Table 1. Individual patients' characteristics, treatment methods and outcome of the patients treated with concurrent chemoradiotherapy (CRT)

No.	Age (years)	Gender	PS	Stage	CTx	Response	RTx (timing)	RTx (Dose/Fr)	CTx compliance	RTx compliance	Failure site	PFS	OS
C-1	75	F	1	IIIA	CB(5)+ETP(80)2c	PR	From c2	39.6/22	Discontinuation +	Discontinuation +	WT	165	971
C-2	75	M	0	IIIA	CB(5)+ETP(80)3c	PR	From c2	44/22	Discontinuation +	4 days omission	Brain	547	1114
C-3	75	M	1	IIB	CD(80)+ETP(100)4c	PR	From c1	45/30	Dose reduction +	7 days omission	Brain	1790	2393+
C-4	76	M	1	IIB	CD(80)+ETP(100)4c	PR	From c1	45/30	Completed	2 days omission	Brain	214	2485
C-5	77	F	1	IIIB	CD(25)x3+ETP(80)1c→ CB(5)+ETP(80)3c	Near CR	From c2	45/30	Changed CTx regimen and dose reduction	Completed	Liver	201	359

No., number; PS, performance status; CTx, chemotherapy; RTx, radiotherapy; Fr, fraction; PFS, progression-free survival; OS, overall survival; F, female; M, male; CB, carboplatin; ETP, etoposide; c, cycle; CD, cisplatin; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; NA, not available; WT, within the thorax; PF, progression-free.
The dose of carboplatin was indicated by area under the curve in parentheses.
The doses of etoposide and cisplatin were indicated by per body surface area in parentheses.

Patients tended to be female, have lower stage and have a poorer PS in concurrent CRT, although there is no significant difference.

All five patients treated with concurrent CRT exhibited a partial response (PR) and the response rate was 100%. Of the 15 patients treated with sequential CRT, 3 had a complete response (CR), 9 exhibited PR, 1 showed stable disease (SD), 1 developed progressive disease (PD) and 1 was not evaluable (NE). The response rate was 80%. The median PFS of concurrent and sequential CRT were 208 and 216 days, respectively (Fig. 1). There was no statistically significant difference between the PFS of the two treatment methods (log-rank $P = 0.9715$) and the two PFS curves almost overlapped each other.

Of the five patients treated with concurrent CRT, discontinuation of chemotherapy occurred in two (40%) and dose reductions were needed in two due to adverse events (40%). Moreover, discontinuation of radiotherapy occurred in one patient (20%) and omissions were needed in three (60%). Among the 15 patients treated with sequential CRT, 11 completed the whole treatment method without discontinuation, dose reduction and omission of chemotherapy/TRT. Dose reductions of chemotherapy were needed in two patients (13%), and one of the two patients was treated with etoposide (100 mg/m^2, days 1–3) plus cisplatin (25 mg/m^2, days 1–3). Discontinuation of chemotherapy occurred in two patients (13%) due to toxicities. Radiotherapy was completed without omission in all 11 patients who received sequential radiotherapy.

Table 4 shows the adverse events in patients treated with concurrent CRT and sequential CRT. Hematological toxicities, febrile neutropenia, fatigue and anorexia tended to be more frequent and severe in concurrent CRT than in sequential CRT. However, Grade 3 or more severe pneumonitis tended to be frequent in sequential CRT (four patients, 27%).

Patients' Characteristics, Tumor Response, PFS, OS and Toxicity in Patients Treated with Etoposide Plus Carboplatin Followed by Sequential TRT

Twelve patients were treated with etoposide plus carboplatin followed by sequential TRT. The number of male patients, 10 (83%), was larger than that of the female patients, and the median age of the patients was 79 years. Eight patients (67%) had a PS of 0 and the remaining a PS of 1. All were smokers, and 10 patients (83%) were Stage IIIA or IIIB and the remaining Stage IIA or IIB.

With regard to the tumor response, CR was achieved by three patients, PR by eight and one patient was NE. The response rate was 91%.

The median PFS and OS were 244 and 601 days, respectively (Fig. 2). The median follow-up duration was 496 days. In terms of the first failure site during and after CRT, nine patients (75%) had experienced disease relapse at the time of data analyses. Five (42%) and two (17%) patients

Table 2. Individual patients' characteristics, treatment methods and outcome of the patients treated with sequential CRT

No.	Age (years)	Gender	PS	Stage	CTx	Response	RTx (dose/Fr)	CTx compliance	RTx compliance	Failure site	PFS	OS
S-1	75	M	0	IIIA	CB(5)+ETP(80)4c	PR	45/30	Completed	Completed	PF	2754+	2754+
S-2	75	M	0	IIIA	CD(25)x3+ETP(80)4c	SD	45/30	Completed	Completed	Brain	137	578
S-3	75	M	0	IIIA	CD(25)x3+ETP(100)4c	PD	50/25	Dose Reduction +	Completed	WT	143	769
S-4	76	M	1	IIIB	CB(5)+ETP(80)4c	PR	45/30	Dose Reduction +	Completed	WT and liver	414	652
S-5	76	M	1	IIIA	CB(5)+ETP(80)4c	CR	45/30	Completed	Completed	Brain	137	257
S-6	77	M	1	IIA	CB(5)+ETP(80)4c	PR	45/30	Completed	Completed	PF	442+	442+
S-7	77	M	0	IIIB	CD(25)x3+ETP(80)3c	PR	NA	Discontinuation +	NA	WT	243	454
S-8	78	M	1	IIIA	CB(5)+ETP(80)4c	PR	59/32	Completed	Completed	Brain	181+	181+
S-9	78	M	0	IIIA	CB(5)+ETP(80)4c	PR	45/30	Completed	Completed	Brain	181	550+
S-10	80	F	1	IIIA	CB(5)+ETP(80)1c	NE	NA	Discontinuation +	NA	WT	70	316+
S-11	80	M	0	IIIB	CB(5)+ETP(80)4c	CR	45/30	Completed	Completed	Brain	152	258
S-12	81	F	1	IIB	CB(5)+ETP(80)4c	PR	50/25	Completed	Completed	PF	1892+	1892+
S-13	83	M	1	IIIB	CB(5)+ETP(80)4c	CR	45/30	Completed	Completed	Brain	269	327
S-14	83	F	1	IIIA	CB(5)+ETP(80)4c	Near CR	50/25	Completed	Completed	Liver and lung	408	415+
S-15	92	M	0	IIIA	CB(5)+ETP(80)4c	PR	45/30	Completed	Completed	WT	218	383

The dose of carboplatin was indicated by area under the curve in parentheses.
The doses of etoposide and cisplatin were indicated by per body surface area in parentheses.

Table 3. Individual patients' characteristics, past history and complications of the patients treated with concurrent CRT and sequential CRT

No	Age (years)	Gender	PS	Stage	Past history	Complications
C-1	75	F	1	IIIA	–	Osteoarthritis
C-2	75	M	0	IIIA	–	Anal stenosis
C-3	75	M	1	IIB	Gastric ulcer	COPD, prostatic hypertrophy
C-4	76	M	1	IIB	Gastric ulcer	–
C-5	77	F	1	IIIB	–	Hypertension, hyperlipidemia, osteoporosis
S-1	75	M	0	IIIA	–	Arrhythmia, prostate cancer
S-2	75	M	0	IIIA	–	Gastric ulcer, hypertension
S-3	75	M	0	IIIA	–	Prostatic hypertrophy, abdominal aortic aneurism
S-4	76	M	1	IIIB	Abdominal aortic aneurism	IHD, DM, hypertension
S-5	76	M	1	IIIA	Abdominal aortic aneurism	Aortic dissection
S-6	77	M	1	IIA	Laryngeal cancer, brain hemorrhage	Hypertension
S-7	77	M	0	IIIB	Gout, gastritis	Hypertension, prostatic hypertrophy
S-8	78	M	1	IIIA	Bladder cancer, brain hemorrhage	Hypertension
S-9	78	M	0	IIIA	ASO, IHD, gastric ulcer	–
S-10	80	F	1	IIIA	IHD, pneumothorax, gout, renal failure	COPD
S-11	80	M	0	IIIB	Rectal cancer	–
S-12	81	F	1	IIB	–	IHD
S-13	83	M	1	IIIB	Asthma, gastric ulcer, colon cancer	Hypertension
S-14	83	F	1	IIIA	Uterine cancer	Hypertension
S-15	92	M	0	IIIA	–	Reflux esophagitis, hypertension

COPD, chronic obstructive pulmonary disease; IHD, ischemic heart disease; DM, diabetes mellitus; ASO, arteriosclerosis obliterans.

experienced disease relapse outside the thorax and within the thorax, respectively. Two patients experienced disease relapse both within and outside the thorax. The most common first failure organ was the brain (five patients, 42%).

Table 5 shows the adverse events in these 12 patients. Although there were moderate levels of hematological toxicities, gastrointestinal toxicities tended to be mild. It is noteworthy that Grade 3 or more severe pneumonitis occurred in four patients (33%).

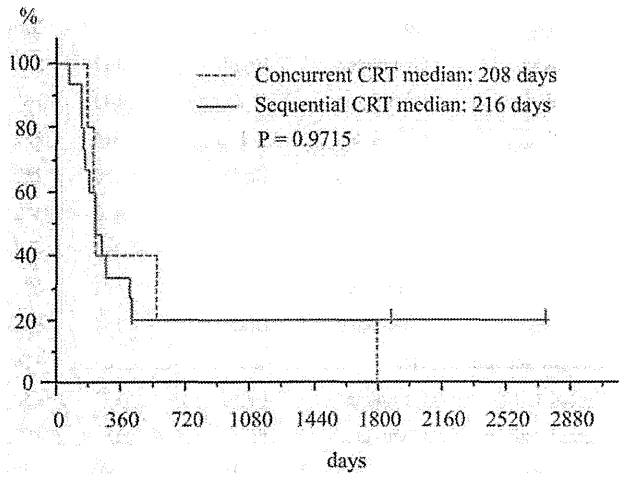

Figure 1. Kaplan–Meier curves for the progression-free survival (PFS) of patients aged 75 years or older treated with concurrent chemoradiotherapy (CRT) and sequential CRT are shown (concurrent CRT, red dashed line; sequential CRT, blue continuous line). The median PFS was 208 days in concurrent CRT and 216 days in sequential CRT. There was no statistically significant difference between the two groups (log-rank $P = 0.9715$).

DISCUSSION

Our investigation is important as it includes a considerable number of LD-SCLC patients aged 75 years or older who have been treated with CRT. Moreover, as this study documents a precise clinical course (i.e. treatment response, PFS, OS, treatment compliance and adverse events), it will enable physicians to determine the optimal treatment strategy for this category of patients.

