Supplemental Data

De Novo Mutations in *GNAO1*, Encoding a Gα$_o$ Subunit of

Heterotrimeric G Proteins, Cause Epileptic Encephalopathy

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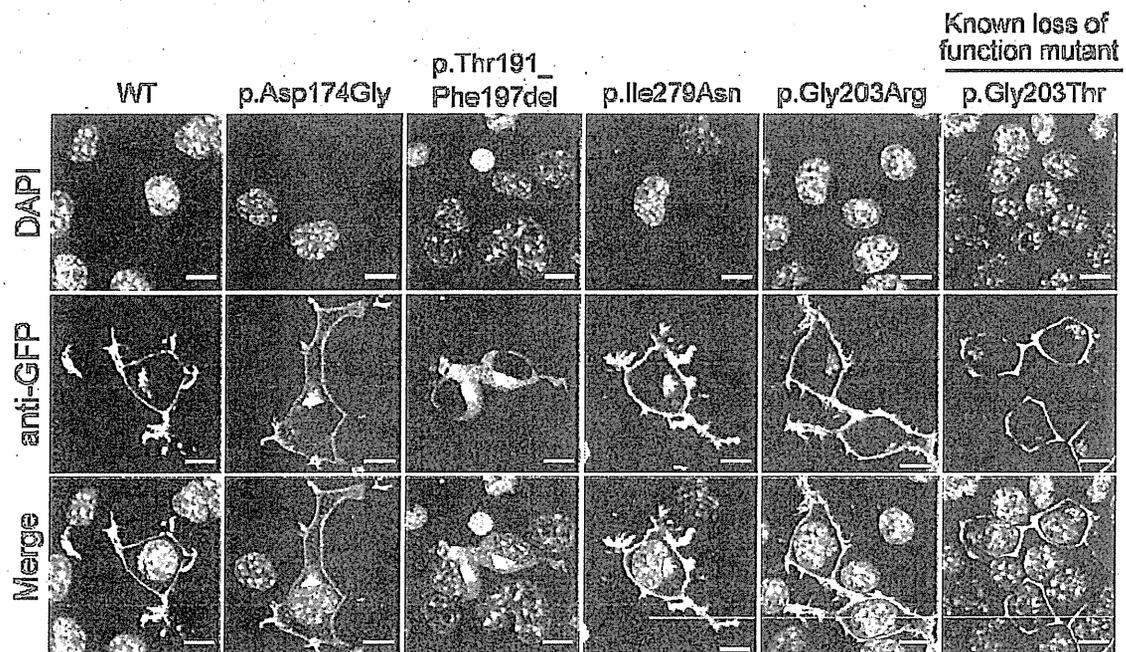
Figure S1. Expression of AcGFP1-Tagged Gα_{o1} in N2A Cells

Human *GNAO1* cDNA was inserted into pAcGFP1-N2 vector (Clontech, Mountain View, CA, U.S.A.) to generate C terminal AcGFP1-tagged Gα_{o1} expression constructs. Expression of AcGFP1-tagged wild-type Gα_{o1} (WT), p.Gly203Arg, and p.Gly203Thr mutants were observed at the cell periphery. In contrast, p.Thr191_Phe197del was expressed in the cytosolic compartment. The p.Asp174Gly and p.Ile279Asn mutants were localized to the cell periphery with weak signal in the cytosol. The nucleus was stained with DAPI. The scale bars represent 10 μm.
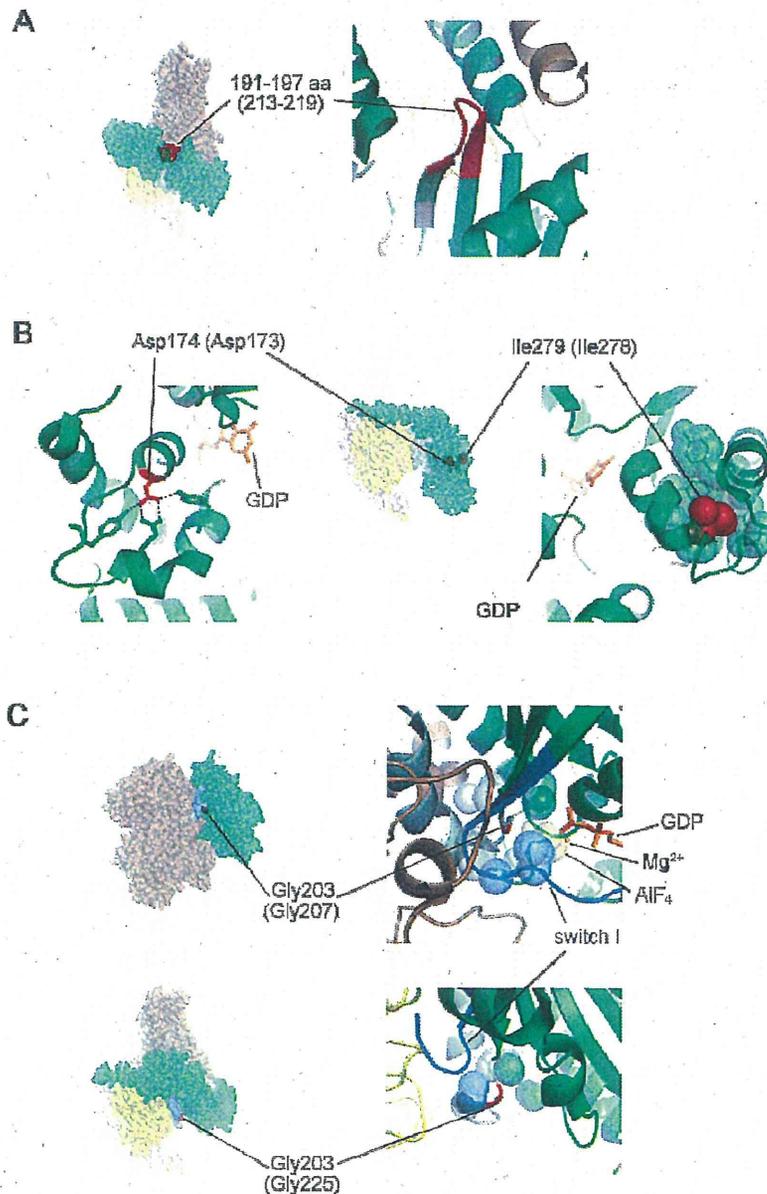
Figure S2. Structural Consideration of the Gα Mutations in Some Gα-Containing Complexes

