RESULTS

Patient Characteristics

Between July 2000 and July 2002, 59 chemonaive patients (43 male, 16 female) with NSCLC were enrolled in phase I and II portions from the five hospitals after approval by the IRB. Twenty-five patients were enrolled in the phase I portion of the study, and 34 patients were enrolled in phase II. Baseline patient characteristics for all patients and patients who received the recommended regimen are summarized in Table 1.

Phase I

Twenty-five patients were enrolled into the phase I portion of the study. The number of patients treated and the DLTs observed in the first cycle at each dose level of gemcitabine and docetaxel are shown in Table 2.

In Arm 1, 50% of patients had DLTs at dose level 1 and dose level 0, therefore Arm 1 could not be the recommended regimen: there were 2/6 and 3/6 patients who achieved partial response (PR) at dose level 1 and 0 in Arm 1, respectively.

Table 1. Baseline characteristics

Patient characteristics	All patients ($n = 59$), n (%)	Patients who received the recommended regimen ($n = 40$), n (%)
Gender		
Male	43 (72.9%)	26 (65.0%)
Female	16 (27.1%)	14 (35.0%)
Age		
Median	62	64
Range	38–74	38–74
ECOG performance status		
0	5 (8.5%)	2 (5.0%)
1	54 (91.5%)	38 (95.0%)
Stage		
IIIB	14 (23.7%)	8 (20.0%)
IV	33 (55.9%)	23 (57.5%)
Postsurgical recurrence	12 (20.3%)	9 (22.5%)
Histological type		
Adenocarcinoma	34 (57.6%)	25 (62.5%)
Squamous cell carcinoma	19 (32.2%)	14 (35.0%)
Large cell carcinoma	5 (8.5%)	1 (2.5%)
Other	1 (1.7%)	0 (0%)
Prior therapy		
None	45 (76.3%)	29 (72.5%)
Surgery	13 (22.0%)	11 (27.5%)
Radiotherapy	0 (0%)	0 (0%)
Radiotherapy and surgery	1 (1.7%)	0 (0%)

ECOG, Eastern Cooperative Oncology Group.

In Arm 2, no DLT was observed at dose level 1: 3/6 patients achieved PR. At dose level 2, one patient discontinued due to progressive disease; therefore, one patient was added. However, another patient discontinued due to grade 3 hypersensitivity (not a DLT). In this regimen, two DLTs had already been observed in five other patients, but the sponsors (Aventis Pharma Japan and Eli Lilly Japan K.K.) and investigators decided not to add one more patient to dose level 2 in Arm 2 in consideration of patients' safety. PRs were observed in 2/7 patients at dose level 2 of Arm 2.

Therefore, the recommended regimen was determined as gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 due to the incidence of DLT.

Dose Administration

In Arm 1, a total of 49 cycles were accomplished. One case delayed the date of administration on day 1 (defined as more than 8 days) as a matter of convenience; seven and four cases delayed their dates of administration on day 8 (defined as more than 1 day) because of adverse events and non-medical reasons, respectively; and four cases could not be treated on day 8 because of adverse events. In Arm 2, including phase I and II portions, a total of 145 cycles were accomplished. Four and five cases delayed their dates of administration on day 1 because of adverse events and non-medical reasons, respectively; 21 and nine cases delayed their dates of administration on day 8 because of adverse events and non-medical reasons, respectively; and two cases could not be treated on day 8 because of

Table 2. Phase I dose-limiting toxicities

Dose level	GEM/DOC (mg/m^2)	Arm 1	Arm 2
0	800/50	3/6 patients: • G3 ALT increased • G1 fever, G3 neutropenia • G2 infection, G3 neutropenia	N/A
1	1000/50	3/6 patients: • G3 infection, G3 neutropenia • G4 neutropenia, G1 fever, G3 infection • G3 neutropenia, G2 infection, G3 arrhythmia, G3 diarrhea	0/6 patients
2	1000/60	N/A	2/5 patients: • G3 ALT increased • G2 fever, G3 neutropenia

GEM, gemcitabine; DOC, docetaxel; G, grade; ALT, alanine aminotransferase; N/A, not applicable.

adverse events. The most common adverse event for a dose delay was neutropenia.

EFFICACY

All 59 patients were involved in the analysis for efficacy, and 19 of 59 patients achieved PR for an overall response rate of 32.2% [95% confidence interval (CI) 20.6–45.6%]. Of the 40 patients who received the recommended regimen in either phase I or phase II, 12 patients achieved PRs for a response rate of 30.0% (95% CI 16.6–46.5%).

The median time to progressive disease in all 59 patients was 111 days (95% CI 71–154 days). Median survival time was 11.9 months (95% CI 7.0–15.0 months), with 1-year survival rate at 47.1% (95% CI 34.0–60.2%).

SAFETY

All 59 patients were evaluable for safety. Grade 3 and 4 drug-related toxicities observed in all 59 patients are shown in Table 3. Grade 3 and 4 drug-related toxicities observed in 40 patients who received the recommended regimen are also shown in Table 4.

In all 59 patients, grade 3 and 4 neutropenia were observed in 19 (32.2%) and 20 (33.9%) patients, respectively. Grade 3 and 4 leukopenia were observed in 24 (40.7%) and four (6.8%) patients, respectively. Grade 3 non-hematological toxicities included infection in four patients (6.8%), anorexia in four patients (6.8%), and nausea, diarrhea, rash and constipation in three patients (5.1%) each. After starting docetaxel administration, grade 3 interstitial pneumonia was reported in three patients (5.1%), all of whom recovered shortly after steroid treatment; grade 4 anaphylaxis was reported in two patients (3.4%). There were no toxic deaths.

Table 3. NCI–CTC grade 3/4 toxicities ($n = 59$)

Toxicities	Grade 3		Grade 4	
	n	%	*n*	%
Hematological toxicities				
Leukopenia	24	40.7	4	6.8
Neutropenia	19	32.2	20	33.9
Lymphopenia	10	16.9	0	0.0
Hemoglobin decreased	4	6.8	0	0.0
Thrombocytopenia	1	1.7	0	0.0
Thrombocytosis	1	1.7	0	0.0
Non-hematological toxicities				
ALT increased	5	8.5	0	0.0
Infection	4	6.8	0	0.0
Anorexia	4	6.8	0	0.0
Nausea	4	6.8	0	0.0
Diarrhea	3	5.1	0	0.0
Interstitial pneumonia	3	5.1	0	0.0
Rash	3	5.1	0	0.0
Constipation	3	5.1	0	0.0
AST increased	2	3.4	0	0.0
Fatigue	2	3.4	0	0.0
Vomiting	2	3.4	0	0.0
Hyperglycemia	1	1.7	0	0.0
Hyponatremia	1	1.7	0	0.0
Allergic reaction	1	1.7	0	0.0
Vasovagal reaction	1	1.7	0	0.0
Body temperature decrease	1	1.7	0	0.0
Weight increase	1	1.7	0	0.0
Hypotension	1	1.7	0	0.0
Pneumonia	1	1.7	0	0.0
Arrhythmia	1	1.7	0	0.0
Edema	1	1.7	0	0.0
Neuropathy peripheral	1	1.7	0	0.0
Anaphylaxis	0	0.0	2	3.4