Two previous research papers have detailed clinical course data in studies similar to ours. In one study, seven LD-SCLC patients aged 75 years or older were treated with etoposide plus cisplatin or carboplatin and with concurrent TRT (14). TRT treatment was delayed for more than 7 days in three of the seven patients. Three experienced Grade 3 or more severe febrile neutropenia, and three experienced

Table 4. Adverse events in patients treated with concurrent CRT and sequential CRT

	Concurrent chemoradiotherapy ($n = 5$)						Sequential chemoradiotherapy ($n = 15$)					
	Gr 1	Gr 2	Gr 3	Gr 4	≥Gr 3 (%)	All (%)	Gr 1	Gr2	Gr 3	Gr 4	≥Gr 3 (%)	All (%)
Leukopenia	0	0	3	2	100	100	1	6	8	0	53	100
Neutropenia	0	0	0	5	100	100	1	0	3	11	93	100
Anemia	0	4	1	0	20	100	3	7	2	0	13	80
Thrombocytopenia	2	2	1	0	20	100	6	3	3	1	27	87
Fatigue	1	1	1	0	20	60	7	2	0	0	0	60
Anorexia	2	1	1	0	20	80	6	5	0	0	0	73
Constipation	2	2	0	0	0	80	12	1	0	0	0	87
Nausea	2	2	0	0	0	80	6	1	0	0	0	47
Infection	0	2	0	0	0	40	1	1	1	0	7	20
Febrile neutropenia	0	0	3	0	60	60	0	0	2	0	13	13
Bilirubin	1	0	0	0	0	20	2	1	0	0	0	20
AST	0	0	0	0	0	0	2	0	0	0	0	13
ALT	1	0	0	0	0	20	3	0	0	0	0	20
Hyponatremia	2	0	0	1	20	60	4	0	1	1	13	40
Creatinine elevation	1	0	0	0	0	20	3	2	0	0	0	33
Pneumonitis	4	0	0	0	0	80	7	0	3	1	27	73
Esophagitis	1	3	1	0	20	100	5	4	0	0	0	60
Dermatitis	4	0	0	0	0	80	9	0	0	0	0	60
Eruption	2	0	0	0	0	40	1	1	0	0	0	13

Gr, grade; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

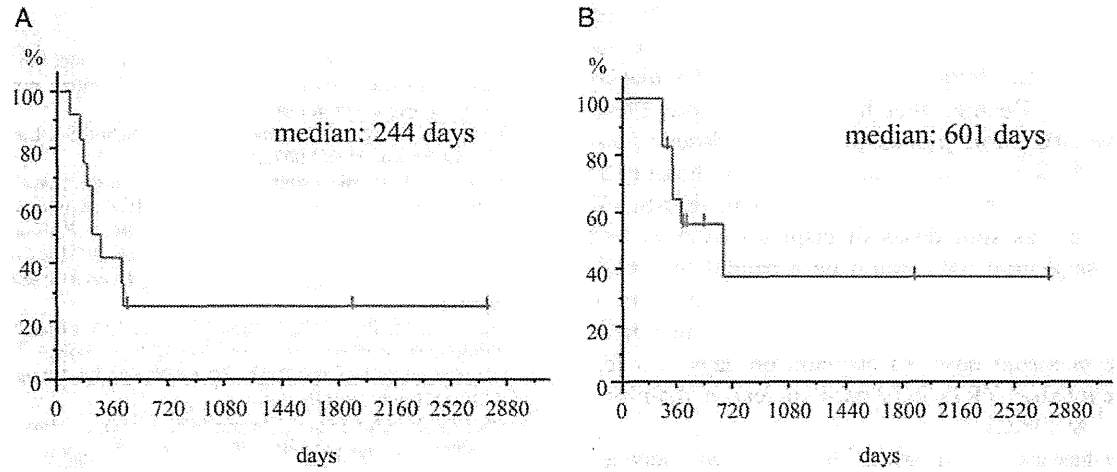

Figure 2. The Kaplan–Meier curve for the PFS (A) and overall survival (OS) (B) of 12 patients aged 75 years or older, treated with etoposide plus carboplatin followed by sequential thoracic radiotherapy is shown. The median PFS and OS were 244 and 601 days, respectively.

Table 5. Adverse events in patients treated by etoposide plus carboplatin and sequential radiotherapy, $n = 12$

	Gr 1	Gr 2	Gr 3	Gr 4	≥Gr 3 (%)	All (%)
Leukopenia	0	6	6	0	50	100
Neutropenia	0	0	3	9	100	100
Anemia	2	5	2	0	17	75
Thrombocytopenia	4	3	2	1	25	83
Fatigue	5	2	0	0	0	58
Anorexia	5	4	0	0	0	75
Constipation	9	1	0	0	0	83
Nausea	5	0	0	0	0	42
Infection	1	1	1	0	8	25
Febrile neutropenia	0	0	2	0	17	17
Bilirubin	1	1	0	0	0	17
AST	1	0	0	0	0	8
ALT	3	0	0	0	0	25
Hyponatremia	3	0	0	0	0	25
Creatinine elevation	2	2	0	0	0	33
Pneumonitis	5	0	3	1	33	75
Esophagitis	5	3	0	0	0	67

Grade 4 thrombocytopenia. One patient died due to radiation pneumonitis and this was judged as treatment-related death. In the second study, the outcome of elderly patients aged 70 years or older, five of whom were 75 years or older, who received early concurrent CRT with four cycles of etoposide plus cisplatin, was reported (15). Of the 12 patients in this report, 8 (67%) experienced Grade 3 or more severe febrile neutropenia. Of the five patients aged 75 years or older, three could not complete the four cycles of chemotherapy and all five experienced delayed TRT for more than 7 days. In our study, five patients received concurrent CRT and two could not complete the chemotherapy course due to toxicities. TRT was discontinued in one patient and another experienced delayed TRT for more than 7 days due to toxicities. These patients suffered from prolonged toxicities and their quality of life decreased for a long time. Moreover, it is speculated that fitter patients were treated by concurrent CRT and more fragile patients were treated by sequential CRT. Therefore, it is suggested that concurrent CRT is not feasible for all LD-SCLC patients aged 75 years or older. Moreover, a high frequency of discontinuation, dose reduction and omission of chemotherapy/TRT in concurrent CRT may lead to a similar PFS as that achieved with sequential CRT.

Based on the previous Phase III study which investigated chemotherapeutic regimen for elderly or poor-risk patients with ED (extensive disease)-SCLC (16) and the convenient administration schedule of carboplatin, etoposide (80 mg/m^2) on days 1–3 plus carboplatin (AUC 5) on day 1 followed by sequential TRT 45Gy in twice-daily fractions or 50 Gy in a once-daily fraction was the most frequently used treatment method for LD-SCLC patients aged 75 years or older in our institute. In our study, the major adverse events of etoposide plus carboplatin followed by sequential TRT were hematological toxicities, including neutropenia and thrombocytopenia. Gastrointestinal toxicities such as anorexia, nausea, vomiting and constipation were very mild. All of the toxicities were manageable and no treatment-related death occurred. The response rate, OS and PFS were satisfactory, when taking the patients' characteristics in our study and the results of the previous Phase II studies that evaluated CRT for LD-SCLC patients aged 70 years or older, into account (17, 18). However, as Grade 3 or more severe pneumonitis occurred in 4 of 12 patients (33%) similar to a retrospective subset analysis of LD-SCLC patients treated with etoposide plus cisplatin and concurrent early CRT in a Phase III trial (10), attention should be paid to the occurrence of radiation

pneumonitis. It may be appropriate to set the radiation field based on the tumor volume after induction chemotherapy to reduce the frequency and severity of radiation pneumonitis (19). On the other hand, the previous Phase III study have also shown etoposide plus split doses of cisplatin seems to be another standard chemotherapeutic regimen for elderly or poor-risk patients with ED-SCLC (16). Etoposide plus split doses of cisplatin on days 1–3 followed by sequential TRT could be a candidate for the standard treatment of LD-SCLC patients aged 75 years or older. However, because only three patients were treated by etoposide plus split doses of cisplatin on days 1–3 followed by sequential TRT, it is hard to lead a definitive conclusion in this study.

Our study has a few limitations. The intervals between evaluations for lesions in this study were not as accurate as those in a prospective study. The severity of non-hematological toxicities, in particular, may have been underestimated in the present study due to its retrospective nature. Patients were treated as inpatients during most of the treatment period, and the toxicity data were recorded in detail in the patients' medical records. The sample size in this study is not very large; therefore, it is difficult to reach a definitive conclusion. However, as it is not easy to collect data on a large number of LD-SCLC patients aged 75 years or older who have received CRT, this study may be useful for physicians trying to determine the optimal treatment strategy for LD-SCLC patients aged 75 years or older.

In conclusion, it is suggested that concurrent CRT is not feasible for all LD-SCLC patients aged 75 years or older. Etoposide (80 mg/m^2) on days 1–3 plus carboplatin (AUC 5) on day 1 followed by sequential TRT is one of the candidates for the standard treatment of these elderly LD-SCLC patients. A further prospective clinical trial is warranted to develop and evaluate the optimal treatment method for LD-SCLC patients aged 75 years or older.

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Conflict of interest statement

None declared.

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Case report

An extremely rare case of small-cell lung cancer harboring variant 2 of the *EML4-ALK* fusion gene

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ABSTRACT

Anaplastic lymphoma kinase (*ALK*) fuses *echinoderm microtubule-associated protein-like 4* (*EML4*) to acquire a transforming activity in lung adenocarcinomas. However, the presence of an *EML4-ALK* fusion gene in other lung cancer histologies is an extremely rare phenomenon. A 43-year-old female was referred to our department due to dyspnea on effort and left back pain. Computed tomography (CT) showed a large mass in the upper lobe of the left lung and a massive left pleural effusion, while a CT-guided needle biopsy confirmed a diagnosis of small-cell lung cancer (SCLC). Surprisingly, the tumor was genetically considered to harbor the *EML4-ALK* fusion gene (variant 2). Although the patient underwent two regimens of cytotoxic chemotherapy for SCLC, she died approximately seven months after the administration of first-line chemotherapy. Our analysis of 30 consecutive patients with SCLC for *EML4-ALK* revealed that two patients, including the current patient and a patient we previously reported, harbored the *EML4-ALK* fusion gene.

1. Introduction

Oncogenic driver mutations, such as *epidermal growth factor receptor* (*EGFR*), *anaplastic lymphoma kinase* (*ALK*) and so on, have been shown to play essential roles in tumorigenesis, survival and proliferation in lung cancer, especially adenocarcinoma [1,2]. Driver mutations have attracted attention as potential targets of kinase inhibitors [2,3]. In addition to the molecular pathogenesis of lung adenocarcinomas, genetic insights into the pathogenesis of squamous cell carcinoma and small-cell lung cancer (SCLC) have recently been reported [4,5]. However, to the best of our knowledge, there is only one case of *echinoderm microtubule-associated protein-like 4* (*EML4*)-*ALK*-positive SCLC combined with adenocarcinoma, which we previously reported [6]. We herein report a genetically rare case of SCLC harboring an *EML4-ALK* fusion gene and describe the patient's clinical course.