Overall and close-up views of the positions in the Gα subunit-containing complexes corresponding to the mutation sites found in human *GNAO1*; p.191_197del in the Gα$_s$βγ-β2AR complex (PDB code 3SN6) (A), p.Asp174Gly and p.Ile279Asn in the GDP-bound Gα$_i$βγ heterotrimer (PDB code 1GG2) (B), and p.Gly203Arg in the transition-state analogue of GTP (GDP+AlF$_4^-$)-bound Gα$_q$-PLCβ complex (PDB code 3OHM) (top) and the Gα$_s$βγ-β2AR complex (bottom) (C). Overall views are shown in the space-filling representation as in Figure 4. In the close-up views, α helices, β strands

and loops are depicted as ribbons, arrows and threads, respectively. Colors are as in Figure 4 except for nucleotides in orange and the mutated residues in red. The amino acid numbering shown is for human *GNAO1* with bovine $G\alpha_s$ (A and C), rat $G\alpha_i1$ (B), and mouse $G\alpha_q$ (C) in parentheses. In B, side chains of some residues involved in a hydrophobic core are shown in the space-filling representation, and hydrogen bonds are shown as black dotted lines. In C, the switch I region is colored cyan, and heavy atoms within 5 Å distance from the atom corresponding to the α carbon of Gly203 in the human $G\alpha_o1$ are shown in the space-filling representation.

Table S1. Primers and PCR Conditions

Exon	Forward primer (5'>3')	Reverse primer (5'>3')	Product size (bp)	PCR conditions
Ex1	TTTGTTTCCAGCCCAGGAGAGGATA	GATCCTGGGCACATGGTGGTG	386	KOD FX with 2 step PCR 70-64
Ex2	CCCCTGTTCCCTTAAGCTG	GAGGACCCAGAACCAACGTA	286	KOD FX 64-56
Ex3	GACCTGGCCCACAGTCAG	AGGCAGCCCAGCACTATAAA	326	HotStartTaq MM Kit 64-56
Ex4	CTTGGCTGGCAGAGGTCTT	CATCACCAGTCCCTTCCACT	295	HotStartTaq MM Kit 64-56
Ex5	ACAGTGTCCAGGCATTTGGT	CCTTGGCAGAAACACAGACA	347	HotStartTaq MM Kit 64-56
Ex6	CAGCGTGCTCACAGCTTAAT	CTCAGAGGGCTGGCCTATC	280	HotStartTaq MM Kit 64-56
Ex7-variant 1	CCAGTCCCTCTCTGTCAAGC	GAGCAGCCTGTTCTCTGAGC	329	KOD FX 64-56
Ex7-variant 2	AGCCACATTGGTGGACCTT	CATCCACAGAGCACAGGAAG	349	HotStartTaq MM Kit 64-56
Ex8-variant 1	ATCCCACTTCCTGGGACAC	TCTGTGGGTGCTGGAATCAT	350	HotStartTaq MM Kit 64-56
Ex8-variant 2	GTCCATGCCAAGCAGTCC	CAAGTGCAAAGAGTGGTCTGA	348	HotStartTaq MM Kit 64-56

The column "PCR conditions" shows the PCR enzymes used and the range of annealing temperatures. The temperature was lowered by 2°C every 3 cycles to the lowest annealing temperature. Then, the PCR reaction was cycled 30 times.

The details of the PCR conditions are as follows:
Hot Start Taq MM Kit: 95°C for 10 s, 64°C, 60°C, 58°C, 56°C for 30 s, and 72°C for 30 s
KOD-FX: 95°C for 10 s, 64°C, 60°C, 58°C, 56°C for 30 s, and 72°C for 30 s
KOD-FX with 2 step PCR: 98°C for 10 s and 70°C, 68°C, 66°C, 64°C for 30 s

The following PCR amplification enzymes were used: Hot Start Taq MM Kit, Hot Start Taq Master Mix Kit (Qiagen, Tokyo, Japan); KOD FX, KOD FX Neo (Toyobo, Osaka, Japan)