NCI–CTC, National Cancer Institute–Common Toxicity Criteria version 2.0; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

DISCUSSION

In this phase I/II study, we examined the activity and tolerability of gemcitabine and docetaxel. In phase I, the recommended regimen was determined as gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8. The response rate of all 59 patients was 32.2% (95% CI 20.6–45.6%). When re-evaluated in the 40 patients who received the recommended regimen, the response rate was 30.0% (95% CI 16.6–46.5%). Although the number of patients was limited, Arm 1 (docetaxel on day 1) had a numerically better response: for the 12 patients in Arm 1, five PRs were recorded for a response rate of 42%. However, Arm 1 had more toxicities than the docetaxel on day-8 schedule.

Overall, the toxicity associated with the gemcitabine–docetaxel regimen was manageable. In Arm 1, five patients (42%) had grade 3/4 neutropenia supervened with infection or fever, while only one patient (9%) had grade 3 neutropenia with infection or fever in Arm 2. This indicated that docetaxel was better tolerated on day 8 than on day 1 in a 21-day cycle. It is speculated that the influence of time to nadir of neutropenia is different in each agent: 14–20 days with gemcitabine and 9 days with docetaxel. The time to recover from nadir is 7–8 days with gemcitabine and 8 days with docetaxel. This could explain why docetaxel on day 8 was better tolerated.

Meta-analysis studies have reported that cisplatin-based regimens produce a significant survival benefit in NSCLC (20–23), improve median survival time by 6–8 weeks and 1-year survival rate from 15% to 25% when compared with the best supportive care (24). But studies with platinum-based combinations have also reported severe toxicities, so the deterioration of patients' quality of life is a major problem to be solved (3).

New effective non-platinum-based therapies have been used in various combinations in recent years, and the combination of gemcitabine and docetaxel has been established as one of the

Table 4. NCI–CTC grade 3/4 toxicities ($n = 40$, recommended regimen)

Toxicities	Grade 3		Grade 4	
	n	%	*n*	%
Hematological toxicities				
Leukopenia	13	32.5	2	5.0
Neutropenia	12	30.0	11	27.5
Lymphopenia	5	12.5	0	0.0
Hemoglobin decreased	2	5.0	0	0.0
Thrombocytopenia	1	2.5	0	0.0
Thrombocytosis	1	2.5	0	0.0
Non-hematological toxicities				
ALT increased	2	5.0	0	0.0
Diarrhea	2	5.0	0	0.0
Infection	2	5.0	0	0.0
Interstitial pneumonia	2	5.0	0	0.0
Rash	2	5.0	0	0.0
Fatigue	2	5.0	0	0.0
Nausea	2	5.0	0	0.0
Vomiting	2	5.0	0	0.0
Hyperglycemia	1	2.5	0	0.0
Hyponatremia	1	2.5	0	0.0
AST increased	1	2.5	0	0.0
Allergic reaction	1	2.5	0	0.0
Vasovagal reaction	1	2.5	0	0.0
Anorexia	1	2.5	0	0.0
Body temperature decrease	1	2.5	0	0.0
Weight increase	1	2.5	0	0.0
Hypotension	1	2.5	0	0.0
Pneumonia	1	2.5	0	0.0
Edema	1	2.5	0	0.0
Constipation	1	2.5	0	0.0
Peripheral neuropathy	1	2.5	0	0.0
Anaphylaxis	0	0.0	2	5.0

NCI–CTC, National Cancer Institute–Common Toxicity Criteria version 2.0; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

well-examined regimens. In recent studies using gemcitabine–docetaxel in NSCLC, response rates of 25–50% (19,25–29) and time-to-progression of disease of 106–132 days (31,32) have been reported. Georgoulias et al. (16) reported that the gemcitabine–docetaxel and docetaxel–cisplatin regimens they compared were equivalent in efficacy, but toxicity was severe in the latter. While docetaxel–cisplatin regimens showed severe toxicities of grade 3 anemia (5%), grade 3/4 neutropenia (13%/21%), grade 3 nausea/vomiting (10%) and grade 3 diarrhea (8%), gemcitabine–docetaxel regimens had grade 3/4 anemia (1%/1%), grade 3/4 neutropenia (11%/11%), grade 3 nausea/vomiting (2%) and grade 3/4 diarrhea (2%/1%) in 441 patients. However, the difference of efficacy and safety by the administration schedule and dosage of gemcitabine and docetaxel has not been well documented.

There are some studies that have examined the efficacy and safety of the same schedule as the recommended regimen in our study, namely gemcitabine on days 1 and 8 plus docetaxel on day 1. In these studies dosages were various: gemcitabine was 800–1100 mg/m^2 and docetaxel was 60–100 mg/m^2 (18,19,27–30). Response rates in these studies also varied from 16 to 38%, which indicates that the response rate of the recommended regimen in our study (30.0%) was clinically meaningful because the dosage of docetaxel (50 mg/m^2) in our study is less than that in any other studies. This might have contributed to the relatively mild toxicities of our recommended regimen.

In another study (26), a high response rate (50.0%) was achieved in patients with another administering schedule: gemcitabine 1000 mg/m^2 on days 1 and 10 plus docetaxel 80 mg/m^2 on day 1, administered every 21 days. The most common treatment-related toxicity was myelosuppression. Grade 3/4 leukopoenia and neutropenia occurred in only six (18%) and eight (24%) patients, respectively.

The median survival was 11.9 months in our study, being slightly better than the result from the median survival of the phase III study with gemcitabine and cisplatin, which was 8.7–9.1 months (33,34). This result suggests that the regimen we selected in the phase II portion of this study is comparable in survival with the cisplatin-based regimen.

In conclusion, the combination of gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 is suggested to be better tolerated and has equivalent efficacy to cisplatin-based therapy. These results should be verified by a phase III study in Japanese patients.

CONCLUSION

In this phase I/II study, we studied the activity and tolerability of gemcitabine and docetaxel in Japanese patients. The combination of gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 is suggested to be well tolerated and has equivalent efficacy to cisplatin-based therapy.