2. Case report

A 43-year-old female ex-smoker of five pack-years was referred to our hospital due to dyspnea on effort and left back pain. A chest X-ray showed a large mass shadow in the left upper lung field and decreased transparency in the left lower lung field. Computed tomography (CT) revealed a large, irregular mass with a maximum diameter of 10 cm in the left upper lobe invading the 4th rib (Fig. 1A) and a massive left pleural effusion. Laboratory examinations revealed elevations in the levels of neuron specific enolase (NSE; 37.7 ng/ml) and pro-gastrin-releasing peptide (ProGRP; 1740 ng/ml), whereas no abnormalities were observed in other tumor markers. A CT-guided tumor biopsy was then performed, and the tumor was pathologically diagnosed as small-cell lung cancer (SCLC) with immunoreactivity to synaptophysin and CD56 (Fig. 2A and B), while no immunoreactivity against thyroid transcription factor-1 (TTF-1) was observed (Fig. 2C). The clinical stage was ultimately determined to be IV (cT3N0M1a: extensive disease). Multiplex reverse transcription-polymerase chain reaction (RT-PCR) and direct sequencing methods revealed the tumor to harbor variant 2 of the *EML4ALK* fusion gene (Fig. 2D), whereas no mutations of *epidermal growth factor receptor* (*EGFR*) or *TP53* were observed (data not shown). As the performance status of the patient

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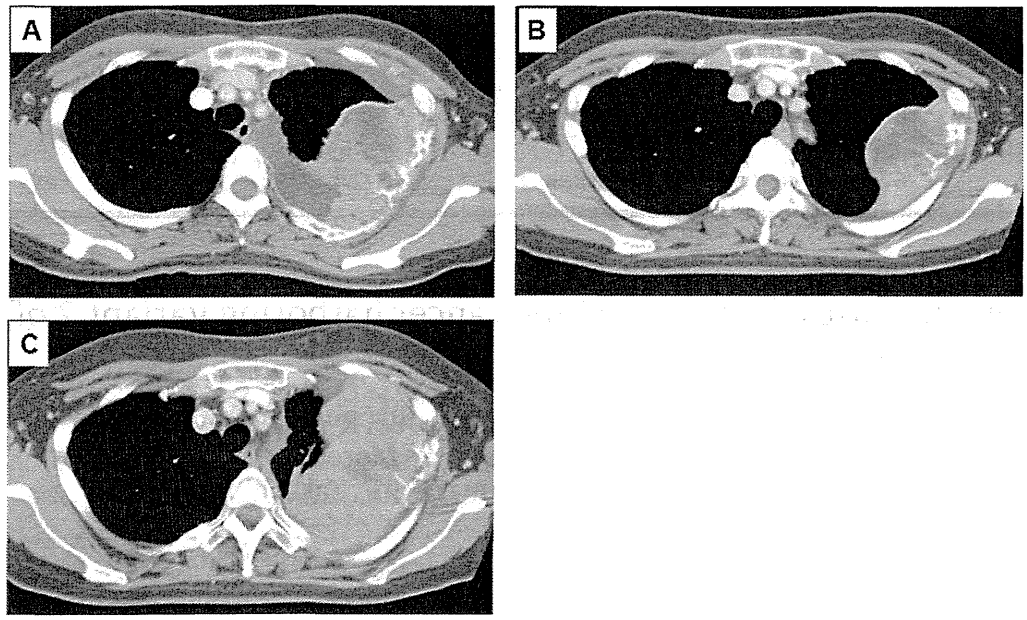

Fig. 1. Computed tomography showed a large mass invading the left 4th rib. (A) CT showed the mass approximately 2.5 (B) and four months (C) after the administration of first-line chemotherapy.

was 3, carboplatin (CBDCA) in combination with etoposide (VP-16) was administered as a first-line regimen with daily thoracocentesis of the pleural effusion. Since the PS improved from 3 to 0 following the administration of one cycle of CBDCA + VP-16, the patient underwent three cycles of cisplatin (CDDP) + VP-16. Although a partial response (PR) was achieved (Fig. 1B) and the levels of NSE and ProGRP decreased (9.9 and 409 ng/ml, respectively) after four cycles of chemotherapy, progressive disease was observed 1.5 months

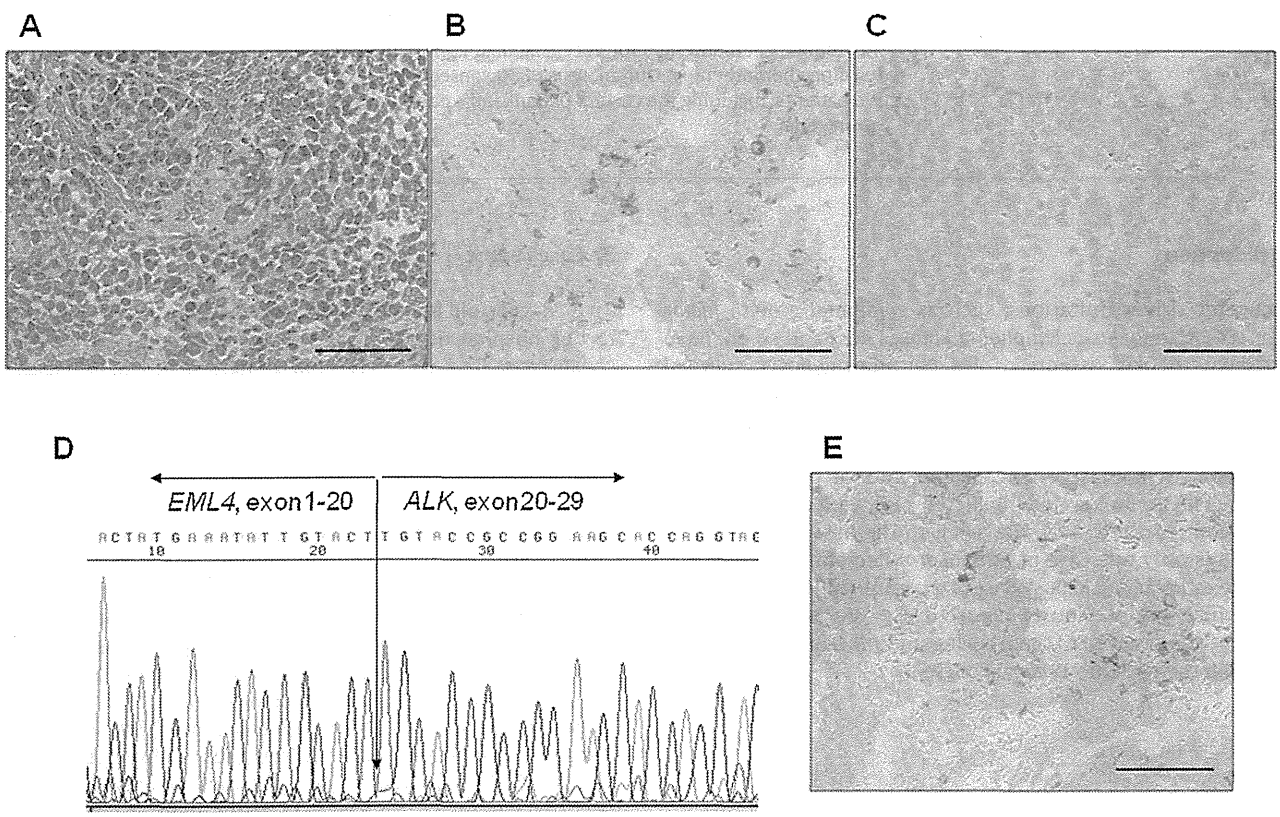

Fig. 2. (A) Microscopic findings of the tumor indicated small, round cells with abundant chromatin. (B) Immunohistochemistry using a specific antibody against synaptophysin (27G12, Novocastra) showed the tumor to be positively stained. (C) Immunohistochemistry using an antibody with specificity for thyroid transcription factor-1 (TTF-1; 8G7G3/1, Dako) showed that the tumor did not have immunoreactivity for TTF-1. (D) The direct sequencing method identified variant 2 of the *echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase* (*ALK*) fusion gene. (E) Immunostaining using an antibody that specifically detects ALK (5A4, Nichirei) revealed immunopositivity of the tumor for ALK. Scale bar (A–C, E): 50 μm.

after the confirmation of a PR (Fig. 1C). Thereafter, a CT-guided biopsy was performed again, and the SCLC histology was reconfirmed. Furthermore, the presence of the *EML4-ALK* fusion gene was confirmed on immunohistochemistry (IHC) using an antibody that specifically detects ALK (Fig. 2E). Although amrubicin was then administered, the disease continued to progress. Approximately six months after the administration of the first-line chemotherapy, the patient was transferred to another hospital for hospice care and died 18 days after the transfer. Based on her clinical course, the progression-free survival (PFS) and overall survival (OS) from the administration of the first-line therapy were approximately four and seven months, respectively.

3. Discussion

Gene mutations in tyrosine kinases play essential roles in the pathogenesis of lung adenocarcinoma and have attracted much attention as potential therapeutic targets in the treatment of adenocarcinoma. The *ALK* gene has been shown to fuse the *EML4* gene, and as a consequence, to possess a transforming activity [1]. Importantly, tumors with the *EML4-ALK* fusion gene, the second most well-known tyrosine kinase in lung adenocarcinoma, can be successfully treated with ALK inhibitors [7]. Mutations of the *EGFR* gene in SCLC have already been identified (5/122: 4%) [8], and integrative genomic analyses have revealed mutations of tumor suppressor genes (TP53 and RB1), histone modifiers (MLL1) and so on in SCLC. However, to the best of our knowledge, there has been only one case of an SCLC patient harboring the *EML4-ALK* fusion gene [6]. In our previous case, fusion of the *ALK* gene to the *EML4* gene was intriguingly detected only in the SCLC component of the resected combined adenocarcinoma with SCLC. Although this previous patient harbored variant 1 of the *EML4-ALK* fusion gene, variant 2 of the fusion gene was identified in the current case. Based on these findings, there are considered to be multiple *EML4-ALK* variants in SCLC patients as well as adenocarcinoma patients. We analyzed 30 consecutive SCLC patients whose RNAs were available for RT-PCR and direct sequencing methods between April 2010 and March 2012. Two of the patients, the present patient and the patient we previously reported [6], were found to harbor the fusion gene. Although a positive reaction of IHC for the ALK protein expression without *ALK* fusion was reported to be found in a patient with SCLC [9], this does not apply to the current case because the fusion was detected using RT-PCR and direct sequencing methods. Furthermore, the possibility of the transformation of adenocarcinoma into SCLC, which is associated with the acquisition of resistance to EGFR-tyrosine kinase inhibitors (TKIs), should be taken into consideration [10]. However, this mechanism does not apply to the present patient, since no EGFR-TKIs were administered because of the absence of the *EGFR* mutations.

