30 min
CDDP	⑥	Normal saline 300 mL		⑥	Normal saline 300 mL	
		Cisplatin	3 hr		cisplatin	1 hr
Post hydration	⑦	ST3® 500 mL		⑦	ST3® 500 mL	2 hr
		metoclopramide 10 mg	2 hr	⑧	ST1® 500 mL	2 hr
	⑧	Mannitol 300 mL	1 hr			
	⑨	furosemide 20 mg	iv			
	⑩	Normal saline 500 mL				
		5HT3 receptor antagonist	2 hr			
total		3,100 mL	13 hr		2,700 mL	9 hr

Figure 1. The chemotherapy hydration regimens used in this study. ST3 (Solita T3®) is a solution containing Na (35 m Eq/L), K (20 mEq/L), Cl (35 mEq/L), L-lactate (20 mEq/L) and glucose (4.3 g/dL). ST1 (Solita T1®) is a solution containing Na (90 mEq/L), Cl (70 mEq/L), L-lactate (20 mEq/L) and glucose (13 g/dL). PEM, pemetrexed; CDDP, cisplatin.

the protective effect of furosemide against nephrotoxicity is still controversial (10). In fact, Lehane *et al.* reported that high doses of furosemide cause nephrotoxicity, and it has been suggested that its use with cisplatin may aggravate the nephrotoxicity (11). To the best of our knowledge, there have been no studies that have examined the protective effect of hydration using saline lacking furosemide supplemented with magnesium and mannitol on the nephrotoxicity induced by cisplatin and pemetrexed in patients with advanced non-squamous NSCLC.

We recently established a new hydration method using 2,700 mL of saline supplemented with magnesium and mannitol without furosemide, and examined the effect of this hydration protocol on the protection against the nephrotoxicity induced by cisplatin and pemetrexed in non-squamous NSCLC patients.

Subjects and methods

Fifty patients with NSCLC who received cisplatin (75 mg/m^2) plus pemetrexed (500 mg/m^2) from May 2009 to March 2012 were retrospectively analyzed in this study. Chemotherapy was repeated every three weeks unless otherwise noted. Until July 2010, the old hydration protocol, comprising 3,100 mL of normal saline with mannitol (300 mL) and furosemide (20 mg), was used for 30 patients (old group). Because of the relatively high incidence of renal toxicity for old hydration group, the old hydration protocol was replaced with a new protocol, which contained 2,700 mL of normal saline lacking furosemide with mannitol (200 mL) and magnesium sulfate (8 mEq), and was given to 20 patients (new group). Both regimens are shown in Figure 1. Nephrotoxicity was evaluated by the both serum creatinine level and creatinine clearance (Ccr) and were compared between patients treated with the old and new protocols. The serum creatinine level was measured by an enzymatic method. The Ccr was calculated with Cockcroft & Gault's formula (12). Hematological toxicities and non-hematological toxicities except renal toxicity were defined according to the Common Terminology Criteria for Adverse Event (CTCAE) version 4.0. The efficacy of chemotherapy was evaluated based on the response rate, disease control rate and overall survival according to the RECIST criteria, version 1.1. Comprehensive informed consent was obtained from all patients.

Statistical analyses

To evaluate the differences in the patients' characteristics, toxicities, and chemotherapeutic responses, the chi-square test was used. The differences in the nephrotoxicity (evidenced by

Table 1. The characteristics of all patients, the patients in the old regimen group and those in the new regimen group.

		All (n=50)	Old regimen (n=30)	New regimen (n=20)	P value
Age	Median (range)	61 (38-74)	60 (38-74)	63 (41-72)	0.4513
Sex	Male	30 (60)	20 (66.7)	10 (50.0)	0.2386
	Female	20 (40)	10 (33.3)	10 (50.0)	
Histology	Adenocarcinoma	46 (92)	28 (93.3)	18 (90.0)	0.6704
	Others	4 (8)	2 (6.7)	2 (10.0)	
Stage	I, II	2 (4)	2 (6.7)	0 (0.0)	0.0337
	III	7 (14)	6 (20.0)	1 (5.0)	
	IV	33 (66)	15 (50.0)	18 (90.0)	
	Postoperative recurrence	8 (16)	7 (23.3)	1 (5.0)	
line	1st	41 (82)	24 (80.0)	17 (85.0)	0.6521
	2nd	9 (18)	6 (20.0)	3 (15.0)	
PS	0	32 (64)	19 (63.3)	13 (65.0)	0.9043
	1	18 (36)	11 (36.7)	7 (35.0)	
HT	+	12 (24)	10 (33.3)	2 (10.0)	0.0584
	–	38 (76)	20 (66.7)	18 (90.0)	
DM	+	7 (14)	4 (13.3)	3 (15.0)	0.8679
	–	43 (86)	26 (76.9)	17 (85.0)	