Table S2. Prediction of the Pathogenicity of the *GNAO1* Mutations

Case	Diagnosis	cDNA change	Amino acid change	Inheritance	SIFT	Polyphen2	Mutation Taster	In-house database
1	Ohtahara syndrome	c.836T>A	p.Ile279Asn	*de novo*	0.00	Probably damaging 1.000	Disease causing 0.999	0 / 408
2	Ohtahara syndrome	c.521A>G	p.Asp174GLy	*de novo* mosaic	0.00	Probably damaging 1.000	Disease causing 0.999	0 / 408
3	Ohtahara syndrome	c.572_592del	p.Thr191_Phe197del	*de novo*	N/A	N/A	N/A	0 / 408
4	Epileptic encephalopathy	c.607G>A	p.GLy203Arg	*de novo*	0.00	Probably damaging 1.000	Disease causing 0.999	0 / 408

N/A = not applicable.
SIFT (http://sift.jcvi.org/): scores of less than 0.05% indicate substitutions that are predicted as intolerant.
PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/): scores are evaluated as 0.000 (most probably benign) to 0.999 (most probably damaging).
Mutation Taster (http://www.mutationtaster.org/): rapid evaluation of DNA sequence alterations. The alterations are classified as disease causing or polymorphism.

Table S3. Read Counts in Family of Individual 2 with a Mosaic Mutation in GNAO1 (c.521A>G)

Methods &samples	Read counts		% of Mutant alleles
	Mutant alleles	Wild type alleles	
Whole exome			
Blood #1	6	29	17.14
Deep sequencing			
Blood #1	2,996	13,430	18.24
Blood #2	2,659	11,784	18.41
Nail #1	4,187	12,174	25.59
Nail #2	2,975	14,948	16.60
Saliva	3,073	14,255	17.73
Father Blood	23*	18,154	0.13
Mother Blood	27*	14,872	0.18

Samples of blood and nail have been independently obtained twice (#1 and #2).

*These small numbers of mutant reads were considered to be PCR or sequencing errors.

Original article

Serum and CSF biomarkers in acute pediatric neurological disorders

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Abstract

Background: There have been numerous reports regarding serum or cerebrospinal fluid (CSF) biomarkers in various disorders; however, the validities of such biomarkers for more precise diagnoses and prognosis estimates remain to be determined, especially in pediatric patients with neurological disorders. *Methods:* Serum/CSF S100B, neuron-specific enolase, and total tau (tTau) were measured in various acute pediatric neurological disorders, and their usefulness for diagnostic and prognostic predictions was validated using receiver operating characteristic curves and area under the curve (AUC) analysis. *Results:* A total of 336 serum and 200 CSF specimens from 313 patients were examined, and we identified statistically significant differences that were relevant from diagnostic and prognostic viewpoints. CSF and serum tTau levels could be good predictors for diagnosis (CSF tTau; AUC = 0.76) and prognosis (serum tTau; AUC = 0.78). *Conclusions:* Both CSF and serum tTau levels could be useful for precise diagnostic and prognostic estimations in acute pediatric neurological disorders. Further studies are needed to clarify the clinical significance of such biomarkers.
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Keywords: S100B; Neuron-specific enolase; Total tau; Receiver operating characteristic curves; Area under the curve

1. Introduction

There are many kinds of pediatric neurological disorders with acute symptoms, such as headache, altered consciousness, seizures, paralysis, and ataxia [1]. Pediatricians or pediatric neurologists who treat these patients use various approaches, such as history taking, physical/neurological examinations, routine laboratory tests, conventional cerebrospinal fluid (CSF) examinations, electroencephalography, and brain imaging to identify the underlying cause. Due to limited time and resources,

it would be useful to develop more precise diagnostic and prognostic predictions, which are sometimes difficult to attain, especially in the early stages of these disorders. For more than a decade, there have been reports about serum/CSF biomarkers that are useful in identifying various neurological disorders, at least in study settings [2–9]. We examined serum and CSF S100B, neuron-specific enolase (NSE), and total tau (tTau), which are glial, neuronal, and axonal damage markers, respectively, in patients with acute encephalopathy with biphasic seizures and late reduced diffusion. We found that all 3 biomarker levels were significantly increased and useful for diagnosis [3]. We hope to evaluate the usefulness of these markers as diagnostic and prognostic predictors in other diseases.

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2. Methods

2.1. Patients

From June 2007 to August 2012, patients were enrolled in the study mainly via mailing lists for Japanese pediatric neurologists or pediatricians, such as the Zao Seminar Mailing List (available at: https://sites.google.com/site/zaoseminar/) and the Japanese Pediatric Conferences Mailing List (available at: https://jpmlc.org/). Diagnoses were made by the attending physicians and later confirmed for the purpose of this study by examination of the available clinico–radiological information. We asked the attending physicians to provide each patient's clinical course and prognosis at the most recent visit. To evaluate the prognosis, the degree of disability was scored with the modified Rankin scale (mRS), which ranged from 1: no residual disability to 4: death (Table 1) [10]. Ethics approval was obtained from the Gunma Children's Medical Center institutional review board, and written informed consent was provided by the patients' parents.

2.2. Biomarker assays

Serum and CSF samples were obtained from each patient at any point during the disease and immediately stored at −80 °C until they were analyzed. Commercially available sandwich enzyme-linked immunosorbent assays (ELISA) for human S100B (BioVendor, Modrice, Czech Republic), NSE (Alpha Diagnostic International, Inc., San Antonio, Texas, USA), and tTau (Invitrogen Corp., Carlsbad, California, USA) were carried out according to the manufacturers' protocols [3]. The detectable range for each ELISA kit was 50–2000 pg/ml, 5–200 ng/ml, and 31.2–2000 pg/ml for S100B, NSE, and tTau protein, respectively.