One of the limitations of the current case report is that the tumor was diagnosed to be SCLC by a biopsy sample. Although biopsy samples do not always reflect the exact histology of the whole tumor, and the absence of lymphadenopathy and *p53* mutations, which occur in more than 90% of all SCLCs [11], is relatively rare, the SCLC histology was confirmed by several findings in the present case. First, a CT-guided biopsy before and after the first-line chemotherapy diagnosed the tumor to be morphologically SCLC. Second, immunoreactivity of the tumor for synaptophysin and CD56 was observed. Third, the levels of tumor markers associated with SCLC, i.e., NSE and ProGRP, were elevated, while no elevation was observed in the levels of carcinoembryonic antigen and cytokeratin 19 fragment. Finally, combination chemotherapy with platinum plus VP-16, one of the standard regimens for patients with SCLC, led to a partial response. With regard to TTF-1 expression, TTF-1 was reported to be expressed in all adenocarcinomas harboring the *ALK* rearrangement [12], and TTF-1 expression was also observed in about 80% of SCLCs [13]; however, the current patient showed no expression of TTF-1, as shown in Fig. 2C, which was different from the results we previously reported in Ref. [6], and no definite correlation between TTF-1 expression and the *EML4-ALK* rearrangement in SCLC has been demonstrated so far. Although these findings show an apparently rare presentation of SCLC in the current patient, future studies would help to elucidate the characteristics of patients with SCLC harboring the *EML4-ALK* rearrangement.

Although SCLC manifests with aggressive features, such as rapid progression, these tumors are generally sensitive to chemotherapy. For first-line therapy, the response rate, median PFS and OS range from 67.5 to 84.4%, 4.7–6.9 months and 9.4–12.8 months, respectively [14,15]. Although the current patient achieved a PR after undergoing four cycles of platinum-based chemotherapy, the PFS and OS were much worse than those of historical controls. As a reason for the poor clinical course of the present patient, there is a possibility that the fusion gene affects sensitivity to chemotherapy. There have been two reports on chemosensitivity in patients with the *EML4-ALK* fusion gene [16,17]. Lee et al. reported that *ALK*-positive non-SCLC patients would benefit significantly from pemetrexed chemotherapy, whereas Takeda et al. demonstrated that the efficacy of first-line platinum-based chemotherapy does not depend on the presence or absence of the *EML4-ALK* fusion gene. Therefore, although the significance of *ALK*-positivity for chemosensitivity has yet to be clarified, *EML4-ALK* fusion may be involved in the sensitivity of platinum-based chemotherapy.

4. Conclusion

We herein reported a very rare case of SCLC in which the patient harbored variant 2 of the *EML4-ALK* fusion gene. Although the frequency and significance of the fusion gene in SCLC patients has not been determined, this phenomenon suggests that SCLC patients harboring the *EML4-ALK* fusion gene can be successfully treated with ALK inhibitors.

Conflict of interest statement

Drs. Takenoyama, Shiraishi, Hirai, Yamaguchi, Seto and Ichionose have conflicts of interest with Pfizer, AstraZeneca and Chugai to disclose as shown in the attached file. The other authors have no conflicts of interest to declare.

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Safety and Efficacy of Platinum Agents plus Etoposide for Patients with Small Cell Lung Cancer with Interstitial Lung Disease

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Abstract. *Background: The safety and efficacy of combination of platinum agents plus etoposide for patients with small cell lung cancer (SCLC) with pre-existing interstitial lung disease (ILD) is uncertain. Patients and Methods: Fifty-two patients received platinum agents plus etoposide as first-line chemotherapy for SCLC with pre-existing ILD. The clinical characteristics, treatment outcome and survival of these patients were retrospectively reviewed. Results: During first-line chemotherapy, only one (2%) out of the 52 patients developed an acute exacerbation of ILD. The median number of treatment cycles was four. The overall response rate was 69%. The median progression-free survival period was 4.5 months. The median survival time was 9.4 months. Thirty-three patients (63%) received at least one subsequent chemotherapy regimen, and five of these patients developed acute exacerbation of ILD. Conclusion: The combination of platinum agents plus etoposide is feasible and effective in SCLC patients with pre-existing ILD, compared with regimens after second-line chemotherapy.*

Small cell lung cancer (SCLC) accounts for 15% to 20% of all lung cancer cases (1). SCLC is characterized by rapid growth and widespread metastatic disease, and most patients have extensive disease at the time of diagnosis. SCLC is significantly sensitive to chemotherapy or radiation therapy, and therefore systemic chemotherapy is recognized as a standard treatment (2). The standard chemotherapy regimen for SCLC patients is the combination of platinum agents plus etoposide or platinum agents plus irinotecan, which is the most frequently used combination and yields a median survival period of approximately 9-12 months in clinical trials (3, 4).

Pre-existing interstitial lung disease (ILD) is one of the most common complications in patients with lung cancer. ILD, also known as diffuse parenchymal lung disease, is a diverse group of pulmonary disorders classified together because of similar clinical, radiographical, physiological, and pathological features (5). In patients with cancer, pre-existing ILD is considered to be a risk factor for acute exacerbation, which is a fatal complication of treatments such as chemotherapy, surgery, and radiation therapy (6, 7). The incidence of lung cancer in patients with ILD is reported to be 20-30% and is higher than that in the general population (8). Kudoh *et al.* reported recently that pre-existing ILD was confirmed to be an important determinant of the development of the acute exacerbation of ILD after chemotherapy for patients with advanced non-small cell lung cancer (NSCLC) (6). However, few reports exist on the association between pre-existing ILD and the safety and efficacy of chemotherapy in patients with SCLC. Whether chemotherapy for patients with SCLC with pre-existing ILD is feasible remains unclear because patients with severe complications, such as pre-existing ILD, have been excluded from most prospective clinical trials.

In this retrospective study, we investigated the safety and efficacy of the combination of platinum agents plus etoposide as a first-line chemotherapy for patients with SCLC with preexisting ILD.

Patients and Methods

Between January 2001 and December 2009, a total of 557 consecutive patients were diagnosed as having SCLC at the National Cancer Center Hospital East. Overall, 52 (11%) of these patients had pre-existing ILD and received first-line chemotherapy. The clinical characteristics, treatment outcome, and survival of these patients were retrospectively reviewed using data obtained from

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Key Words: Interstitial lung disease, small cell lung cancer, chemotherapy, cisplatin, carboplatin, etoposide, acute exacerbation.

Table I. *Patients' characteristics.*

Characteristic	N	%
Number of patients	52	
Age (years)		
Median (range)	71 (50-85)	
Gender		
Male	50	96
Female	2	4
Performance status		
0/1	9/37	88
2/3	5/1	12
Smoking status		
Never smoker	0	0
Current/former	25/27	100
Brinkman index median (range)	1050 (315-2940)	
Clinical stage		
Limited disease	29	56
Extensive disease	23	44

Table II. *Treatment outcome of first-line chemotherapy in 52 patients with small cell lung cancer with pre-existing Interstitial Lung Disease.*

	N	%
Number of patients	52	
First-line chemotherapy regimen		
Carboplatin plus etoposide	22	42
Cisplatin plus etoposide	30	58
Number of cycles (1/2/3/4)	8/6/6/32	15/12/12/61
Objective response		
CR	1	
PR	35	
SD	9	
PD	3	
NE	4	
Overall response rate (LD/ED)		72/65

CR, Complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; LD, limited disease; ED, extensive disease.

their medical records. The patients were staged according to the staging system of the Veterans Administration Lung Cancer Study Group as limited disease (LD) or extensive disease (ED) (9). In this study, two independent pulmonologists (T.Y. and K.Y.) who had no knowledge of the patients' outcome diagnosed pre-existing lung conditions based on pre-treatment chest computed tomography (CT) findings obtained before first-line chemotherapy. Pre-treatment conventional CT or high-resolution CT (HRCT) films of the chest were used in our analysis. Pre-existing ILD was diagnosed when diffuse ground-glass opacity, peripheral reticular opacity, and consolidation without segmental distribution and a honeycomb pattern were detected in bilateral lung fields on the chest X-ray and CT findings. The acute exacerbation of ILD was diagnosed based on the chest X-ray and/or CT findings, which showed newly-developed diffuse pulmonary opacities, physical findings, and serum levels of markers of damaged pneumocytes [*i.e.* lactate dehydrogenase (LDH), C-reactive protein (CRP), Krebs von den Lungen-6 (KL-6)] and the lack of a response to antibiotics. Patients with pulmonary infection, pneumothorax, pulmonary embolism, or heart failure were excluded.

The objective tumor response was assessed according to the Response Evaluation Criteria Solid Tumor (RECIST) (10). The objective response rate (ORR) was calculated as the total percentage of patients with a complete response (CR) or a partial response (PR). Toxicity was graded using the Common Terminology Criteria for Adverse Events (CTCAE), ver. 3.0 (11). A univariate analysis was performed to identify risk factors for the acute exacerbation of ILD in patients with SCLC with pre-existing ILD. All the variables were analyzed using the Fisher's exact test. Multivariate analyses were performed using logistic regression. A clinical evaluation of progression-free survival (PFS) was measured from the start of the first-line chemotherapy to the identifiable time for progression. The overall survival (OS) was measured as the period from the start of first-line chemotherapy until death from all causes. The PFS and OS were plotted using the Kaplan-Meier method. All the p-values were two-sided, and a level of 5% was considered statistically significant, unless otherwise specified.

Results

Patients' characteristics. The pre-treatment characteristics of the patients are shown in Table I. The median age at the time of first-line chemotherapy for SCLC was 71 years (range=50-85 years), 96% of them were men, and 88% had an Eastern Cooperative Oncology Group performance status (PS) of 0 or 1. All the patients were current or former smokers. None of the patients had histologically-confirmed interstitial pneumonia. Overall, 56% of the patients had LD and 44% had ED. As the first-line chemotherapy, 22 (42%) out of the 52 patients received carboplatin plus etoposide, and 30 (58%) patients received cisplatin plus etoposide (every 3 to 4 weeks). In the three cases, radiation therapy was performed after four cycles of chemotherapy. After progression, 33 patients received second-line chemotherapy. Subsequent chemotherapy regimens were amrubicin in 17 patients, cisplatin plus irinotecan in nine, irinotecan in six, carboplatin plus etoposide in five, topotecan in four, the combination of irinotecan, cisplatin plus etoposide in two, and carboplatin plus paclitaxel in one patient.