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Original article

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Phase I–II study of amrubicin and cisplatin in previously untreated patients with extensive-stage small-cell lung cancer

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Background: Amrubicin, a totally synthetic 9-amino-anthracycline, demonstrated excellent single-agent activity for extensive-stage small-cell lung cancer (ED-SCLC). The aims of this trial were to determine the maximum-tolerated doses (MTD) of combination therapy with amrubicin and cisplatin, and to assess the efficacy and safety at their recommended doses (RD).

Patients and methods: Eligibility criteria were patients having histologically or cytologically proven measurable ED-SCLC, no previous systemic therapy, an Eastern Cooperative Oncology Group performance status of 0–2 and adequate organ function. Amrubicin was administered on days 1–3 and cisplatin on day 1, every 3 weeks.

Results: Four patients were enrolled at dose level 1 (amrubicin 40 mg/m^2/day and cisplatin 60 mg/m^2) and three patients at level 2 (amrubicin 45 mg/m^2/day and cisplatin 60 mg/m^2). Consequently, the MTD and RD were determined to be at level 2 and level 1, respectively. The response rate at the RD was 87.8% (36/41). The median survival time (MST) was 13.6 months and the 1-year survival rate was 56.1%. Grade 3/4 neutropenia and leukopenia occurred in 95.1% and 65.9% of patients, respectively.

Conclusions: The combination of amrubicin and cisplatin has demonstrated an impressive response rate and MST in patients with previously untreated ED-SCLC.

Key words: anthracycline, cisplatin, phase I–II, small-cell lung cancer

Introduction

Small-cell lung cancer (SCLC) is one of the most chemosensitive solid tumors, and the outcome of SCLC patients is slowly but surely improving. Combination chemotherapy consisting of cisplatin plus etoposide and concurrent twice-daily thoracic radiotherapy has yielded a 26% 5-year survival rate in limited-stage (LD) patients [1]. Despite the high response rate to combination chemotherapy, however, local and distant failure is very common, especially in extensive-stage (ED) patients. Moreover, resistance to chemotherapeutic agents develops easily after failure of initial treatment. Thus, long-term survivors are still very rare among patients with ED-SCLC. To improve the outcome of SCLC patients, several strategies, such as high-dose chemotherapy, dose-intensive chemotherapy, alternating chemotherapy and introduction of new drugs, have been investigated [2–6]. However, only the introduction of new agents has improved the outcome of SCLC patients. Combination chemotherapy with etoposide plus cisplatin or etoposide plus cisplatin alternating cyclophosphamide, doxorubicin and vincristine had been mainly used for SCLC in North America. Recently, a Japanese trial [Japan Clinical Oncology Group (JCOG) 9511] demonstrated the superiority of the combination of irinotecan and cisplatin for ED-SCLC patients over the combination of etoposide and cisplatin [6]. The development of more active chemotherapy, and especially the introduction of effective new drugs, is therefore essential to improve the survival of SCLC patients.

Amrubicin (SM-5887) is a totally synthetic anthracycline and a potent topoisomerase II inhibitor [7–14]. It has antitumor activity, and is more potent than doxorubicin against various mouse experimental tumors and human tumor

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xenografts. Amrubicin and its 13-hydroxy metabolite, amrubicinol, inhibit purified human DNA topoisomerase II [11]. Amrubicinol is 10–100 times more cytotoxic than amrubicin [9]. The potent therapeutic activity of amrubicin is caused by the selective distribution of its highly active metabolite, amrubicinol, in tumors [9]. In an experimental animal model, amrubicin did not exhibit any chronic cardiotoxicity potential, and no deleterious effects on doxorubicin-induced cardiotoxicity in dogs was observed [14]. In a phase II study of amrubicin using a schedule of 45 mg/m^2 on days 1–3 every 3 weeks, in 33 previously untreated ED-SCLC patients, an overall response rate of 76% and a complete response (CR) rate of 9% were reported [15]. Moreover, median survival time (MST) was 11.7 months in the single-agent phase II study of amrubicin. Amrubicin is one of the most active new agents for SCLC. Thus, we conducted a phase I/II study of amrubicin plus cisplatin for untreated ED-SCLC, because cisplatin is considered as one of the most important drugs in the treatment of SCLC. The aims of this trial were to determine the maximum-tolerated doses (MTD) of combination therapy of amrubicin with cisplatin, to assess the efficacy and safety for ED-SCLC at their recommended doses (RD), and to examine the pharmacokinetics of the drug combination.

Patients and methods

Patient selection

Patients with histologically and/or cytologically documented SCLC were eligible for this study. Each patient was required to meet the following criteria: extensive-stage disease [16]; no prior therapy for primary lesion; measurable lesion; Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0–2; expected survival time >2 months; age 20–74 years; adequate hematological function [white blood cell (WBC) count 4000–12 000/mm^3, neutrophils ≥2000/mm^3, platelets ≥100 000/mm^3, hemoglobin ≥10 g/dl]; adequate hepatic function [total bilirubin within 1.5× the upper limit of normal; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) within 2.5× the upper limit of normal]; adequate renal function (creatinine within the upper limit of normal); partial pressure of arterial oxygen 60 torr; no abnormality requiring treatment on electrocardiogram; left ventricle ejection fraction >60%; written informed consent. Patients with symptomatic brain metastasis, pleural effusion that required drainage, non-steroidal anti-inflammatory drug or glucocorticoid use for >50 days, pericarditis carcinomatous, active infection, varicella, superior vena cava syndrome, syndrome of inappropriate secretion of antidiuretic hormone (SIADH), gastric and/or duodenal ulcer, severe heart disease, severe renal disease, active concomitant malignancy, symptomatic pneumonitis and/or pulmonary fibrosis and pregnant/nursing women were excluded. This study was approved by the Institutional Review Board at each hospital.

Patient evaluation

Pretreatment evaluation consisted of complete blood cell counts, differential, routine chemistry measurements, progastrin-releasing peptide (ProGRP), neuron-specific enolase, electrocardiogram, echocardiography, chest radiograph, chest and abdominal computed tomography (CT) scan, whole-brain magnetic resonance imaging (MRI) or CT scan, and isotope bone scan. Complete blood cell counts, differential and routine chemistry measurements were performed at least once a week during the chemotherapy.

Treatment schedule

At level 1, chemotherapy consisted of cisplatin 60 mg/m^2 on day 1 and amrubicin 40 mg/m^2 on days 1–3. Amrubicin was administered as an intravenous injection over 5 min and cisplatin was administered as a drip infusion over 60–120 min with adequate hydration. At level 2 the dose of amrubicin was increased to 45 mg/m^2 on days 1–3. Level 3 was planned with cisplatin 80 mg/m^2 on day 1 and amrubicin 45 mg/m^2 on days 1–3. The chemotherapy was repeated every 3 weeks for four to six courses. Intrapatient dose escalation was not allowed. Administration of granulocyte colony-stimulating factor (G-CSF) was permitted prophylactically for patients expected to experience grade 3 neutropenia during the first course. Prophylactic administration of G-CSF was only permitted a second or later courses.