2.3. Statistical analysis

Data were expressed as the median and interquartile range (IQR) unless otherwise specified. Statistical analysis was performed with the statistical package R (version 2.15.2, available as a free download from http://www.

r-project.org). Comparisons were performed between numerical variables with the Wilcoxon rank-sum test and between proportions with the proportion test. For multiple comparisons between numerical variables or proportions, the Kruskal–Wallis rank sum test or the proportion test were performed, then, if there were significant differences, the pairwise Wilcoxon rank-sum tests or the pairwise proportion tests were performed, adjusted with Holm's method. A P-value < 0.05 was considered statistically significant. As a measure of binary decision performance, the receiver operating characteristic (ROC) curves were assessed, using the area under the curve (AUC) with Bootstrap method [11]. An optimal threshold value (cutoff point) was selected as the situation maximizing the Youden index (Youden index = sensitivity + specificity−1) [12].

3. Results

We collected 497 serum and 274 CSF specimens from 372 patients with various disorders. To evaluate the usefulness of serum/CSF biomarkers as diagnostic or prognostic predictors in the early phase of acute pediatric neurological disorders, we only used specimens taken within 5 days of illness (DOI; the first day of neurological symptoms was regarded as DOI 0) and only investigated diagnostic categories with specimens from more than 5 patients. Thus, 336 serum and 200 CSF specimens from 313 patients were available for evaluation (median age, 2 years; IQR, 1–5 years; male: female ratio, 160:153). Diagnostic categories were as follows (in alphabetical order); acute encephalitis/encephalopathy (AEE), aseptic meningitis (AM), afebrile seizures (AS), controls (CTR), febrile seizures (FS), and septic meningitis (SM). AEE comprised various types of acute encephalitis and encephalopathy, such as acute disseminated encephalomyelitis, acute encephalopathy with biphasic seizures and late reduced diffusion, and acute encephalitis with refractory repetitive partial seizures [13,14]. AS included epileptic seizures or gastroenteritis-related convulsions [15]. CTR included patients who were suspected to have a neurological disorder or involvement, but testing revealed that they did not, such as extra-cerebral infections, Kawasaki disease, and blood disorders. The study

Table 1
Modified Rankin scale.

Score	Description
1	No residual disability; the child attends regular education and does not need remedial teaching[a]
2	Mild residual disability; the child is able to attend regular education but needs remedial teaching because of mild motor disturbances, mild learning disability, or both
3	Severe residual disability; the child has a severe motor deficit (needs braces or wheelchair), severe learning disability, or both, attends a school for special education or is confined to a daily care centre
4	Death

[a] For patient who has underlying condition and disability, the score is determined as 1, unless the disability is worsened after the event.

Table 2
Characteristics of study populations.

Diagnosis	Number	Age (year)	Gender (M:F)	Sampling time (DOI)	mRS
AEE	88	3 (1–6)	37:51	1 (0–3)	2 (1–3)
AM	15	4 (2.5–8.5)	10:5	0 (0–1)	1 (1–1)
AS	52	2 (0.75–4)	20:32	0 (0–0)	1 (1–1)
CTR	85	3.5 (1–8)	40:45	Not available	1 (1–1)
FS	51	1 (1–3)	40:11	0 (0 – 1)	1 (1–1)
SM	22	1 (0–2.5)	13:9	1 (0–2)	1 (1–1)

AEE, Acute encephalitis/encephalopathy; AM, aseptic meningitis; AS, Afebrile seizures; CTR, control; FS, Febrile seizures; SM, septic meningitis; DOI, day of illness, mRS, modified Rankin scale.
Numerical variables are expressed as median (inter quartile range). There were statistically significant differences in age (between AEE and SM*), mRS (between AEE and AM**, AS**, CTR**, FS**, SM*; SM and CTR*), Gender (between FS and AEE**, AS**, CTR**), and sampling time (between AEE and AS**, FS**; SM and AS**) (* denoting P-value less than 0.05, ** less than 0.01).

population characteristics were summarized, and we found statistically significant differences in age, mRS, gender, and sampling time between diagnostic groups (Table 2).

3.1. Serum and CSF biomarkers between each diagnostic group

Initially, the serum and CSF levels of S100B, NSE, and tTau were compared between each diagnostic group (Fig. 1). There were statistically significant differences in serum NSE between AEE (median, 14.5 ng/ml; IQR, 6.0–35.8) and AS (median, 5.0 ng/ml; IQR, 5.0–8.0; $P < 0.001$), AEE and CTR (median, 8.0 ng/ml; IQR, 5.0–13.1; $P = 0.011$), AEE and FS (median, 5.9 ng/ml; IQR, 5.0–11.2; $P = 0.001$), AS and CTR ($P = 0.030$), and AS and SM (median, 9.0 ng/ml; IQR, 5.5–18.0; $P = 0.017$). Significant difference were also observed from serum tTau: AEE (median, 31.2 pg/ml; IQR, 31.2–292.5) and AS (median, 31.2 pg/ml; IQR, 31.2–31.2; $P < 0.001$), AEE and CTR (median, 31.2 pg/ml; IQR, 31.2–31.2; $P < 0.001$), AEE and FS (median, 31.2 pg/ml; IQR 31.2–50.0; $P = 0.006$), AS and SM (median, 40.0 pg/ml; IQR, 31.2–122.5; $P = 0.009$), CTR and SM ($P < 0.001$). For CSF S100B, we observed significant differences between AEE (median, 90.0 pg/ml; IQR 58.7–300.0) and CTR (median, 50.0 pg/ml; IQR, 50.0–50.0; $P < 0.001$), AEE and FS (median, 51.5 pg/ml; IQR, 50.0–77.4; $P = 0.011$), CTR and SM (median, 130.0 pg/ml; IQR, 79.6–272.2; $P < 0.001$), and FS and SM ($P = 0.006$). For CSF tTau, we found significant differences between AEE (median, 230.0 pg/ml; IQR, 116.0–800.0) and FS (median, 100.0 pg/ml; IQR, 50.0–141.8; $P = 0.002$).