Treatment efficacy. Table II summarizes the treatment outcome of first-line chemotherapy. Regarding treatment delivery, the median number of administered cycles was four (range=1-4). Overall, 32 (61%) of the patients completed all four of the planned cycles. The treatment was discontinued because of progressive disease (PD) in seven patients, toxicity in nine, and other reasons in four patients. The ORR was 69% (72% in LD and 65% in ED, respectively), comprising of one CR and 35 PR. The response was not evaluable in four patients because of death before the first

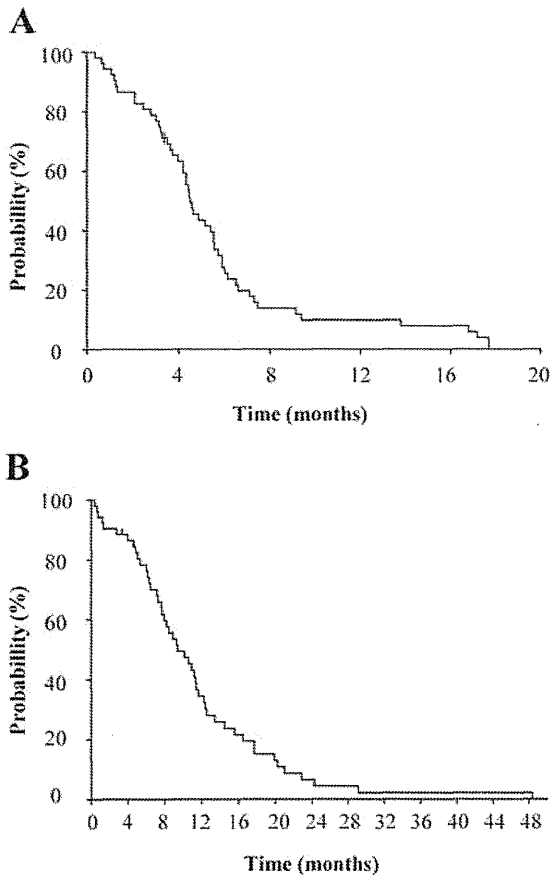

Figure 1. *Progression-free survival (PFS) (A) and overall survival (OS) (B) of patients with SCLC with Interstitial Lung Disease who received platinum agents plus etoposide as first-line chemotherapy. The vertical bars indicate the censored cases at the cut-off date. The median PFS, median OS, and 1-year survival times were 4.5 months [5.4 months, limited disease (LD) stage; 3.7 months, extensive disease (ED) stage], 9.4 months (10.6 months, LD stage; 8.2 months, ED stage), and 32%, respectively.*

tumor response evaluation. The median PFS after first-line chemotherapy and the median OS were 4.5 months (5.4 months, LD stage; 3.7 months, ED stage) (Figure 1A) and 9.4 months (10.6 months, LD stage; 8.2 months, ED stage), respectively (Figure 1B). Regarding the PFS and OS, the differences between the LD and ED stages were not statistically significant.

Incidence of acute exacerbation of ILD. Only one patient (2%) developed an acute exacerbation of ILD during first-line chemotherapy (carboplatin plus etoposide). During second- or third-line chemotherapy, five patients developed acute exacerbation of ILD. The regimens immediately before the development of the acute exacerbation of ILD were amrubicin in three patients, a combination of irinotecan, cisplatin, plus etoposide in one, and topotecan in one patient. The characteristics of all six patients with acute exacerbations of ILD, are listed in Table III. All the patients were smokers and men with a good PS before chemotherapy. The median time from the last administration of chemotherapy to the development of the acute exacerbation of ILD was 37 days. Although all the patients with acute exacerbation of ILD were treated using steroids, three out of the six patients did not improve and died. The results of univariate analyses of risk factors (age, sex, Brinkman index, LDH levels, and PS) for the acute exacerbation of ILD are listed in Table IV. No significant risk factors for acute exacerbation of ILD were identified. The results of the multivariate analysis for the acute exacerbation also showed that none of the variables were significant.

Discussion

In our study, the results for the 52 patients with SCLC with pre-existing ILD indicated that the combination of platinum agents plus etoposide as first-line chemotherapy yielded an ORR of 69%, a median PFS of 4.5 months, and a median OS of 9.4 months. Although directly comparable historical control data were not available, the observed efficacy in our study was the same as the results of two previous randomized phase III trials with platinum agents plus etoposide for ED stage patients with SCLC [Japan Clinical Oncology Group (JCOG) 9511: ORR=67.5%; PFS=4.8 months; median OS=9.4 months; and JCOG 9702: ORR=73%; PFS=5.2 months; median OS=10.6 months] (3, 4). Furthermore, the incidence of acute exacerbation of ILD during first-line chemotherapy observed in our study, was 2% (1/52). The combination of platinum agents plus etoposide seems to be effective and tolerable as a first-line chemotherapy for patients with SCLC with preexisting ILD.

The incidence of lung cancer is reported to be higher in patients with ILD than in patients without (8). In patients with lung cancer, pre-existing ILD has been reported to be a risk factor for the development of anticancer agent-associated acute exacerbation of ILD, which is a fatal complication of treatment. There are some reports regarding the safety and efficacy of chemotherapy for advanced or recurrent NSCLC with pre-existing ILD (6, 12, 13), and the incidence of acute exacerbation of ILD in NSCLC is reported to range from 20% to 24% in Japan, although the chemotherapeutic regimens that were administered were not the same (14, 15). Minegishi *et al.* reported the results of feasibility study for carboplatin plus etoposide in 17 SCLC patients with idiopathic interstitial pneumonias (IIPs) (16). The results indicated that the acute exacerbation of IIP occurred in one (5.9%) out of the 17 patients, with a median PFS of 5.5 months and a median OS of 8.7 months. However, that study was limited in that it included a small number of patients. It

Table III. *Summary of date for six patients who developed acute exacerbation of Interstitial Lung Disease.*

No.	Age (years)	Gender	PS	BI index	Prior chemotherapy	Time to AE after prior chemotherapy	Initial manifestations	AE status	Time to death after last chemotherapy (days)
1	71	Male	1	940	Carboplatin, Etoposide (1st line)	5 (day 5 in cycle 1)	Dyspnea	Died	19
2	53	Male	2	1490	Amrubicin (3rd line)	17 (day 17 in cycle 1)	Dyspnea, fever	Died	30
3	70	Male	1	1150	Cisplatin, Etoposide, Irinotecan (2nd line)	140 (day 93 in cycle 3)	Dyspnea	Died	123
4	50	Male	1	330	Topotecan (2nd line)	52 (day 24 in cycle 2)	Dyspnea	Improved	–
5	63	Male	1	960	Amrubicin (2nd line)	23 (day 23 in cycle 1)	Dyspnea, fever	Improved	–
6	62	Male	1	620	Amrubicin (3rd line)	73 (day 17 in cycle 3)	Dyspnea	Improved	–

PS, Performance status; BI, Brinkman index; AE, acute exacerbation.

was also unclear whether chemotherapy regimens such as platinum agents plus etoposide, which is the most frequently used regimen worldwide (4, 17), were feasible in patients with SCLC, with pre-existing ILD at the time of the start of our study.

In our study, acute exacerbation of ILD during second- or third-line chemotherapy occurred in five (16%) out of the 33 patients who received subsequent chemotherapy, compared with 2% (1/52) of the patients who received platinum agents plus etoposide as first-line chemotherapy. Previous reports have also shown that second-line chemotherapy has a high frequency and risk of the acute exacerbation of ILD, consistent with the results of the present study (15, 16). We speculated that the difference in the incidence of acute exacerbation of ILD between the first-line and subsequent chemotherapy regimens can be accounted for by some of the effective agents used for refractory SCLC, such as amrubicin and irinotecan, which are reportedly associated with a high incidence of acute exacerbation in patients with pre-existing ILD (18, 19).

Our study has a major limitation in that the diagnosis of acute exacerbation of ILD was not based on pathological findings but only on results of chest CT findings and the clinical course. We cannot completely exclude the possibility that the patients had developed lymphangitic carcinomatosis or some other disease, rather than acute exacerbation of ILD. However, pathological findings for the diagnosis of acute exacerbation of ILD are difficult to obtain. Therefore, we diagnosed acute exacerbation of ILD based on clinical and radiographic findings that were consistent with drug-induced ILD. Moreover, the pathological diagnosis of ILD using an open lung biopsy before treatment is extremely difficult and impractical, since chemotherapy should be started as soon as possible after the diagnosis of SCLC, which is characterized by rapid growth and widespread metastatic disease. We consider that the diagnosis of pre-existing ILD and the acute exacerbation of ILD based on clinical and radiological findings is appropriate in clinical practice.

Table IV. *Relationship between clinical variables and acute exacerbation of Interstitial Lung Disease.*

	No. of patients	Incidence of AE (%)	*p*-Value
Total	52	12	
Age			
<70 years	18	22	0.17
≥70 years	34	6	
Gender			
Male	50	12	>0.99
Female	2	0	
PS			
0/1	46	0	
2/3	6	13	
Smoking index			
<1000	21	14	0.68
≥1000	31	10	
LDH			
Normal	30	7	0.38
High (more than upper limit of normal)	22	18	

PS, Performance status; LDH, lactate dehydrogenase.

Our findings indicated that the combination of platinum agents plus etoposide is feasible and effective for the treatment of patients with SCLC with pre-existing ILD. A further large study is warranted to enable definitive conclusions to be drawn.

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Subpopulation of small-cell lung cancer cells expressing CD133 and CD87 show resistance to chemotherapy

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Tumors are presumed to contain a small population of cancer stem cells (CSCs) that initiate tumor growth and promote tumor spreading. Multidrug resistance in CSCs is thought to allow the tumor to evade conventional therapy. This study focused on expression of CD133 and CD87 because CD133 is a putative marker of CSCs in some cancers including lung, and CD87 is associated with a stem-cell-like property in small-cell lung cancer (SCLC). Six SCLC cell lines were used. The expression levels of CD133 and CD87 were analyzed by real-time quantitative reverse transcription-polymerase chain reaction and flow cytometry. CD133+/− and CD87+/− cells were isolated by flow cytometry. The drug sensitivities were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Non-obese diabetic/severe combined immunodeficiency mice were used for the tumor formation assay. SBC-7 cells showed the highest expression levels of both CD133 and CD87 among the cell lines. CD133−/CD87−, CD133+/CD87−, and CD133−/CD87+ cells were isolated from SBC-7 cells; however, CD133+/CD87+ cells could not be obtained. Both CD133+/CD87− and CD133−/CD87+ subpopulations showed a higher resistance to etoposide and paclitaxel and greater re-populating ability than the CD133−/CD87− subpopulation. CD133+/CD87− cells contained more G0 quiescent cells than CD133−/CD87− cells. By contrast, CD133−/CD87− cells showed the highest tumorigenic potential. In conclusion, both CD133 and CD87 proved to be inadequate markers for CSCs; however, they might be beneficial for predicting resistance to chemotherapy. (*Cancer Sci* 2013; 104: 78–84)

Small-cell lung cancer (SCLC) is highly sensitive to chemotherapy. More than 80% of patients achieve an objective response; however, most responders eventually relapse because of drug resistance. Less than 30% of patients with limited disease and 1–2% of patients with extensive disease survive to 5 years.[1]

Cancer stem cells (CSCs) have been proposed as one of the causes of treatment resistibility. Cancer stem cells are a rare population of undifferentiated cells that are responsible for tumor initiation, maintenance, and spreading. They are resistant to anticancer agents and can self-renew and generate progeny in the form of differentiated cells that constitute most of the cells in tumors.[2,3] Because a surviving population of CSCs after conventional treatment might be responsible for tumor regrowth, identifying and eradicating the CSC population are very important.