PS, performance status; HT, hypertension; DM, diabetis mellitus. Patients characteristics according to the chemotherapy hydration regimens is also shown.

the ⊿serum creatinine and ⊿Ccr) between the old regimen and new regimen were analyzed with Mann-Whitney's U test. To analyze the overall survival (OS), survival curves were drawn by the Kaplan-Meier method. The OS was calculated from the date of initiation of chemotherapy to the date of death. The OS rates were compared using the log-rank test according to the regimen (old regimen vs. new regimen). The univariate and multivariate analyses using a multiple regression analysis were performed to identify the independent factors associated with protection against nephrotoxicity in patients with non-squamous NSCLC who received cisplatin plus pemetrexed using the SPSS (IBM, USA, New York), software program, version 19. P values <0.05 were considered to be statistically significant.

Results

The characteristics of all patients, the patients in the old regimen group and those in the new regimen group are shown in Table 1. The median age of all patients was 61 years old (range, 38-74 years old). Of the total of 50 patients, 30 patients were males (60%), 47 patients had adenocarcinoma (94%). The clinical stage was I and II in two, III in seven, IV in 33 and postoperative recurrence in eight patients. The ECOG performance status was 0 in 32 patients and 1 in 18 patients. As a co-morbidity related to the renal toxicity, 12 patients had hypertension (HT) and seven had diabetes mellitus (DM). There were no significant differences in the patient age, gender, performance status, histology, administered lines of treatment or incidence of DM between the groups. With regard to HT, patients in the new regimen group tended to less frequently have HT than those in the old group. However, there were no significant differences between the groups. In contrast, the old regimen group included more advanced stage patients compared to the new regimen group.

The completion rate of four cycles was 43.5% and 46.7% for the old and new regimen groups, respectively (data not shown). This difference was not statistically significant. The change in the serum creatinine level (maximum serum creatinine during one course of chemotherapy - serum creatinine before chemotherapy) and the change in the Ccr (Ccr before chemotherapy - nadir Ccr during one course of chemotherapy) were calculated. Figure 2A and B show the comparison of the change in the serum creatinine and the change in the Ccr in both groups. The nephrotoxicity in the old regimen group was more severe than that in the new regimen group.

We also compared other toxicities, besides renal dysfunction, between the groups. Both groups demonstrated similar toxicity profiles, and there were no significant differences in any of the other toxicities between the groups (data not shown). Of note, the response rate and disease control rate in the two groups were also not significantly different (Table 2). Even though the overall survival in the new regimen group did not reach the median survival time, the overall survival in the new regimen group appears to be