3.2. Serum and CSF biomarkers between patients with good and poor prognoses

Next, the serum and CSF levels of S100B, NSE, and tTau were compared between patients with good and poor prognoses (defined as mRS of 1–2 and 3–4,

respectively) (Fig. 2). The levels of all the measured biomarkers were significantly higher in patients with poor prognosis than in those with good prognosis, i.e., serum S100B, good (median, 50.0 pg/ml; IQR 50.0–70.4) vs. poor (median, 66.6 pg/ml; IQR 50.0–581.0; $P < 0.001$); serum NSE, good (median, 7.5 ng/ml; IQR, 5.0–15.0) vs. poor (median, 16.3 ng/ml; IQR, 7.3–132.9; $P < 0.001$); serum tTau, good (median, 31.2 pg/ml; IQR 31.2–40.0) vs. poor (median, 227.7 pg/ml; IQR, 31.2–1603.0; $P < 0.001$); CSF S100B, good (median, 60.0 pg/ml; IQR, 50.0–120.0) vs. poor (median, 182.8 pg/ml; IQR, 81.7–340.0; $P < 0.001$); CSF NSE, good (median, 5.0 ng/ml; IQR 5.0–5.0) vs. poor (median, 5.0 ng/ml; IQR, 5.0–7.2; $P = 0.020$); and CSF tTau, good (median, 118.7 pg/ml; IQR, 50.0–294.2) vs. poor (median, 319.3 pg/ml; IQR, 120.8–1900.0; $P = 0.002$).