Cancer stem cells were isolated initially from leukemia and subsequently from solid tumors, including brain, breast, prostate, colon, and liver cancer.[2–6] The methods used to isolate CSCs include cell surface marker analysis,[2–6] side-population analysis,[7] and the sphere-formation assay.[5,8] Putative CSC markers were reported to be CD34-positive/CD38-negative for acute myeloid leukemia, CD44-positive/CD24-negative/α2β1-low/Lin-negative for breast cancer, CD44-positive/α2β1-high/CD133-positive for prostate cancer, and CD133-positive/nestin-positive for brain cancer.[9] The present study focused on expression of CD133 and CD87 as putative cell-surface markers. CD133 is reported to be a marker of CSCs in some cancers, such as brain, prostate, and colorectal cancer.[3–5] Freshly dissociated human SCLC and non-small-cell lung cancer contain CD133-positive cells, which could generate long-term lung tumor spheres *in vitro* that could both differentiate and preferentially form tumors *in vivo*.[8] However, CD133 was reported to be both a positive and a negative marker of CSCs in lung cancer.[10,11] Meanwhile, in human SCLC cell lines, a small population of urokinase plasminogen activator receptor (uPAR/CD87)-positive cells was identified, of which a subset demonstrated enhanced clonogenic activity *in vitro*.[12] CD87 has been implicated in the growth, metastasis, and angiogenesis of several solid and hematologic malignancies, and its increase was associated with a poor clinical outcome.[13] Targeting CD87 can have broad-spectrum antitumor effects.[14]

We hypothesized that both CD133 and CD87 might be useful as CSCs markers in SCLC. To test this hypothesis, we investigated the expression levels of CD133 and CD87 using six SCLC cell lines. Additionally, we examined whether amrubicin might be effective for such cancer stem-like cells because it was demonstrated to be effective for refractory SCLC patients.[15]

Material and Methods

Drugs. Drugs were obtained from the following sources: cisplatin and amrubicinol from Nippon Kayaku (Tokyo, Japan); etoposide and paclitaxel from Bristol-Myers Squibb (Tokyo, Japan); 7-ethyl-10-hydroxy-campthothecin (SN-38), an active metabolite of irinotecan, from Yakult Honsha Co. Ltd. (Tokyo, Japan); and 3-[4,5-dimethyl-thizol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture. The SBC-3, 4, 5, 6, 7, and 9 cell lines were established in our laboratory from SCLC patients.[16] The SBC-3 cell line was derived from bone marrow aspirates of an untreated patient.[17] The other cell lines were established from pleural effusion or pericardial effusion of patients who had

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received chemotherapy. All cell lines were characterized by Tsuchida *et al.*,[18] and some were stored at the Japanese Collection of Research Bioresources (http://cellbank.nibio.go.jp/cellbank.html). These cell lines were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin in a tissue culture incubator at 37°C under 5% CO_2.

Reverse transcription-polymerase chain reaction. RNA samples were prepared for reverse transcription-polymerase chain reaction (RT-PCR) using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol, and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Duplex TaqMan real-time PCR was used to analyze the CD133 and CD87 expression levels in each cell line using an ABI PRISM 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Sequences of the Taqman probe and primers for CD133, CD87, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: CD133: Taqman probe (5′-FAM-TGGCATCGTGCAAACCTGTGGCC-TAMRA-3′), forward primer (5′-AGTGGATCGAGTTCTCTATCA GTG-3′), reverse primer (5′-CAGTAGCTTTTCCTATGCCAA ACC-3′); CD87: Taqman probe (5′-FAM-ACAGCCCGGCC AGAGTTGCCCT-TAMRA-3′), forward primer (5′-CCACTCA GAGAAGACCAACAGG-3′), reverse primer (5′-GGTAACGG CTTCGGGAATAGG-3′). GAPDH was co-amplified in the same reaction mixture as an endogenous reference gene. Sequences of the probe and primers for GAPDH were as follows: Taqman probe: 5′-FAM-CGTCGCCAGCCGAGCCA-CATCG-TAMRA-3′; forward primer: 5′-CGACAGTCAGCC GCATCTTC-3′; and reverse primer: 5′-CGACCTTCACCTT CCCCATG-3′. The average levels of CD133 and CD87 expression were determined from differences in the threshold amplification cycles between CD133 and CD87 and GAPDH.

Flow cytometry. Cells were harvested and re-suspended at 1×10^6 cells/mL of staining buffer. Fluorescent-labeled monoclonal antibodies were added in concentrations recommended by the manufacturer. After washing, the labeled cells were analyzed and sorted using a FACS Aria flow cytometer (Becton Dickinson, Mountain View, CA, USA). The antibodies used were allophycocyanin (APC)-conjugated mouse anti-human CD133 (Clone AC 133; Miltenyi Biotec, Auburn, CA, USA) and FITC-conjugated mouse anti-human uPAR (CD87; American Diagnostica, Stamford, CT, USA) and phycoerythrin (PE)-conjugated mouse anti-human MDR1 (eBioscience, San Diego, CA, USA). Gating was implemented on the basis of negative-control staining profiles. The sort was performed in four-way purity mode (the purity was >98%). The cell-cycle analysis was performed after staining with Hoechst 33342 and Pyronin Y (Sigma-Aldrich, St. Louis, MO, USA). Cells were stained according to the manufacturer's instructions.

Limiting dilution assay. To determine the clonogenicity and regenerative ability of single cells, a limiting dilution assay was carried out. The cells were resuspended in fresh medium, diluted to 3 cells/mL, and seeded at approximately 0.3 cells/well with 100 μL of medium into 96-well plates. Wells containing no cells or more than one cell were excluded after careful microscopic examinations, and those containing a single cell were marked and monitored daily under a microscope. After colony formation, the colonies were counted, dissociated, harvested, and cultured again.

Cell proliferation assay. Cell proliferation was examined on days 1, 2, 3, and 4. Isolated cells (1×10^5) were seeded in a cell culture flask at a final volume of 5 mL. After incubation, proliferation was evaluated by enumerating cells. Growth inhibition was determined using a modified MTT dye reduction assay with Cell Counting Kit-8 (Dojindo, Tokyo, Japan). Briefly, cells were plated on 96-well plates at a density of 3000 cells per well with RPMI 1640 with 10% FBS. Several concentrations of each drug were added to wells, and incubation was continued for 72 h. MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was then added to all wells, and incubation was continued for a further 2 h. After the dark blue crystals had dissolved, the absorbance was measured with a microplate reader. The percentage of growth is shown relative to that of untreated controls. Each assay was performed in triplicate or quadruplicate. The mean ± standard error of the 50% inhibitory concentration (IC_{50}) of the drugs in cells was determined.

Immunoblotting. Proteins were extracted from each cell line and incubated in lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerol phosphate, 10 mM NaF, and 1 mM Na-orthovanadate] containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) and centrifuged at 20 630*g* for 20 min at 4°C. Proteins were separated by SDS-PAGE using 5–15% precast gels (Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes. Specific proteins were detected by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) using the antibodies to aldehyde dehydrogenase 1A1 (1:100 dilution; Abcam, Cambridge, MA) and β-actin (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA). The secondary antibody; anti rabbit IgG (HRP-linked, species-specific whole antibody) (GE Healthcare), was used at a 1:5000 dilution.

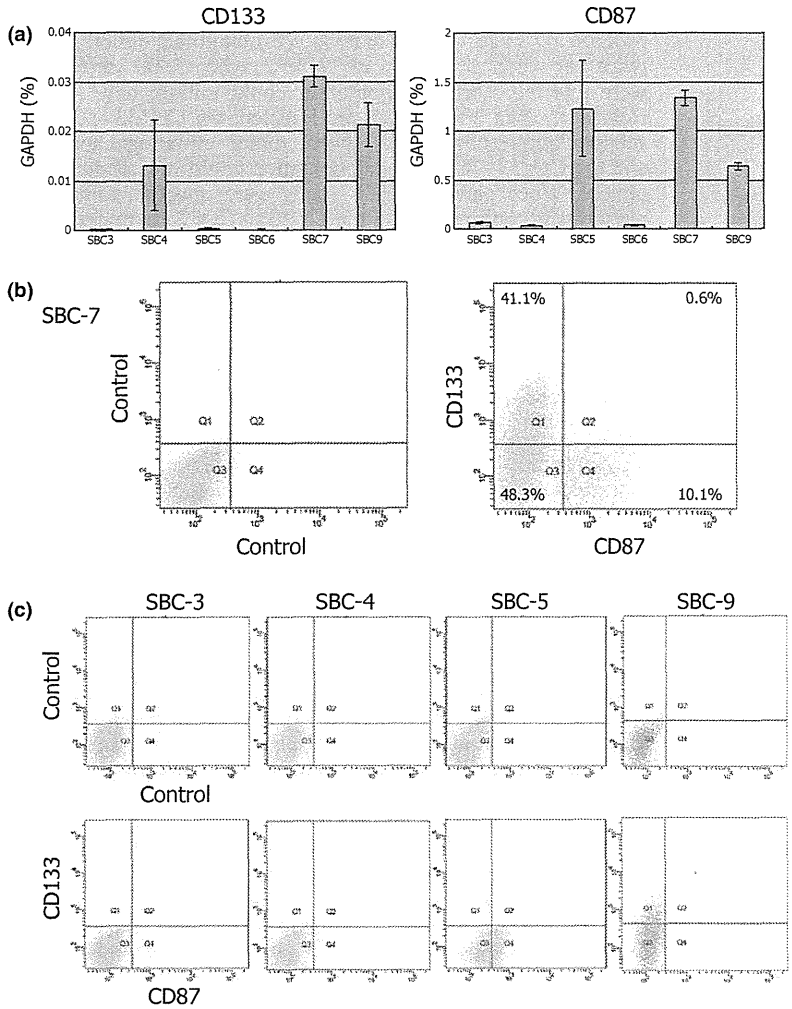
Xenograft model. Sorted cells were injected subcutaneously into the backs of 7-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Charles River, Yokohama, Japan). Groups of mice were inoculated with CD133+/CD87−, CD133−/CD87+, or CD133−/CD87− cells at 5×10^3 and 2×10^3 cells. Tumor growth was monitored twice per week, and tumor volume (width^2 × length/2) was determined periodically. A lack of tumor formation at 8 weeks after sorted-cell injection was described as "no tumor formation".