The administrations of both cisplatin and amrubicin were postponed if patients met the following criteria: WBC <3000/mm^3; neutrophils <1500/mm^3; platelets <100 000/mm^3; AST and ALT >5× the upper limit of normal; total bilirubin >1.5× the upper limit of normal; creatinine >1.3× the upper limit of normal; ECOG PS 3 or 4; active infection; grade 2 or worse non-hematological toxicity, except for alopecia, anorexia, nausea, vomiting or fatigue.

The administrations of both cisplatin and amrubicin were withdrawn if patients met the following criteria: tumor regression <15% after first course or <30% after second course; WBC <3000/mm^3; neutrophils <1500/mm^3; platelets <100 000/mm^3; no recovery from grade 3 or 4 non-hematological toxicity at 6 weeks after the start of previous chemotherapy; abnormality of electrocardiogram requiring treatment for more than 6 weeks; left ventricle ejection fraction <48%; treatment delay of >4 weeks.

The dose of amrubicin was decreased 5 mg/m^2/day if patients met the following criteria: grade 4 leukopenia or neutropenia for ≥4 days; grade 3 neutropenia with fever; platelets <20 000/mm^3 during the previous course. The dose of cisplatin was decreased to 75% if creatinine increased to >1.5× the upper limit of normal during the previous course.

The dose-limiting toxicity (DLT) was defined as follows: grade 4 leukopenia or neutropenia for ≥4 days; grade 3 febrile neutropenia; platelets <20 000/mm^3; grade 3 or worse non-hematological toxicity except for nausea, vomiting, anorexia, fatigue, hyponatremia and infection. Initially, three patients were treated at each dose level. If DLT was not observed in any of the three patients, dose escalation was carried out. If DLT was observed in one of three patients, an additional three patients were entered at the same dose level. If DLT was observed in three or more of six patients, or two or three of the initial three patients, we considered that dose to be the MTD. If DLT was observed in one or two of six patients, dose escalation was also carried out. Dose escalation was determined based only on the data from the first course of chemotherapy.

Response and toxicity evaluation

Response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) and tumor markers were excluded from the criteria [17]. CR was defined as the complete disappearance of all clinically detectable tumors for at least 4 weeks and no new lesions. Partial response (PR) was defined as at least a 30% decrease in the sum of the longest diameters of target lesion, taking as reference the baseline sum longest diameter, the required non-progression in non-target lesions and no new lesions for at least 4 weeks. Stable disease (SD) included: regression of target lesions insufficient to meet the criteria for PR, a <20% increase in the sum of the longest diameter of target lesion, taking as reference the smallest sum longest diameters recorded since the treatment started, the required non-progression in non-target lesions and no new lesions for at least 6 weeks. Progressive disease (PD) indicated a >20% increase in the sum of the longest diameters of target lesion, taking as reference the smallest sum longest diameter recorded since the treatment started

and/or unequivocal progression of existing non-target lesions and/or appearance of new lesions. The evaluation of objective tumor response for all patients was performed by an external review committee.

Toxicity grading criteria of the National Cancer Institute Common Toxicity Criteria (version 2.0) was used for evaluation of toxicity.

Statistical analysis

This study was designed to reject response rates of 70% (P0) at a significance level of 0.05 (one-tailed) with a statistical power of 80% to assess the activity of the regimen as a 85% response rate (P1) at the recommended dose. The upper limit of rejection was 29 responses (CR+PR) among 37 evaluable patients. Overall survival was defined as the interval between the first administration of the drugs in this study and death or the last follow-up visit. Median overall survival was estimated using the Kaplan–Meier method [18].

Table 1. Characteristics of treated patients

	Phase I	Phase II	Total
Number of patients	7	37	44
Gender			
Male	5	31	36
Female	2	6	8
Age (years)			
Median	65	64	64.5
Range	54–73	50–74	50–74
ECOG PS			
0	0	5	5
1	7	32	39
2	0	0	0
Stage			
IIIB	0	2	2
IV	7	35	42
Prior therapy			
Yes	0	1	1
No	7	36	43
Serum ALP			
Normal	7	29	36
Elevated	0	7	7
Serum LDH			
Normal	3	14	17
Elevated	4	23	27
Na			
Normal	6	35	41
Decreased	1	2	3
Number of metastases			
0	0	2	2
1	4	27	31
2	3	6	9
3	0	1	1
4 or more	0	1	1

In one patient, serum ALP level could not be measured.
ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; ALP, alkaline phosphatase.

Pharmacokinetic analysis

Pharmocokinetic analysis was performed in patients entering the phase I section of this study. One milliliter of the blood was taken from the patients before administration of amrubicin, and at 0 min, 15 min, 1, 2, 3, 4, 8 and 24 h after administration on days 1 and 3 in the first course of chemotherapy. Concentrations of amrubicin and its active metabolite, amrubicinol, in plasma and red blood cells were measured as reported elsewhere [9].

Results

Patient characteristics

Between April 2001 and December 2002, 45 patients with ED-SCLC were enrolled and 44 were treated in this study (Table 1). One patient did not receive the protocol treatment because atrial fibrillation was observed just before administration on day 1 of the first course. All treated patients were assessed for response, survival and toxicity. The median age of the treated patients was 64.5 years (range 50–74). There were 36 males and eight females. Five patients had an ECOG PS 0 and 39 patients had PS 1. Only one patient received surgery for brain metastasis as a prior therapy.

MTD and DLT in the phase I study

Four patients were enrolled at dose level 1 (amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1) and three patients at level 2 (amrubicin 45 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1). Toxicities in the phase I study are listed in Table 2. No DLT were observed during the first course of level 1. At level 2, grade 4 neutropenia for $\geq$4 days and febrile neutropenia occurred in one patient, and febrile neutropenia and grade 3 constipation occurred in another patient. Consequently, the MTD and RD were determined to be level 2 and level 1, respectively.

Pharmacokinetics of amrubicin and its active metabolite, amrubicinol

Pharmacokinetic parameters of amrubicin in plasma were almost identical on days 1 and 3 at the two dose levels (Table 3). No clear dose relationship in the area under the concentration–time curve (AUC) of amrubicin in the plasma was observed. The AUC of amrubicinol in red blood cells tended to increase on day 3 at both doses (Table 4). No clear dose relationship in the AUC of amrubicinol in red blood cells was observed. Combination with cisplatin did not alter the pharmacokinetics of amrubicin and amrubicinol (data not shown).