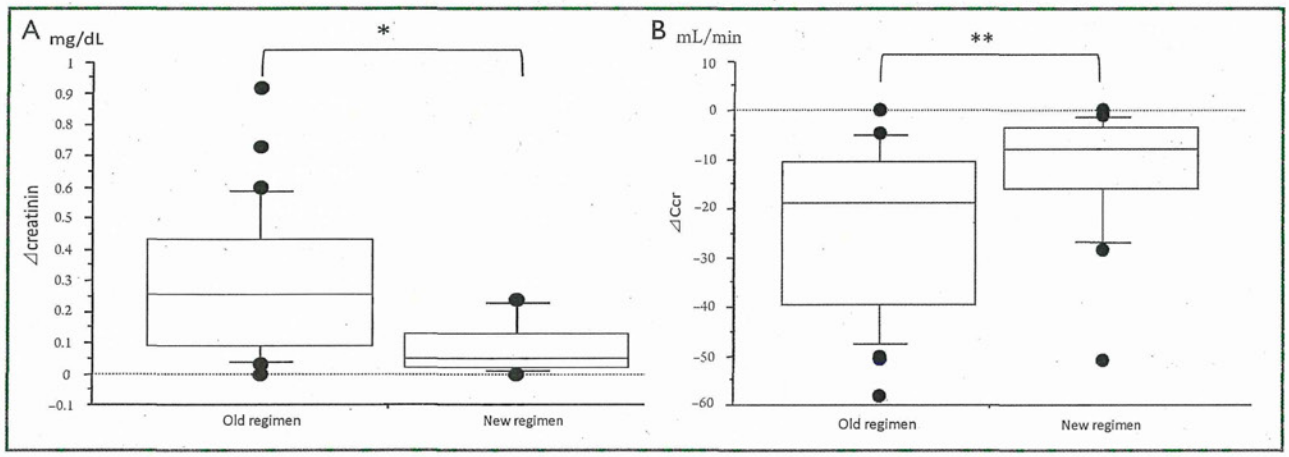

Figure 2. Differences in nephrotoxicity between patients treated using the old and new regimens. The Δserum creatinine and ΔCcr were calculated as described in the Subjects and Methods. The Δserum creatinine and ΔCcr in both groups were compared. The Δserum creatinine and ΔCcr are shown by vertical bars in A and B, respectively. *P=0.0004, **P=0.0148 *vs.* the new regimen.

Table 2. Treatment response (n=50).

Response	n	Old Regimen n=30	New Regimen n=20
CR	0	0	0
PR	10	7	3
SD	21	11	10
PD	8	4	4
NE	11*	8	3
Response rate	25%	23.3%	15.0%
Disease control rate	62%	60.0%	65.0%
			P=0.5723

CR. complete response, PR. partial response, SD. stable disease, PD. progressive disease, NE. not evaluable, *. Eleven patients with NE contain 6 patients with discontinuation of treatment due to toxicity, 4 patients with no evaluable lesion, and 1 patient with hospital tranceference.

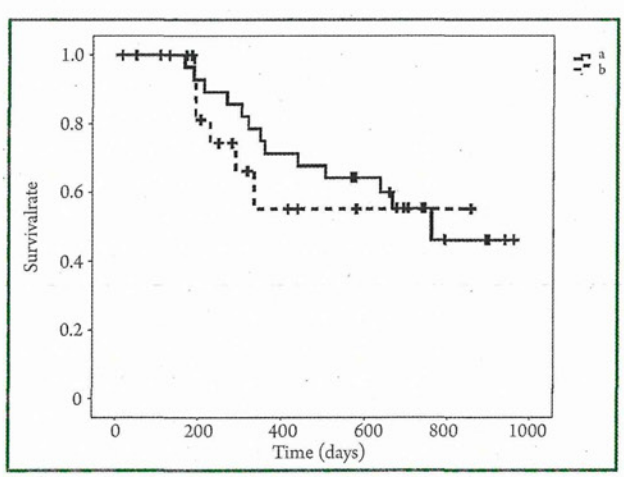

Figure 3. Overall survival. A comparison of the overall survival of patients with advanced non-squamous NSCLC between those treated using the old regimen group (a) and new regimen (b). There were no significant differences between the groups (P=0.4722).

equivalent to that in the old regimen group (Figure 3).

We next performed a univariate analysis to identify the factors influencing renal toxicity. As shown in Table 3, renal toxicity was more frequently observed in males and in the old regimen group than in females and in the new regimen group. Interestingly, there were no differences in nephrotoxicity based on the patient age, histology, stage, performance status, or co-morbidities (HT and DM) in the two groups. According to the multivariate analysis (Table 4), only the new chemotherapy regimen was an independent factor predicting the protection of renal toxicity caused by cisplatin and pemetrexed.

Discussion

In this study, we clearly demonstrated that a new regimen utilizing 2,700 mL of saline lacking furosemide supplemented with magnesium and mannitol with rapid cisplatin infusion (300 mL of saline containing 75 mg/m^2 cisplatin in 1 hour)

Table 3. Patient characteristics according to nephrotoxicity.