Statistical analysis. The differences between the groups were compared using Student's *t*-test and χ^2 test. $P < 0.05$ was considered statistically significant. All data were analyzed using Microsoft Office Excel 2007 (Microsoft Japan Corporation, Tokyo, Japan).

Results

SBC-7 cells showed high expression levels of both CD133 and CD87. Expression levels of CD133 and CD87 mRNA by real-time quantitative RT-PCR were determined. SBC-7 cells showed the highest expression of both CD133 and CD87 among the six cell lines. SBC-9 cells also showed both CD133 and CD87 expression, and SBC-4 and SBC-5 cells showed expression of only CD133 and CD87, respectively. SBC-3 cells demonstrated neither CD133 nor CD87 expression (Fig. 1a). We confirmed expression of CD133 and CD87 in each cell line by flow cytometry (Fig. 1b,c). SBC-7 cells displayed some subpopulations: CD133+/CD87− (41.1%), CD133−/CD87+ (10.1%), and CD133−/CD87− (48.3%); however, CD133+/87+ double-positive cells were very rare (0.6%). The cell-surface expression of CD133 was confirmed in SBC-7 and SBC-9, and that of CD87 was in SBC-5 and SBC-7, respectively. Although there seemed to be a correlation between the mRNA levels and cell surface expressions, cell surface expression was not detected at moderate mRNA levels, such as CD133 in SBC-4 and CD87 in SBC-9. Because only SBC-7 cells showed both CD133 and CD87 expressions in flow cytometry analysis, we selected SBC-7 cells and investigated their characteristics as CSCs.

CD133+/CD87− and CD133−/CD87+ subpopulations showed re-populating ability. We used SBC-7 cell lines and examined the properties of each subpopulation. To compare the re-populating ability of each subpopulation, we sorted the CD133+/



	SBC-3	SBC-4	SBC-5	SBC-9
CD133+ / 87+	0 %	0.1 %	0.1 %	0.1 %
CD133+ / 87–	0 %	0 %	0.1 %	14.4 %
CD133–/ 87+	0.1 %	0.3 %	26.5 %	0.3 %
CD133–/ 87-	99.9 %	99.6 %	73.4 %	85.2 %

Fig. 1. (a) The mRNA expression levels of CD133 and CD87 in each cell line using real-time quantitative reverse transcription-polymerase chain reaction. SBC-7 cells showed the highest expression levels of both CD133 and CD87 among the six cell lines. SBC-4 cells expressed only CD133, and SBC-5 cells expressed only CD87. SBC-3 cells expressed neither CD133 nor CD87. Bars indicate the standard deviation. (b) Flow cytometry analysis of SBC-7 cells stained with CD133 and CD87 antibodies. SBC-7 cells showed CD133+/CD87–, CD133–/CD87+, and CD133–/CD87– subpopulations; however, a CD133+/CD87+ subpopulation was not obtained. (c) Flow cytometry analysis of SBC-3, 4, 5, and 9 cells stained with CD133 and CD87 antibodies. SBC-5 showed a CD133–/CD87+ subpopulation. SBC-9 cells showed a CD133+/CD87– but not a CD133–/CD87+ subpopulation.

CD87–, CD133–/CD87+, CD133–/CD87–, and CD133+/CD87+ cells by flow cytometry (Suppl. Fig. S1), cloned the sorted cells with limiting dilutions, and cultured them separately under the same conditions for 6 weeks. Although we attempted to select CD133+/CD87+ cells several times, no double-positive cells could be obtained for further examination, including *in vivo* study. Therefore, we investigated the characteristics of three subpopulations: CD133+/CD87–, CD133–/CD87+, and CD133–/CD87–. We then re-stained the cultured cells with CD133 and CD87 antibodies and analyzed them by flow cytometry. The CD133+/CD87– population generated both CD133+/CD87– and CD133–/CD87– subpopulations, and the CD133–/CD87+ population generated both CD133–/CD87+ and CD133–/CD87– subpopulations. However, the CD133–/CD87– population produced only CD133–/CD87– cells. CD133+/CD87+ were not obtained from any cultured subpopulation (Fig. 2).

Drug sensitivity, cell cycle and aldehyde dehydrogenase 1A1 expression in the subpopulations. Next, we examined the sensitivity of each subpopulation to the chemotherapeutic drugs cisplatin, etoposide, paclitaxel, and 7-ethyl-10-hydroxy-camptothecin (SN-38: active metabolite of irinotecan). Cells expressing either CD133 or CD87 were more resistant to etoposide and paclitaxel than were double-negative cells (Table 1). In addition, CD133+/CD87– cells showed the highest resistance to etoposide among the three groups ($P < 0.05$). The IC_{50}s (μM) to cisplatin were 5.19 ± 0.19 in CD133–/CD87–, 3.49 ± 0.68 in CD133+/CD87–, 4.72 ± 0.64 in CD133–/CD87+, and 2.14 ± 0.22 in parent SBC-7 (Table 1). Although CD133- and CD87-positive cells tended to be more sensitive to cisplatin than double-negative cells, there was no significant difference among the cell lines tested. When compared with SBC-7 parental cells, CD133+/CD87– cells showed more resistance to etoposide ($P = 0.01$) and paclitaxel ($P = 0.02$), and CD133–/CD87+ cells were more resistance to paclitaxel ($P = 0.03$).

Additionally, we analyzed the cell cycle of each subpopulation by flow cytometry. The CD133+/CD87– subpopulation contained more G0 quiescent cells than did CD133–/CD87+ and CD133–/CD87– subpopulations (Fig. 3). Aldehyde dehydrogenase 1A1 levels seemed similar among the three subpopulations (Suppl. Fig. S2).

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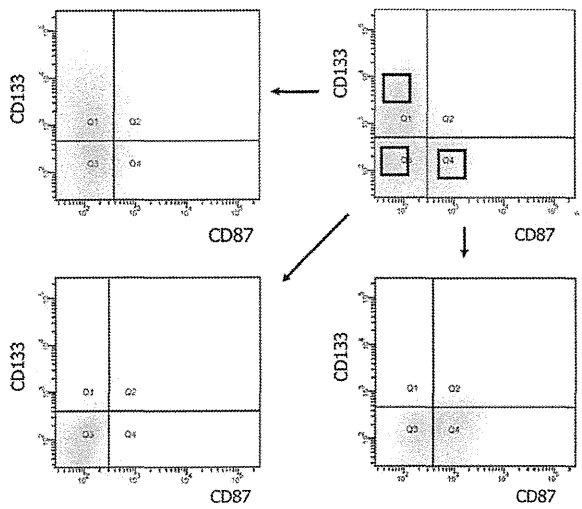

Fig. 2. Re-analysis of each subpopulation after limiting dilatation by flow cytometry. CD133+/CD87− and CD133−/CD87+ subpopulations in SBC-7 cells showed re-populating ability. However, the CD133−/CD87− subpopulation could produce only CD133−/CD87− cells.

Growth rate and MDR1 expression in the subpopulations. We also investigated the cell proliferation rates of each subpopulation (Suppl. Fig. S3). The growth rate of CD133−/CD87+ cells was greater than that of CD133−/CD87− and CD133+/CD87− cells. The growth rates of CD133−/CD87− and CD133+/CD87− cells were similar. Although rapid proliferation makes a cell line appear more drug-sensitive compared with a more-slowly growing cell line, the drug sensitivity of the SBC-7 subclones could not be explained by the growth rate alone. Next, we examined the expression levels of MDR1 on each subpopulation by flow cytometry. The expression of MDR1 was higher in CD133−/CD87+ cells than that in CD133−/CD87− cells (8.1% *vs* 3.1%) (Suppl. Fig. S4).

Drug exposure did not induce CD133 or CD87 expression. We investigated whether the expression levels of CD133 and CD87 were upregulated in cells resistant to chemotherapeutic drugs. We used the SBC-3 cell line as a parent cell, which expressed neither CD133 nor CD87, and its resistant cell lines to cisplatin, SN-38, or etoposide (SBC-3/CDDP, SBC-3/SN-38, or SBC-3/ETP, respectively).[19–21] The CD133 mRNA levels in SBC-3/CDDP and CD87 in SBC-3/ETP were slightly upregulated compared with those in SBC-3 (Fig. 4a). However, in flow cytometry analysis, there was no significant upregulation of CD133 or CD87 expression in the resistant cells (Fig. 4b). Thus, the surface expression of CD133 or CD87 at least was unlikely to be induced by the chronic exposure of chemotherapeutic drugs *in vitro*.

CD133−/CD87− subpopulations showed high tumor formation ability *in vivo*. The tumorigenic potential of each subpopulation through subcutaneous injection of each sorted cell line in NOD/SCID mice was evaluated. We monitored tumor growth twice per week. As shown in Table 2, when 5000 sorted cells were injected, each subpopulation could initiate new tumors. However, when 2000 cells were injected, the CD133−/CD87− subpopulation showed the highest tumor initiating capability, and the CD133−/CD87+ subpopulation could not produce new tumors. When parental SBC-7 cells were injected, tumor formation was confirmed as in the CD133−/CD87− subpopulation. The pathological feature of the tumors with hematoxylin-eosin staining was similar to parental SBC-7 xenograft tumors (Suppl. Fig. S5). Re-analysis of each derived tumor using CD133 and CD87 antibodies in flow cytometry showed that the surface markers of the tumor cells were similar to those of each subpopulation cultured *in vitro* (data not shown).

CD133-positive cells were also resistant to amrubicinol. Although CD133- and CD87-positive cells could not satisfy the requirements for CSCs, these cells showed chemoresistant characteristics. Additionally, CD133+/CD87− cells had higher tumorigenicity and higher resistance to chemotherapeutic drugs than CD133−/CD87+ cells. The IC_{50}s of amrubicinol in CD133-positive and -negative cells were 0.732 ± 0.119 μM and 0.172 ± 0.038 μM, respectively ($P = 0.009$).

Discussion

The need to target therapies at the self-renewal capacity of the stem-cell compartment, effectively interrupting the source of recurrence in tumors sensitive to conventional therapeutic approaches, has also evolved under the CSC hypothesis in the lung cancer field.[9] However, identifying a phenotypic marker in lung CSCs has been unsuccessful. In this study, we investigated whether CD133 or CD87 might be a putative marker of CSCs. At first, we examined the expression levels of CD133 and CD87 mRNA by real-time quantitative RT–PCR. Then, we confirmed the expression of CD133 and CD87 on cell surface by flow cytometry. Although there were discrepancies between the expression levels of mRNA and protein in some cell lines, such as SBC-4 and SBC-9, only SBC-7 cells displayed both CD133 and CD87 cell-surface markers. The ambivalence might be explained by following reasons. (i) Although mRNA was induced, the protein might not be detected because of small quantity; (ii) The protein might be subject to degradation easily, and (iii) It might stay in the cytoplasm and could not appear on the cell surface.