Treatment received in patients treated at the RD

Forty-one patients were treated at the RD: amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1. Of 41 patients, 32 (78%) patients received more than three

Table 2. Toxicities during the first course in the phase I study

	Level 1 (n=4)					Level 2 (n=3)				
Amrubicin	40 mg/m^2 days 1–3					45 mg/m^2 days 1–3				
Cisplatin	60 mg/m^2 day 1					60 mg/m^2 day 1				
	Grade (NCI CTC)					Grade (NCI CTC)				
	0	1	2	3	4	0	1	2	3	4
Leukopenia	0	1	1	2	0	0	0	1	1	1
Neutropenia	0	0	0	2	2	0	0	0	0	3
Febrile neutropenia	4	–	–	0	0	1	–	–	2	0
Hemoglobin	1	1	2	0	0	2	1	0	0	0
Thrombocytopenia	1	2	0	1	0	0	2	0	1	0
Stomatitis	3	0	1	0	0	3	0	0	0	0
Nausea	1	1	2	0	–	1	1	0	1	–
Constipation	3	0	1	0	0	1	0	1	1	0
Hyponatremia	2	1	0	0	1	1	2	0	0	0
Hypocalcemia	3	0	1	0	0	3	0	0	0	0

Dose limiting toxicity at level 2: febrile neutropenia, two patients; grade 4 neutropenia ≥4 days, one patient; grade 3 constipation, one patient.
NCI CTC, National Cancer Institute Common Toxicity Criteria.

Table 3. Pharmacokinetics of amrubicin in plasma

Dose	n	Day	$T_{1/2\alpha}$ (h)	$T_{1/2\beta}$ (h)	V_d (l)	CL (l/h)	$AUC_{0-24\,h}$ (ng h/ml)
40 mg/m^2	4	1	0.11±0.04	2.29±0.31	46.6±11.0	13.6±1.8	2995±434
	4	3	0.08±0.01	2.89±0.34	50.0±10.6	11.6±1.9	3511±514
45 mg/m^2	3	1	0.13±0.05	2.39±0.34	56.3±10.6	14.9±1.8	3052±402
	3	3	0.09±0.03	2.27±0.18	51.9±3.7	14.2±2.3	3217±479

$T_{1/2\alpha}$, half-life at distribution phase; $T_{1/2\beta}$, half-life at elimination phase; V_d, volume of distribution; CL, clearance; AUC, area under the concentration–time curve.

courses of chemotherapy, and 10 (31%) of these 32 patients needed dose reduction of amrubicin at the fourth course (Table 5). Of 41 patients, 22 (54%) patients completed four courses of chemotherapy without dose modification. The main cause of dose reduction was myelosuppression, especially leukopenia and neutropenia.

Objective tumor response and overall survival

The objective tumor responses are given in Table 6. Four CRs and 32 PRs occurred, for an objective response rate of 87.8% [95% confidence interval (CI) 73.8% to 95.9%] in 41 patients treated at the RD. The objective response rate for all 44 patients was 88.6% (95% CI 75.4% to 96.2%). The overall survival times of the 41 patients treated at the RD are shown in Figure 1. The MST of the 41 patients was 13.6 months (95% CI 11.1–16.6), with a median follow-up time for eight censored patients of 16.4 months (95% CI 14.2–18.8). The 1- and 2-year survival rates were 56.1% and 17.6%, respectively. The MST of all 44 patients was 13.8 months (95% CI 11.1–16.6). The 1- and 2-year survival rates of all 44 patients were 56.8% and 21.4%, respectively.

Table 4. Pharmacokinetics of amrubicinol in red blood cells

Dose	n	Day	$T_{1/2}$ (h)	$AUC_{0-24\,h}$ (ng·h/ml)
40 mg/m^2	4	1	21.0±3.1	1412±314
	4	3	20.7±4.8	2159±622
45 mg/m^2	3	1	19.6±6.1	1098±277
	3	3	18.1±5.7	2027±332

$T_{1/2}$, elimination half-life; AUC, area under the concentration–time curve.

Table 5. Treatment received in patients treated at the recommended dose

Cycle	n	Amrubicin (mg/m^2)			Cisplatin (mg/m^2)	
		40	35	30	60	45
1	41	41			41	
2	36	30	6		36	
3	33	26	5	2	33	
4	32	22	8	2	32	
5	18	9	5	4	18	
6	13	6	3	4	12	1

Table 6. Response rates

	n	CR	PR	SD	PD	NE	Response rate (%) (95% CI)
All	44	4	35	3	0	2	88.6 (75.4–96.2)
Treated at RD	41	4	32	3	0	2	87.8 (73.8–95.9)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated; 95% CI, 95% confidence interval; RD, recommended dose.

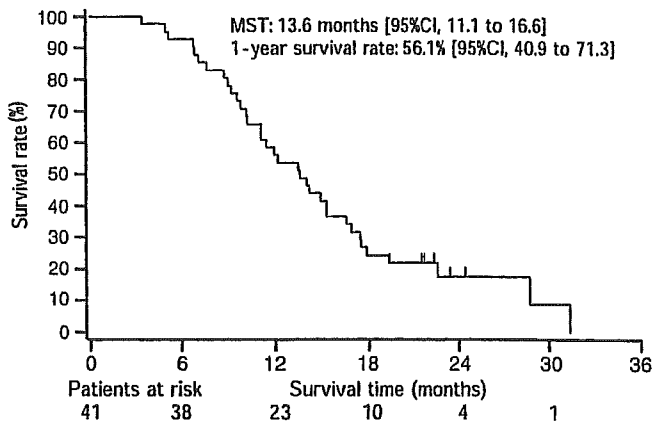

Figure 1. Overall survival of patients with extensive-stage small-cell lung cancer who were treated with amrubicin and cisplatin at the recommended dose. MST, median survival time; 95% CI, 95% confidence interval.

Toxicity in patients treated at the RD

The worst grades of hematological and non-hematological toxicities experienced by each patient are listed in Table 7. Hematological toxicity, especially leukopenia and neutropenia, was common and relatively severe. Grade 3 or worse leukopenia and neutropenia occurred in 65.9% and 95.1% of patients, respectively. Febrile neutropenia was observed in two patients at level 2. Grade 3 or worse anemia and thrombocytopenia occurred in 53.7% and 24.4% of patients, respectively. Four patients received platelet transfusions. Common non-hematological toxicities were gastrointestinal toxicity, such as anorexia, nausea, vomiting, constipation, diarrhea and stomatitis. Gastric ulcers developed in three patients. Hepatic and renal toxicity were not common in this study. Grade 3 or worse hyponatremia and hypokalemia occurred in 22% and 9.8% of patients, respectively. One patient developed myocardial infarction; however, cardiac toxicity was not common. No treatment-related deaths were observed.