		All (n=50)	Nephrotoxicity+, (n=21)	Nephortoxicity-, (n=29)	P value
Age	Median (range)	61 (38-74)	62 (38-74)	60 (39-72)	0.4551
Sex	Male	30 (60)	16 (76.2)	14 (48.3)	0.0467
	Female	20 (40)	5 (23.8)	15 (41.7)	
Histology	Adenocarcinoma	46 (94)	19 (90.5)	27 (93.1)	0.5232
	Others	4 (6)	2 (9.5)	2 (6.9)	
Stage	I	33 (66)	11 (52.4)	22 (75.9)	0.0836
	Other stage	17 (34)	10 (47.6)	7 (17.2)	
line	1st	40 (80)	17 (81.0)	24 (82.8)	0.8697
	2nd	10 (20)	4 (19.0)	5 (17.2)	
PS	0	32 (64)	11 (52.4)	21 (72.4)	0.1452
	1	18 (36)	10 (47.6)	8 (17.6)	
Regimen	old	30 (60)	17 (81.0)	13 (44.8)	0.0101
	new	20 (40)	4 (19.0)	16 (55.2)	
HT	+	12 (24)	6 (28.6)	6 (20.7)	0.3814
	–	38 (76)	15 (71.4)	23 (79.3)	
DM	+	7 (14)	4 (19.0)	3 (10.3)	0.5195
	–	43 (86)	17 (81.0)	26 (89.7)	

Nephrotoxicity+ is defined with the grade 1 and greater of serum creatinin after 1 course of pemetrexed and cisplatin. PS, performance status; HT, hypertention; DM, diabetes mellitus, Parenthesis indicates the percentage.

Table 4. Predictive factors of nephrotoxicity caused by pemetrexed and cisplatin according to the Multivariate analysis.

Factor	95% CI	HR	P value
Sex (male/female)	0.761-11.041	2.899	0.6882
Stage (other stage/4)	0.327-5.452	1.335	0.1109
Chemotherapy regimen (new/old)	0.055-0.986	0.232	0.0393

CI, confidential interval; HR, hazard ratio.

would be useful to avoid the renal toxicity caused by cisplatin and pemetrexed, without any reduction in the efficacy of the regimen in patients with advanced non-squamous NSCLC. To exclude the possibility that the patient background, such as age, gender, stage and co-morbidities, would affect the renal toxicity induced by cisplatin and pemetrexed, a multivariate analysis using a multiple regression method was performed, and revealed that the new regimen was an independent predictive factor for the protection against the nephrotoxicity induced by cisplatin and pemetrexed.

There has been speculation about what ingredients (factors) in the new regimen contributed to protecting against the nephrotoxicity induced by cisplatin and pemetrexed. Several researchers had previously reported that magnesium supplementation protected against cisplatin-induced nephrotoxicity (8,9). Willox *et al.* performed a randomized trial to evaluate the effect of magnesium supplementation in testicular cancer patients receiving cisplatin, and demonstrated its effect on renal protection (9). Bodnar *et al.* have revealed the nephroprotective effect of magnesium supplementation during chemotherapy with cisplatin in patients with epithelial ovarian cancer (8). In addition, Lajer *et al.* reported that magnesium depletion enhances cisplatin-induced nephrotoxicity (13). Based on these previous reports, the magnesium supplementation in the new regimen appears to have been at least partly responsible for the reduced incidence of nephrotoxicity in this study.

Mannitol causes osmotic diuresis. Hayes and Frick revealed that mannitol decreased cisplatin nephrotoxicity (14,15). Clinically, mannitol reduces the urine concentration of cisplatin, and this effect is considered to be the mechanism underlying the amelioration of renal toxicity. Since most of the previous reports supported its effect on nephroprotection, except one controversial paper in which hydration with saline + mannitol was not nephroprotective compared to saline alone, mannitol was included in our new regimen (16).

Although other researchers have already reported the effect of furosemide on reducing the renal toxicity, its effect on the prevention of nephrotoxicity is still controversial (10). In fact, it has been reported that furosemide protects renal function, while

it worsens renal histopathology (11). Moreover, McMurtry *et al.* reported that furosemide enhances rodent nephrotoxicity (11). Therefore, furosemide was not included in the new regimen, resulting in amelioration of the nephrotoxicity induced by cisplatin and pemetrexed. We also employed the rapid infusion of cisplatin (75 mg/m^2/300 mL/1 hour) in the new regimen. The nephrotoxicity induced by cisplatin is related to the contact time of free cisplatin to the proximal tubules in the kidneys (17). Therefore, the rapid cisplatin infusion method used in the new regimen might have contributed to the reduction of cisplatin-induced nephrotoxicity. The new regimen involving the supplementation with magnesium and mannitol, in concert with rapid cisplatin infusion, could prevent cisplatin-induced nephrotoxicity.