3.3. Evaluations of diagnostic and prognostic validities, using ROC curve analyses

Finally, in order to evaluate diagnostic and prognostic validities, we applied ROC curve analyses. To qualify the diagnostic and prognostic validities, we analyzed each biomarker's ability to distinguish AEE from FS and between poor and good prognoses. Then we drew the ROC curves and calculated each AUC (Fig. 3). AUCs for diagnosis were as follows: serum S100B, 0.58, 95% confidence interval (CI) 0.50–0.66; serum NSE, 0.71, 95% CI 0.62–0.79; serum tTau, 0.68, 95% CI 0.60–0.76; CSF S100B, 0.72, 95% CI 0.61–0.82; CSF NSE, 0.62, 95% CI 0.56–0.68; and CSF tTau, 0.76, 95% CI 0.64–0.86. AUCs for prognosis were as follows: serum S100B, 0.64, 95% CI 0.56–0.73; serum NSE, 0.71, 95% CI 0.60–0.81; serum tTau, 0.78, 95% CI 0.70–0.86; CSF S100B, 0.72, 95% CI 0.59–0.82; CSF NSE, 0.61, 95% CI 0.49–0.73; and CSF tTau, 0.72, 95% CI 0.60–0.83. Furthermore, optimal threshold values were calculated for biomarkers with AUC > 0.75. The values for CSF tTau (AUC = 0.76) to distinguish AEE from FS were 156.7 pg/ml (Youden index 0.59), sensitivity
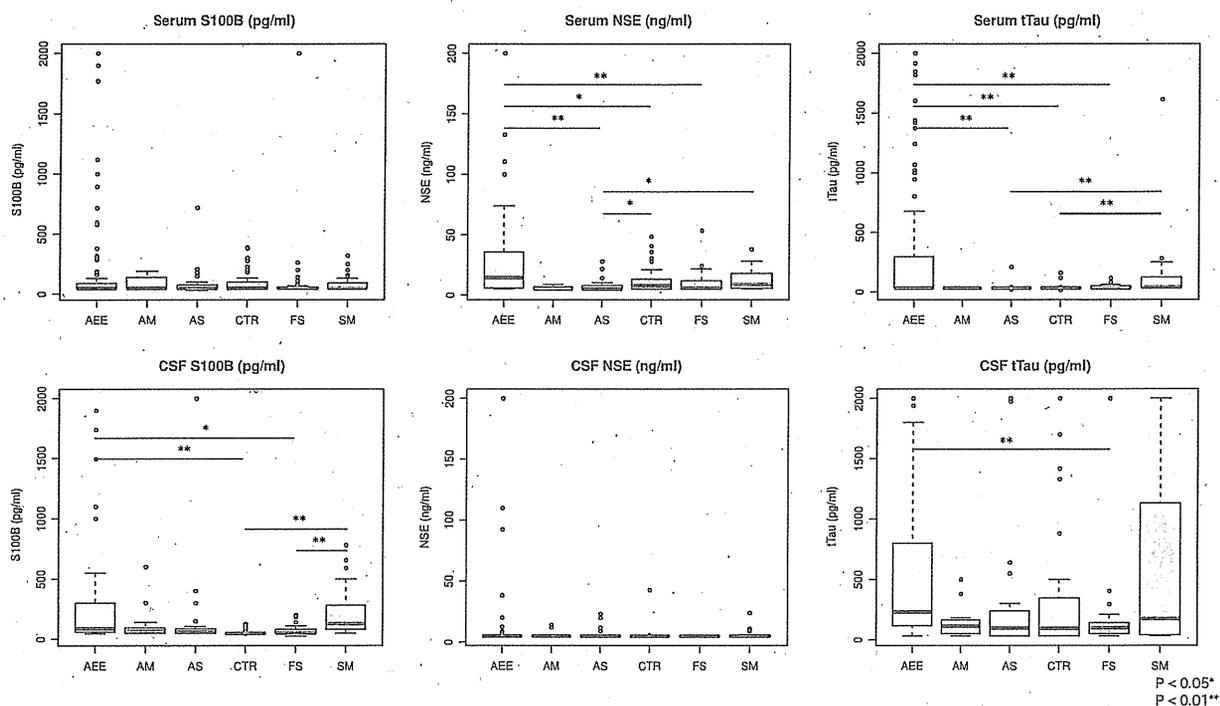
Fig. 1. Boxplot of serum (top) and CSF (bottom) levels of S100B (left), NSE (middle), and tTau (right) from patients with acute encephalitis/encephalopathy, AEE; aseptic meningitis, AM; afebrile seizures, AS; control, CTR; febrile seizures, FS; and septic meningitis, SM. Center lines denote medians, boxes denote 25–75% percentiles, and whiskers denote minimum and maximum values (white circles denote outliers). Parameters with statistically significant differences are noted with asterisks (P < 0.05) or double asterisks (P < 0.01).
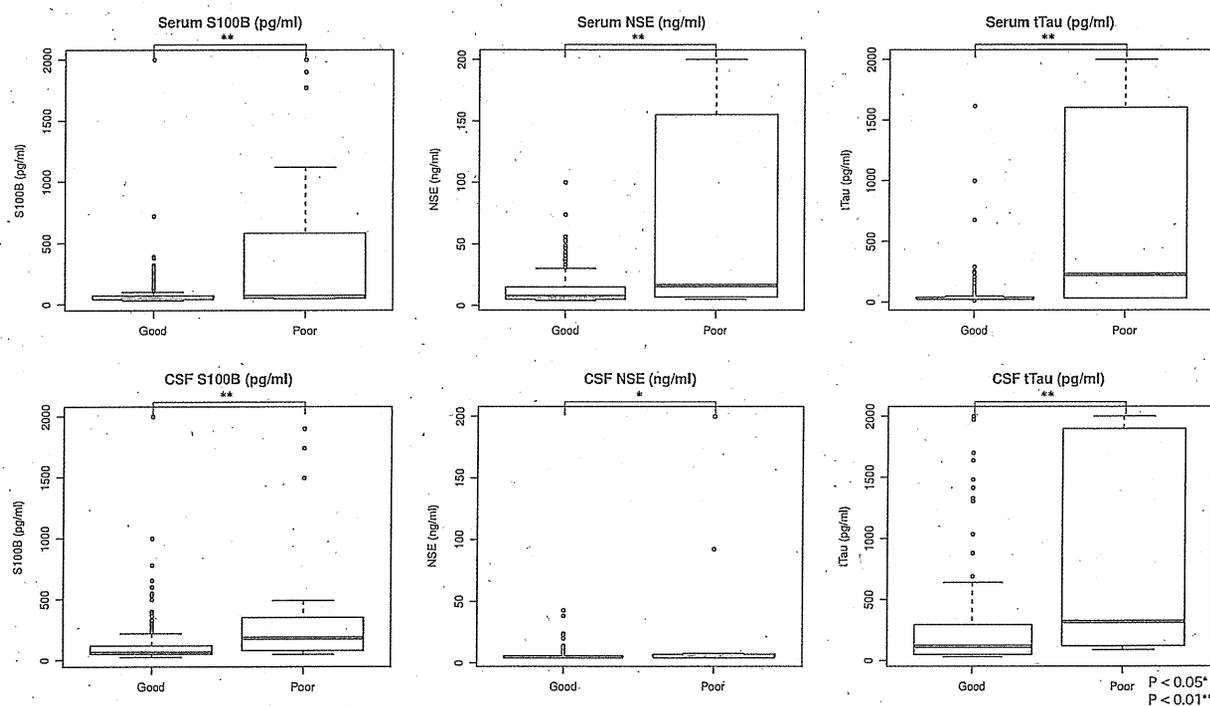