Discussion

Doxorubicin and epirubicin are classified as active agents for SCLC, for which single-agent activity is a >20% response rate [19]. Doxorubicin has been used as a constituent of combination therapy for SCLC in the CAV (cyclophospamide, doxorubicin and vincristine) and CAP (cyclophosphamide, doxorubicin and cisplatin) regimens. Epirubicin has shown

Table 7. Toxicity in patients treated at the recommended dose ($n=41$)

	Grade (NCI CTC)					Grade 3/4 (%)
	0	1	2	3	4	
Leukopenia	1	0	13	20	7	65.9
Neutropenia	0	1	1	7	32	95.1
Febrile neutropenia	41	–	–	0	0	0.0
Hemoglobin	1	8	10	17	5	53.7
Thrombocytopenia	9	14	8	10	0	24.4
Stomatitis	22	13	5	1	0	2.4
Anorexia	1	14	13	13	0	31.7
Nausea	3	15	14	9	0	22.0
Vomiting	20	8	11	2	0	4.9
Constipation	24	1	13	3	0	7.3
Diarrhea	26	12	1	2	0	4.9
Gastric ulcer	38	0	1	2	0	4.9
Bilirubin	24	12	4	1	0	2.4
Hyponatremia	18	14	–	7	2	22.0
Hypokalemia	31	6	–	4	0	9.8
Hyperkalemia	33	3	4	1	0	2.4
Hypocalcemia	31	5	4	0	1	2.4

NCI CTC, National Cancer Institute Common Toxicity Criteria.

50% and 48% response rates in two clinical studies in 41 and 80 previously untreated patients, respectively, with ED-SCLC [20, 21]. However, currently, combination modalities containing doxorubicin or epirubicin are not being used in the therapy of SCLC, in preference to combination therapy with cisplatin and etoposide. Since amrubicin has shown excellent single-agent activity [15], it can be expected to be superior to other anthracyclines in the treatment of SCLC. Additionally, the present results of combination therapy with cisplatin support the view that amrubicin may be a promising agent that overcomes the therapeutic plateau of SCLC.

Amrubicin is one of the most promising new agents for the treatment of SCLC. In a previous phase II study of amrubicin 45 mg/m^2 on days 1–3 every 3 weeks as a monotherapy for chemonaive ED-SCLC, a 76% overall response rate and 11.7 month MST were observed [15]. The overall response rate and MST were comparable to those achieved with standard combination chemotherapy, such as etoposide plus cisplatin [5, 6]. Moreover, only a few patients treated in the phase II study received salvage chemotherapy consisting of cisplatin and etoposide [15]. The major toxicity of amrubicin as a monotherapy was hematological toxicity: grade 4 leukopenia and neutropenia were seen in 12.1% and 39.4% of patients, respectively, and thrombocytopenia and anemia of grade 3 or worse in 21.2%. Hepatic, renal and cardiac toxicities with amrubicin were not common. Cisplatin is a key drug for the treatment of SCLC and its hematological toxicity, such as leukopenia and neutropenia, is not severe. Thus, we conducted a phase I–II study of amrubicin and cisplatin treatment for chemonaive ED-SCLC to determine the MTD of this combination therapy, to

assess the efficacy and safety of the drugs delivered at their RD in chemonaive ED-SCLC, and to examine pharmacokinetics.

The topoisomerase I inhibitor, irinotecan, is also very effective for SCLC [6]. Combinations of topoisomerase I and topoisomerase II inhibitors, such as irinotecan plus etoposide, have been reported as active combination chemotherapy for SCLC [22]. Thus, combination of irinotecan and amrubicin is another candidate for new combination chemotherapy for SCLC. A phase I study of irinotecan and amrubicin for chemonaive non-SCLC was performed in National Cancer Center Hospital (unpublished data). However, the MTD was less than irinotecan 60 mg/m^2 on days 1 and 8 and amrubicin 35 mg/m^2 on days 2–4, due to relatively severe myelotoxicity. We considered that amrubicin <35 mg/m^2 on days 2–4 with irinotecan 60 mg/m^2 on days 1 and 8 was insufficient to treat SCLC.

In this study, we determined the RD to be amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1 every 3 weeks, and 41 patients were treated at the RD. Main toxicities of this combination chemotherapy were myelosuppression, especially leukopenia and neutropenia, and gastrointestinal toxicities including anorexia, nausea, vomiting, constipation, diarrhea, stomatitis and gastric ulcer. Of 41 patients, 32 (78%) patients received four or more courses of chemotherapy, and 22 (54%) patients completed four courses of chemotherapy without dose modification. One patient developed myocardial infarction; however, other cardiac toxicity, including decrease in left ventricle ejection fraction, was not observed in up to six courses of chemotherapy. The total dose of amrubicin was 720 mg/m^2. Grade 3 or 4 hyponatremia occurred in nine (22%) patients; however, most of the patients were asymptomatic. No unexpected toxicities and no treatment-related deaths were observed in this study. Toxicities observed in this study were manageable.

Four CRs and 32 PRs occurred, for an objective response rate of 87.8% (95% CI 73.8% to 95.9%) in 41 patients treated at the RD. In most patients, ProGRP levels changed in parallel with tumor responses. The MST of the 41 patients was 13.6 months, and the 1-year survival rate was 56.1%. These results were better than recently reported results for irinotecan and cisplatin in chemonaive ED-SCLC: an objective response rate of 84% and MST of 12.8 months [6]. The combination of amrubicin and cisplatin has demonstrated an impressive response rate and MST in patients with previously untreated ED-SCLC. A possible reason for the better results is overselection of patients, because we used unusual exclusion criteria such as non-steroidal anti-inflammatory drug or adrenal cortical steroid use for >50 days, and gastric and/or duodenal ulcer. However, in a phase II study, this kind of bias is not uncommon.

Combination chemotherapy with etoposide plus cisplatin or etoposide plus cisplatin, alternating with cyclophosphamide, doxorubicin and vincristine, had been considered as standard chemotherapy for SCLC in North America and Japan. A Japanese phase III trial (JCOG 9511) demonstrated that treatment with four cycles of irinotecan plus cisplatin every 4 weeks yielded a highly significant improvement in survival in ED-SCLC patients over standard etoposide plus cisplatin, with less myelosuppression [6]. Based on the results of the JCOG 9511 trial, irinotecan plus cisplatin is considered to be the reference chemotherapy arm for ED-SCLC in future trials in Japan [23]. The JCOG are preparing a phase III clinical trial of amrubicin and cisplatin for previously untreated ED-SCLC to compare combination therapy of irinotecan with cisplatin.