It has been reported that supplementation with magnesium would affect *in vitro* and *in vivo* tumor growth (18). In fact, Parsons *et al.* have suggested that magnesium depletion decreases tumor growth (19). However, there were no statistically significant differences in the chemotherapeutic response or overall survival between the groups in our study, although the clinical outcomes appeared to be a little bit better in patients treated using the old regimen compared to those treated with the new regimen. Our results are supported by the report by Willox *et al.*, in which the supplementation with magnesium did not modify the chemotherapeutic response or prognosis for patients with testicular cancer (9).

There are several limitations in this study, which need to be addressed. Our study population was relatively small and retrospective, large scale and prospective studies are needed to confirm the utility of this protocol. In addition, there are several differences between the old regimen and the new regimen besides magnesium and furosemide. For instance, dexamethasone is contained in the new regimen, while it is not contained in the old regimen. We could not exclude the possibility that addition of dexamethanose in the new regimen was useful to prevent cisplatin-induced nehrotoxicity.

In conclusion, we clearly demonstrated that saline hydration without furosemide, supplemented with magnesium and mannitol, with rapid cisplatin infusion ameliorated the nephrotoxicity caused by cisplatin and pemetrexed in patients with advanced non-squamous NSCLC.

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ORIGINAL ARTICLE

High prevalence of gene abnormalities in young patients with lung cancer

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ABSTRACT

Background: Recently, driver oncogenes in adenocarcinoma of the lung were identified, and several molecular target agents were introduced in the clinical setting. However, there are few reports on the frequency of gene abnormalities in young patients with lung cancer.

Materials and methods: Twelve patients with lung adenocarcinoma aged 40 or younger at Juntendo University Urayasu Hospital or Juntendo University Hospital from July 2004 to March 2010 were analyzed for driver oncogene status including *EGFR* activating mutation, *EML4-ALK* fusion gene, and *K-ras* mutation.

Results: Four patients showed *EGFR* gene mutation. Five out of 7 *EGFR* mutation-negative patients showed positive results for *EML4-ALK* gene fusion. One case whose *EGFR* mutation was indeterminate.

Conclusions: Driver oncogene including *EGFR* mutation and *EML4-ALK* fusion gene was identified in 9 of 12 cases (75%). Examination of gene abnormalities is essential in young patients with non-small cell lung cancer to provide the best treatment.

KEY WORDS Young patients; driver oncogene; lung cancer; *EGFR*; *EML4-ALK*

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Introduction

Many young patients with lung cancer at the time of diagnosis are already advanced stage and therefore result in a poor prognosis (1,2). For patients harboring the *epidermal growth factor receptor* (*EGFR*) gene mutation, *EGFR* tyrosine kinase inhibitors (*EGFR*-TKIs) have been used effectively to prolong progression-free survival and overall survival (3,4). Recently, the powerful driver oncogene, fusion gene of the *anaplastic lymphoma kinase* (*ALK*) with the *echinoderm microtubule-associated protein-like 4* (*EML4*) was identified in non-small cell lung cancer (5). Prolongation of the survival period is expected with the use of the *ALK*-TKI. However, few studies have analyzed the frequency of driver oncogenes in young patients with non-small cell lung cancer aged 40 or younger. Therefore, we performed gene mutation analyses in young patients with lung cancer.

Methods and materials

We retrospectively reviewed medical records of all hospitalized patients with non-small cell lung cancer aged 40 or younger at Juntendo University Urayasu Hospital or Juntendo University Hospital from July 2004 to March 2010. We examined patient background, treatment modalities, and gene abnormalities. First, we examined *EGFR* mutation by performing direct sequencing for tumor biopsy specimens obtained by bronchoscope, resected tumor samples, or cell blocks of bronchoalveolar fluid or pleural effusion. When the *EGFR* mutation was negative, we next performed immunohistochemical analysis [using an intercalated antibody-enhanced polymer (iAEP)] and fluorescence in situ hybridization (FISH) for detection of the *EML4-ALK* fusion protein and gene (6), respectively. In negative cases for both *EGFR* mutation and *EML4-ALK* fusion gene, we analyzed the samples for presence of the *K-ras* mutation. We did not conduct re-evaluation for the *EGFR* gene mutation after recurrence.

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Table 1. Background characteristics of the 12 patients in whom examination was performed for gene abnormalities.