Fig. 2. Boxplot of serum (top) and CSF (bottom) levels of S100B (left), NSE (middle), and tTau (right) from patients with good prognosis (mRS 1–2) and poor prognosis (mRS 3–4). The centerlines denote medians, boxes denote 25–75% percentiles, and whiskers denote minimum and maximum values (white circles denote outliers). Parameters with statistically significant differences are noted with asterisks (P < 0.05) or double asterisks (P < 0.01).
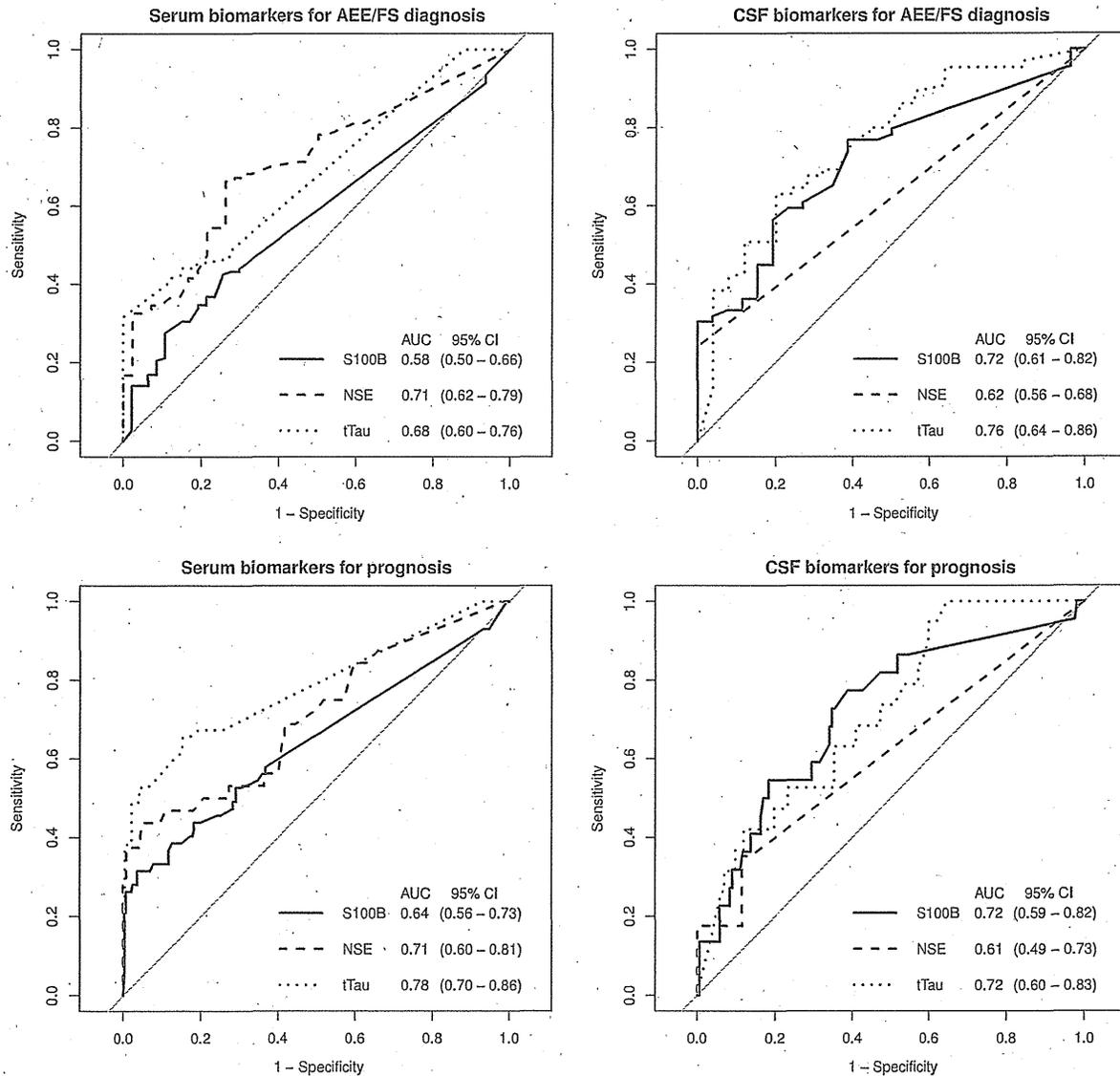
Fig. 3. ROC curves for diagnosis (top) and prognosis (bottom) with serum (left) and CSF (right) biomarkers (solid line, S100B; dashed line, NSE; and dotted line, tTau). AUCs are noted in the right lower corner with 95% CIs.

63.0% (95% CI 52.3–73.9%), and specificity 80.0% (95% CI 64.0–96.0%). The values for serum tTau (AUC 0.78) for distinguishing between poor and good prognoses were 68.8 pg/ml (Youden index 0.50), sensitivity 65.3% (95% CI 51.0–77.6%), and specificity 84.8% (95% CI 79.6–89.5%).

4. Discussion

There have been a many reports regarding the use of serum or CSF biomarkers to monitor various disorders, such as acute ischemic stroke, cerebral hemorrhage, traumatic brain injury, hypoxic ischemic encephalopathy, encephalitis, and meningitis [2–9]. Because different biomarkers were employed across studies and for different disorders, it was challenging to compare findings and establish firm conclusion; however, many biomarkers seemed to be increased in more severe disorders. Alterations in various biomarkers might reflect each central nervous system (CNS) cell damage rather than disease-specific changes. Here, we employed S100B, NSE, and tTau, as astrocytic, neuronal, and axonal damage markers in various acute pediatric neurological disorders to clarify their utility in making more precise diagnostic or prognostic predictions.

There were significant differences in serum NSE, serum tTau, CSF S100B, and CSF tTau (Fig. 1). As a whole, there tended to be the higher levels of the assessed biomarkers levels in AEE, which could reflect

greater CNS damage than other more benign disorders. The level of serum NSE was higher in CTR than AS, however, their CSF NSE levels were not different. The patients in CTR were not healthy controls and NSE is also secreted outside CNS [3,16]. Thus the increased level of serum NSE in CTR must be reflected their extra CNS pathologies. As a prognostic evaluation, all biomarker levels were higher in patients with poor prognoses than in those with good prognoses (Fig. 2).