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EKB-569, a new irreversible epidermal growth factor receptor tyrosine kinase inhibitor, with clinical activity in patients with non-small cell lung cancer with acquired resistance to gefitinib

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KEYWORDS
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Summary EKB-569 is a potent, low molecular weight, selective, and irreversible inhibitor of epidermal growth factor receptor (EGFR) that is being developed as an anticancer agent. A phase 1, dose-escalation study was conducted in Japanese patients. EKB-569 was administered orally, once daily, in 28-day cycles, to patients with advanced-stage malignancies known to overexpress EGFR. Two patients with advanced non-small cell lung cancer with EGFR mutations and acquired gefitinib resistance from the phase 1 study are described in detail. *Case #1* is a 63-year-old man with smoking history. He received treatment from 4 March 2004. Because he had no severe adverse events, a total of 10 courses of therapy were completed through December 16. Grade 2 skin rash and ALT elevation, and grade 1 diarrhea and nail changes developed. A chest CT scan on 4 August 2003 revealed multiple pulmonary metastases that had decreased in size. *Case #2* is a 49-year-old woman with no smoking history. She received therapy from 9 February 2004. She received a total of five courses of the therapy until 22 June 2004. Grade 3 nausea and vomiting

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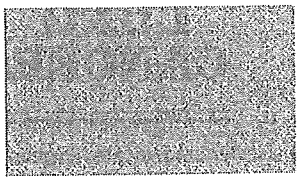

and grade 1 diarrhea and dry skin developed. A chest CT scan on March 3 revealed multiple pulmonary metastases that had decreased in size. A brain MRI on March 4 showed that multiple brain metastases also had decreased in size. Based on RECIST criteria, they had stable disease but radiographic tumor regression was observed.

1. Introduction

1.1. Efficacy of gefitinib

The epidermal growth factor receptor (EGFR) autocrine pathway contributes to a number of processes important to cancer development and progression, including cell proliferation, apotosis, angiogenesis, and metastatic spread [1]. EGFR-tyrosine kinase has become a particularly promising drug targeting for treating non-small cell lung cancer. Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways implicated in proliferation and survival of cancer cells [2]. Responsiveness characteristics include distinct subgroups of women, patients who have never smoked, patients with adenocarcinoma, and Asians [3–5]. Molecular predictive markers have also been investigated. It is suggested that MAPK is a predictive marker for survival after treatment with gefitinib in chemo-naive patients with bronchioloalveolar carcinoma [6]. Patients with P-Akt-positive tumors who received gefitinib had a better response rate, disease control rate, and time to progression than patients with P-Akt-negative tumors, suggesting that gefitinib may be most effective in patients with basal Akt activation [7]. However, it was not possible to predict gefitinib sensitivity by the level of EGFR overexpression as determined by immunohistochemistry [8] or immunoblotting [9]. Recently it has been reported that somatic mutations in the tyrosine kinase domain of the *EGFR* gene occur in a subset of patients with lung cancer who showed a dramatic response to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib [10–12]. All of these mutations were within exons 18 through 21 of the kinase domain of the *EGFR* gene.

1.2. Drug summary

EKB-569 (Wyeth Research, Collegeville, PA) is a potent, low molecular weight, selective, and irreversible inhibitor of EGFR that is being developed as an anticancer agent. EGFR is a receptor tyrosine kinase that is activated by a variety of growth factors. Upon binding ligands, including epidermal growth factor (EGF) or transforming growth factor alpha (TGF-α), EGFR dimerizes and its intracellular kinase domain is activated, leading to the recruitment and phosphorylation of a number of proteins that ultimately lead to cell growth [13,14]. Several features of EKB-569 may provide certain advantages over other EGFR inhibitors. First, EKB-569 is an orally available, small-molecule EGFR inhibitor, whereas antibody-targeted EGFR inhibitors require intravenous (IV) administration. Second, EKB-569 is an irreversible inhibitor of EGFR, while other small-molecule EGFR inhibitors bind EGFR reversibly [15].

1.3. Effects in humans (Japanese)

A phase 1, open-label, dose-escalation study to assess the safety, tolerability, and pharmacokinetics of EKB-569 was conducted in Japanese patients. EKB-569 was administered orally, once daily, in 28-day cycles, to patients (pts) with advanced-stage malignancies known to overexpress EGFR. Enrollment and treatment are completed; 15 pts (six men, nine women) were treated with 25 mg (3 pts), 35 mg (8 pts), or 50 mg (4 pts) of EKB-569. Their median age was 62 years (range 47–72); ECOG performance status varied: 0 = 4/15 (26.7%) or 1 = 11/15 (73.3%).

The most frequently occurring tumor types included non-small cell lung (10 pts) and breast (2 pts). The remaining tumors were renal, leiomyosarcoma, and malignant thymoma (1 pt each). The most frequently reported EKB-569-related adverse events were diarrhea (86.7%), rash (53.3%), anorexia (40.0%), and dry skin (40.0%). Dose-limiting toxicities were observed at the 50-mg dose level with grade 4 interstitial lung disease and grade 3 diarrhea, stomatitis, and increased blood calcium levels. Thus, the maximum tolerated dose was 35 mg EKB-569 per day.

1.4. Molecular analysis of lung cancer specimens

We obtained appropriate approval from the institution and written informed consent from the patients for the comprehensive use of tumor samples for molecular and pathologic analyses. Surgically resected tumor samples were obtained retrospectively before the patients received

any systemic treatment. All of these tumors were formalin fixed and paraffin embedded by the Department of Pathology. To minimize non-neoplastic tissue contamination, the tumor portion was first selected and marked on an H&E-stained tissue section slide by a pathologist. Only the tumor portion was dissected from the unstained tissue section and sent for DNA extraction.

DNA was extracted from the paraffin section containing a representative portion of each tumor, using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). For mutational analysis of the kinase domain of the *EGFR* coding sequence, exons 19, 20, and 21 were amplified with three pairs of primers (exon 19, F: 5′-TCACAATTGCCAGTTAACGTCT-3′-(this is the convention for writing a primer), R: 5#-cagcaaagcagaaactcacatc; exon 20, F: 5#-tgaaact-caagatcgcattcat, R: 5#-catggcaaactcttgctatcc; exon 21, F: 5#-gagcttcttcccatgatgatct, R: 5#-gaaaatgctggctgacctaaag). The PCR conditions were one cycle at 95 °C for 11 min, 46 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s, followed by one cycle at 72 °C for 7 min. PCR products were diluted and cycle-sequenced using the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Forster City, CA) according to the manufacturer's instructions. Sequencing products were electrophoresed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All sequencing reactions were performed in both forward and reverse directions and chromatograms were reviewed manually and analyzed by BLAST (basic local alignment search tool). High-quality sequence variations found in both directions were scored as candidate mutations.

2. Clinical cases

Two patients from the Japanese phase 1 study are described in detail.