No	Age	Sex	BI	Histology	T	N	M	Stage	PS	EGFR	EML4-ALK	1st line	2nd line	Outcome	Survival time
1	33	m	10	adeno	4	0	1	IV	1	+	n.d.	CBDCA+TXL	Gefitinib	death	1,208 days
2	37	m	800	adeno	4	2	1	IV	1	+	n.d.	Gefitinib	CBDCA+TXL	death	461 days
3	37	m	450	adeno	4	3	1	IV	3	+	n.d.	CBDCA+TXL	Gefitinib	death	379 days
4	39	m	400	adeno	3	3	1	IV	0	+	n.d.	CDDP+PEM	Gefitinib	death	364 days
5	31	f	100	adeno	1	3	0	IIIB	0	±	n.d.	CBDCA+TXL	Gefitinib	alive	2,688+α
6	35	f	0	adeno	4	0	0	IIIB	0	-	+	CBDCA+GEM	PEM	alive	1,456+α
7	37	f	0	adeno	2	1	1	IV	0	-	+	CBDCA+PEM		alive	757+α
8	34	f	0	adeno	4	3	1	IV	2	-	+	CBDCA+TXL	GEM	death	568 days
9	33	m	300	adeno	4	3	1	IV	1	-	+	CBDCA+TXL	CBDCA+PEM	death	175 days
10	35	m	0	adeno	4	3	1	IV	1	-	+	CBDCA+TXL	CBDCA+PEM	death	99 days
11	37	f	0	adeno	2	2	0	IIIA	0	-	-	CBDCA+TXL		alive	365+α
12	36	m	340	non-small	2b	3	1b	IV	1	-	-	CBDCA+TXL		alive	280+α

Abbreviations: BI, brinkman index; PS, performance status; ±, EGFR mutaion indeterminate, but responded to gefitinib; n.d., not done; CBDCA, carboplatin; TXL, pacritaxicel; PEM, pemetrexed; GEM, gemcitabine.

Survival analysis was conducted using the Kaplan-Meier method.

Results

Case profile

We retrospectively studied 12 young patients with non-small cell lung cancer (men, 7; women, 5). The mean age of the patients was 35.3 years (Table 1).

Smoking history

Six patients were smokers. Three out of these patients were heavy smokers over 20 pack pear, and had a long history of smoking. One man and 4 women were non-smokers.

Histology and stage of the disease

All of the patients had non-small cell lung cancer. Eleven patients (91.6%) were diagnosed with adenocarcinoma, while one was with histology not otherwise specified. According to the clinical TMN classification, there were 1 patient with stage IIIA, 2 with stage IIIB cancer; and 9 with stage IV.

Examination of the gene abnormalities

Activating EGFR gene mutations, exon 19 deletion, were detected in 4 cases.

One case whose *EGFR* gene mutations were indeterminate because sample size was not enough for direct sequencing. But she seems to harbor *EGFR* activation mutation because she responded to gefitinib remarkably. Therefore, we considered that she harbored an *EGFR* mutation. Subsequently, we conducted iAEP followed by FISH analyses for 7 patients without *EGFR* mutation to determine EML4-ALK fusion protein and gene. Among 7 patients, 5 patients showed positive for *EML4-ALK* protein or gene. Analysis for the presence of *K-ras* mutation was performed in 2 cases that were negative for both the *EGFR* mutation and the *EML4-ALK* fusion gene. One of the cases was *K-ras* mutation-negative, while the other case was not clear for *K-ras* mutation because of inadequate sample (Figure 1).

Median survival time and survival curve

The patients harboring *EGFR* mutation were treated with gefitinib. The median survival time (MST) was 461 days. The MST for the patients harboring *EML4-ALK fusion gene* was 568 days (Figure 2), because these patients could not be treated *ALK* inhibitors.

Discussion

In this study, all patients were diagnosed as non-small cell lung cancer with advanced stage. Development of metastases without symptoms or prolonged neglect of symptoms could be the reasons for this finding. Gene analysis showed that *EGFR* mutation was clearly identified in 4 of our 12 cases.

The frequency of the EGFR mutation in cases of lung adenocarcinoma has been reported by a previous study (7). There were no significant differences in the frequency for *EGFR* mutation depending on the patient age (8). Five of the 7 *EGFR*-negative cases in our study were detected to have the EML4-ALK fusion gene. According to a previous study, the frequency of the *EML4-ALK* fusion gene is in the range of 1.6% to 8.6% (9-12).