AEE can resemble FS, especially in an early stage of disease, in terms of fever, seizure, and consciousness disturbance. Therefore, we performed ROC curve analyses not only to differentiate between diagnoses of AEE and FS, but also to distinguish between poor and good prognoses. When AUC is higher than 0.75, the discriminative performance is thought to be good, and when AUC is higher than 0.90, it is thought to be excellent [11,12]. We found that CSF tTau was useful for discriminating AEE from FS (AUC = 0.76), and Serum tTau could differentiate between poor and good prognoses (AUC = 0.78). tTau was originally examined in CSF and was found to be increased in various neurological disorders; later, serum tTau was demonstrated as a good prognostic predictor [2–4,17–21]. Our results emphasize the usefulness of both CSF and serum tTau levels. tTau is considered more CNS specific than S100B and NSE, which corresponds to our findings [22].

Because they have relative low sensitivities and high specificities, as well as optimal threshold values, serum and CSF tTau could be useful for "ruling in" conditions, i.e., if serum or CSF tTau is higher than a threshold value, the patient is likely to have the target state, more severe disorder, or a more grim prognosis [23].

In this study, we did not employ a strict protocol for sampling timing or frequency. Therefore, it was not possible to do longitudinal analyses with serial specimens. Because CSF sampling is more invasive, serial CSF sampling is impractical. However, blood sampling is less invasive, so serial sampling for serum tTau examinations in various disorders could be a good strategy for further research. Finally, we would like to mention that an obstacle for clinical utilization of these biomarkers was that we used ELISA kits, which would not be suitable for a clinical setting, especially for emergencies in a patient-by-patient manner. Thus, a more convenient way for measuring potentially useful biomarkers is clearly needed.

Acknowledgments

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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*

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).
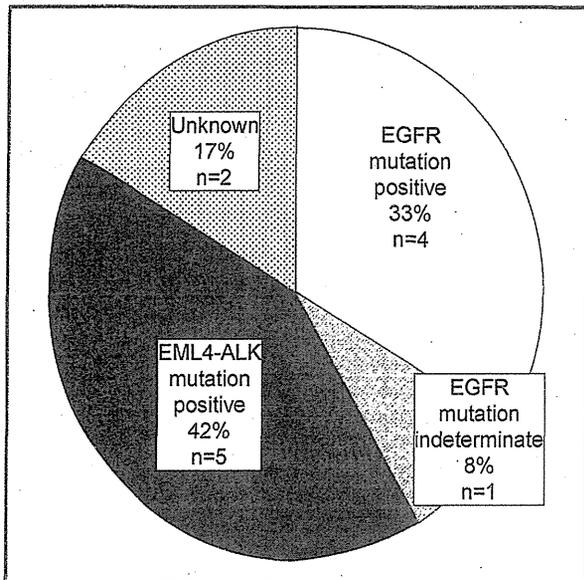
Figure 1. Frequency of gene abnormalities in 12 young patients with lung cancer aged 40 or younger.

The EML4-ALK fusion gene is recognized to be associated with the onset of lung cancer in young patients. EML4-ALK fusion gene has an exclusive relation with EGFR mutation and K-ras mutation (13). The frequency for the EML4-ALK fusion gene in this study was markedly higher than previous studies. Our sample-size was small, but gene abnormalities were identified in 75% in patients aged 40 and younger with lung cancer. Although all patients with EGFR activating mutation were treated with an EGFR-TKI, the overall survival was unsatisfactory. Unfortunately, we did not perform a re-examination for the gene abnormalities in the recurrent tumors. One of the potential mechanisms for short survival for these patients could be explained by the fact that 3 patients were heavy smokers, whose k-ras could be mutated. Moreover, we could evaluate only one case the k-ras status. Furthermore, the overall survival of the patients harboring the EML4-ALK fusion gene was also unsatisfactory, probably because ALK-TKI was not available at that time for these patients.

The results indicated that driver oncogenes were detected in 75% of our cases and that the frequency of EML4-ALK fusion gene was high in the young patients with non-small cell lung cancer. Our finding also suggests that the onset of non-small cell lung cancer in patients aged 40 or younger is more significantly related to gene abnormalities including driver oncogene mutation than to environmental factors.

Conclusions

In this study, we clarified that all 12 patients aged 40 and younger were non-small cell lung cancer and 9 in 12 patients
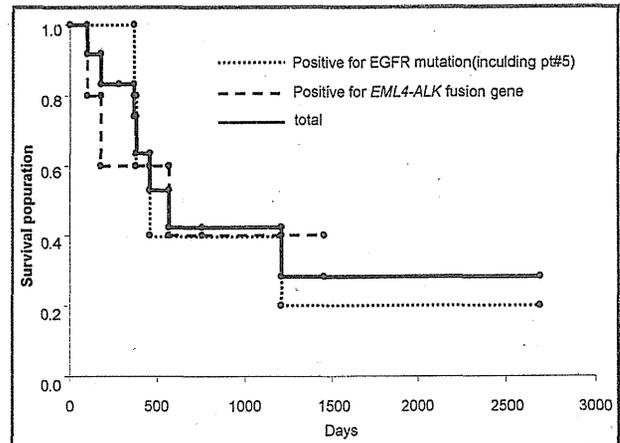

Figure 2. Survival curve of the patients.

were positive for the EGFR gene mutation or the EML4-ALK fusion gene. Our study revealed that ALK fusion gene affected carcinogenesis by the young patients in efficiency more than previous reports. Therefore, examination of gene abnormalities is especially important in young patients with lung cancer to provide a appropriate treatment modality.

Acknowledgements

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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

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.

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