Both CD133- and CD87-positive cells showed higher resistance to chemotherapeutic drugs and a higher re-populating ability and contained more G0 quiescent cells than did the double-negative subpopulation *in vitro*. However, the double-negative subpopulation showed the highest tumor-initiating capability *in vivo*. Thus, CD133 and CD87 did not satisfy the requirements for CSCs in SCLC cells. The reason that double-negative cells showed the highest tumor-initiating capability remains unclear. We used SCLC cell lines to examine the characteristics of CD133- and CD87-positive cells. In cell

Table 1. Sensitivity of each subpopulation to chemotherapeutic drugs

	Cisplatin (μM)	SN-38 (nM)	Etoposide (μM)	Paclitaxel (nM)
CD133−/CD87−	5.19 ± 0.19	37.31 ± 5.88	5.17 ± 0.20	2.87 ± 0.08
CD133+/CD87−	3.49 ± 0.68	107.96 ± 40.24	>100 ($P < 0.01$)*	7.02 ± 0.36 ($P = 0.01$)*
CD133−/CD87+	4.72 ± 0.64	12.66 ± 2.74	23.46 ± 4.00 ($P = 0.04$)*	6.94 ± 0.31 ($P = 0.01$)*
SBC-7	2.14 ± 0.22	22.37 ± 3.59	37.54 ± 7.12	3.66 ± 0.26

SN-38, 7-ethyl-10-hydroxy-campthothecin, an active metabolite of irinotecan. The asterisk indicates that the IC_{50}s (mean ± standard error) of the CD133+/CD87− and CD133−/CD87+ subpopulations were significantly higher than that of the CD133−/CD87− subpopulation ($P < 0.05$). The P-value was calculated with Student's t-test compared with CD133−/CD87− subpopulation.

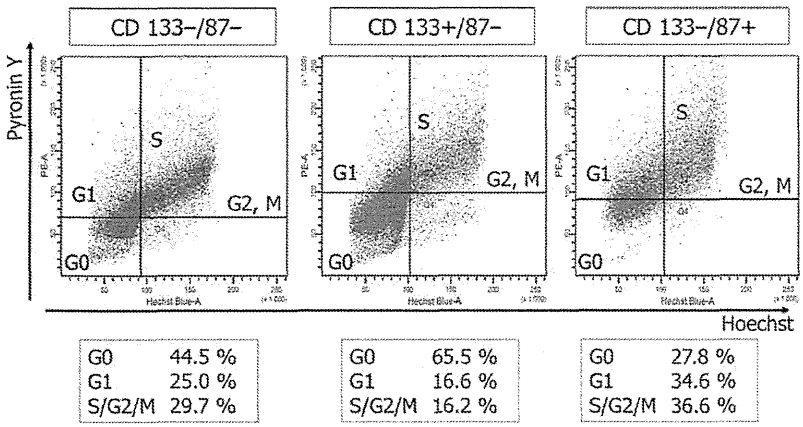

Fig. 3. Cell-cycle analysis of each subpopulation with Hoechst 33342 and Pyronin Y. The CD133+/CD87− subpopulation contained more G0 quiescent cells than did CD133−/CD87+ and CD133−/CD87− subpopulations.

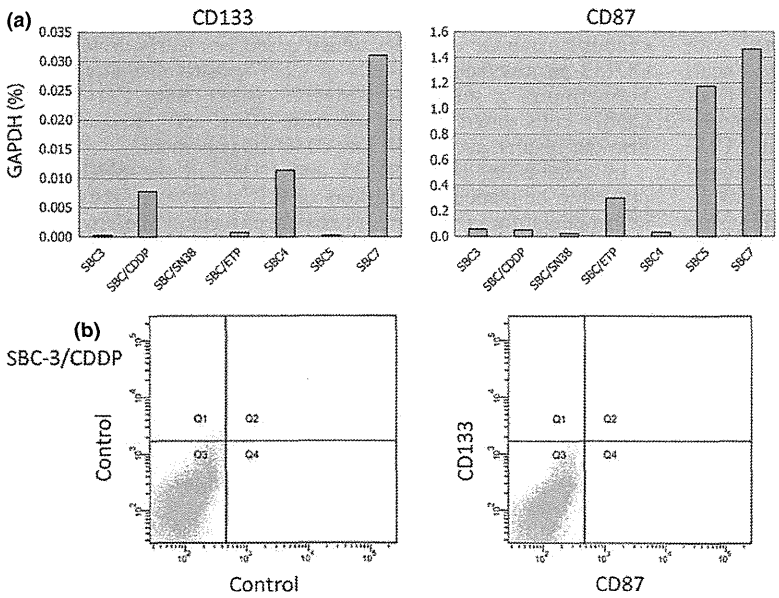

Fig. 4. (a) CD133 and CD87 mRNA levels in parental (SBC-3) and resistant (SBC-3/CDDP, SBC-3/SN38, and SBC-3/ETP) cell lines using real-time quantitative reverse transcription–polymerase chain reaction. CD133 in SBC-3/CDDP and CD87 in SBC-3/ETP were more highly expressed than those in SBC-3. (b) Flow cytometry analysis of SBC-3/CDDP cells stained with CD133 and CD87 antibodies. The expression of CD133 or CD87 was not increased in resistant cells.

Table 2. Tumorigenicity of sorted subpopulations

Injected cell numbers	2×10^3	5×10^3
CD133−/CD87−	3/3 (100%) $P = 0.014$	6/6 (100%) $P = 0.121$
CD133+/CD87−	1/2 (50%) $P = 0.171$	7/7 (100%) $P = 0.089$
CD133−/CD87+	0/3 (0%)	4/6 (67%)

Data are presented as number of tumors/injections of sorted cells. The P-value was calculated with χ-test comparing each sub-population to CD133-/CD87+ population.

lines, the characteristics of tumor cells can be changed from primary cultured cells or fresh cells; thus, the double-negative subpopulations might acquire some specific ability to initiate new tumors. In addition, Meng *et al.* previously reported that lung cancer cell lines regardless of CD133 expression could initiate new tumors in nude mice.[11] Thus, CD133 alone might not be useful as a stem cell marker for lung cancer.

Particularly, because CD133-positive cells showed a higher tumor-initiating capability than CD87-positive cells, we investigated the strategy to overcome the resistance to conventional chemotherapy in CD133-positive cells. Amrubicin, a synthetic 9-aminoanthracycline, is converted to the active metabolite amrubicinol via reduction of its C-13 ketone group to a hydroxyl group by carbonyl reductase.[22] Adriamycin-resistant cells show partial resistance to amrubicin *in vitro*.[23] Phase II studies of previously treated SCLC patients showed that amrubicin was effective in both sensitive and refractory relapse.[15] Unfortunately, CD133-positive cells were 4.3 times more resistant to amrubicinol than were CD133-negative cells.

In the present study, both CD133 and CD87 proved to be inadequate markers for CSCs; however, they seemed to predict resistance to chemotherapy. We could not clarify the mechanism why CD133- or CD87-positive cells showed higher resistance to etoposide and paclitaxel. Etoposide targets the cells in S/G2/M phase. CD133+/CD87− fraction, which harbored 16.2% of S/G2/M fraction, showed a higher level of IC_{50} in etoposide than CD133−/CD87− containing 29.7% of that fraction. However, CD133−/CD87+ fraction, which harbored higher levels S/G2/M phase was also more resistant against etoposide compared with CD133−/CD87−. Therefore, the resistant mechanism of CD133 or CD87 was not clarified only by cell cycle analysis. Gutova *et al.* reported that CD87-positive cells

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showed higher expression of MDR1.[12] In our study, the expression level of MDR1 was higher in CD133−/CD87+ subpopulation. However, the expression rate of MDR1 (8.1%) was lower than that (10–40%) in their report.[12] Chen *et al.* indicated that CD133-positive cells were highly co-expressed with ABCG2 transporter and were significantly resistant to conventional treatment methods compared with CD133-negative non-small-cell lung cancer cells.[24] Thus, the CD133- or CD87-positive subpopulation in SBC-7 might be related to drug resistance. Meanwhile, cisplatin seemed effective irrespective of the CD133 or CD87 status because cisplatin resistance was not associated with MDR1 or ABCG2 overexpression.[25,26] The surface expressions of both CD133 and CD87 were not increased after chronic exposure of SBC-3 cells to chemotherapeutic drugs, resulting in acquisition of resistance. The upregulation of CD133 or CD87 expression might be a part of a complicated chemoresistance mechanism.

Increased levels of urokinase plasminogen activator and its receptor CD87 were strongly correlated with poor prognosis and unfavorable clinical outcome in patients with acute myeloid leukemia and breast cancer.[13] In many solid tumors, such as glioblastoma, the presence of CD133 was correlated with poor survival.[3] In patients with non-small cell lung cancer, CD133 was indicative of a resistance phenotype, but did not represent a prognostic marker for survival.[27] Although the clinical outcome of CD133 or CD87 expression in SCLC patients remains unclear, our data suggested that the tumors expressing CD133 and/or CD87 might be resistant to conventional chemotherapy. To prove the hypothesis, the relationship between CD133 and/or CD87 expression levels on human SCLC materials and corresponding chemosensitivity should be investigated. The drugs should be screened for their ability to overcome the resistant SCLC cells.

The limitation of our study was that we were unable to generate CD133+/CD87+ double-positive cells, which might have true CSC characteristics. Thus efficient sorting of a small population of double-positive cells for *in vivo* experimentation is necessary. Characterization of the CD133+/CD87+ might be relevant for this study and could reveal some remarkable properties of this subset (e.g., an enhanced tumorigenic ability) compared with single-positive CD133 or CD87 fractions. In addition, we extensively examined the SBC-7 line, which was the only cell line that exhibited surface expression of both CD133 and CD87 among the cells we used. We tried to confirm that CD133 or CD87 positive cells showed higher chemoresistance than negative cells using the SBC-9 cells. SBC-9 cells were divided into CD133+/CD87− and CD133−/CD87− subpopulations. Unfortunately, CD87 positive cells in the SBC-9 cells were not obtained because it might be due to the small amount of the cells (0.4%). We investigated cell viability of both subpopulations after 96 h exposure to cisplatin, etoposide and paclitaxel at the IC_{50} of each drug for the SBC-9 cells. CD133+/CD87− cells were resistant to only etoposide than CD133−/CD87− cells (Suppl. Fig. S6). We should further examine using the cell lines which could be clearly divided into CD133-positive/negative cells or CD87-positive/negative cells. Furthermore, a second tumorigenic assay using CD133+and CD87+ cells sorted from an alternate SCLC cell line could confirm our results; such a cell line could be generated.

In conclusion, both CD133 and CD87 in the SBC-7 line proved to be inadequate markers of CSCs; however, they might be beneficial for prediction of resistance to chemotherapy.

Disclosure Statement

The authors have no conflict of interest.

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