2.1. Case #1

A 63-year-old man with smoking history (BI: 720) who was treated for hyperlipidemia and hypertension showed an abnormal chest X-ray in February 1996. Further examinations including a chest computed tomography (CT) scan and bronchoscopy revealed an adenocarcinoma of the lung, c-T1N0M0, stage Ia, in the right upper lobe. He had undergone a right upper lobectomy with mediastinal lymph node dissection in July 1996 and was proven to have a well-differentiated adenocarcinoma, p-T1N0M0, stage Ia. After further follow-up, multiple pulmonary metastases in both lungs were found in January 2000. Then he was given first-line chemotherapy of cisplatin and docetaxel beginning in May 2000. After two courses of this regimen, multiple pulmonary metastases had not increased in size by CT scan; however skin metastases were found. He was started on oral gefitinib 250 mg/day on November 2000. After 4 weeks, a CT scan indicated a reduction of multiple pulmonary metastases. During this treatment, grade 2 rash and grade 1 nail changes, AST/ALT elevations, and diarrhea were observed. On June 2002, multiple pulmonary metastases had increased, and this treatment was discontinued. The patient entered a phase I study of a new EGFR tyrosine kinase inhibitor (TAK-165), starting treatment on October 2002. After 2 weeks of treatment, grade 3 anorexia was observed and the therapy was stopped. On February 2003, multiple pulmonary metastases had more increased, and on March 2003, he entered a phase I study of EKB-569, receiving treatment from 4 March 2004. EKB-569 (25 mg) was administered orally, once daily, in 28-day cycles. Because he had no severe adverse events, a total of 10 courses of therapy were completed through December 16. Grade 2 skin rash and ALT elevation, and grade 1 diarrhea and nail changes developed during this therapy. Based on RECIST criteria, the patient had stable disease (SD) but radiographic tumor regression was observed on 4 August 2003 (day 27 in the sixth course) (Fig. 1). The size of multiple pulmonary metastases increase by CT scan on 8 December 2003, and the treatment was stopped on 17 December 2003.

A lung cancer specimen was obtained at surgery and studied by immunohistochemistry. EGFR overexpression was detected. In addition, we found the heterozygous in-frame deletion E746-A750 in exon 19 of the *EGFR* gene by direct sequencing of the specimen.

2.2. Case #2

A 49-year-old woman with no smoking history, who was treated for Basedow's disease, insomnia, and bronchial asthma, had an abnormal chest X-ray in October 2000. Further examinations including a chest CT scan and bronchoscopy revealed lung cancer in the left upper lobe. She was diagnosed with adenocarcinoma, c-T1N0M0, stage Ia. She had a left-upper lobectomy with mediastinal lymph node dissection, which revealed a well-differentiated adenocarcinoma, p-T4N2M1, stage IV. She was then given first-line chemotherapy of carboplatin and paclitaxel beginning in January 2001. After two courses of therapy, she discontinued treatment because of adverse events. Right supraclavicular lymph node metastases were found on August

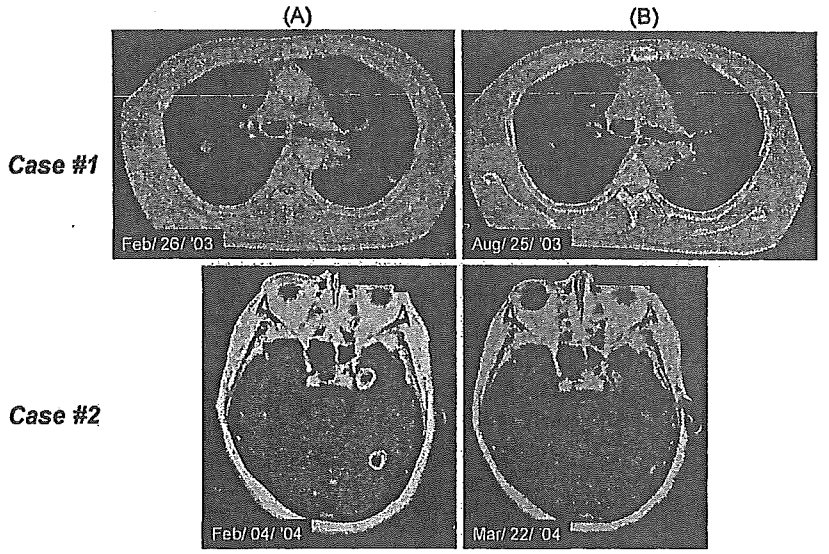

Fig. 1 *Clinical case #1*: a 63-year-old man with adenocarcinoma of lung. CT scan before treatment (A) and after initiation of EKB-569 (B). *Clinical case #2*: a 49-year-old woman with adenocarcinoma of brain metastasis. MRI scan before treatment (A) and after initiation of EKB-569 (B).

2001. Radiotherapy for the metastases (60 Gy/30 fractions) was done, and they decreased in size. On March 2002, right supraclavicular lymph node metastases increased and left clavicular lymph node metastases were found. On April 2002, the patient enrolled in a phase II trial of cisplatin, gemcitabine, and irinotecan for non-small-cell lung cancer. After two courses of therapy, bone metastases were found and pulmonary metastases had grown slowly so the treatment was stopped. She entered a phase I study of a new EGFR tyrosine kinase inhibitor (TAK-165) and started treatment on July 2002. The treatment was stopped after a week later due to grade 3 fatigue. In September 2002, the patient was started on oral gefitinib 250 mg/day. While she was taking 250 mg gefitinib daily for 15 months, the size of multiple pulmonary and bone metastases did not increase by CT scan and she had SD. On December 2003, the patient developed grade 3 oral mucositis and discontinued treatment. On January 2004, the size of multiple pulmonary and bone metastases increase by CT scan. She then entered a phase I study of EKB-569 and received therapy from 9 February 2004. EKB-569 (35 mg) was administered orally, once daily, in 28-day cycles. She received a total of five courses of the therapy until 22 June 2004. Grade 3 nausea and vomiting and grade 1 diarrhea and dry skin developed during the therapy. A chest CT scan on March 3 (day 24 in the first course) revealed multiple pulmonary metastases that had decreased in size. A brain MRI on March 4 (day 25 in the first course) showed that multiple brain metastases also had decreased in size (Fig. 1). The response was SD by RECIST criteria, although tumor regression was observed. The size of bone metastases increase by CT scan on 18 June 2004, and the treatment was stopped on 22 June 2004.

A lung cancer specimen was obtained by surgery and studied by immunohistochemistry. EGFR overexpression was detected. This lung cancer specimen had a heterozygous point mutation in exon 21 (L858R, CTG to CGG) of the *EGFR* gene.

3. Discussion

This is the first case report to describe the effects of EKB-569 on patients with adenocarcinoma of the lung. Case 1 is a 63-year-old man with a smoking history (BI: 720), and case 2 is a 49-year-old woman with no smoking history. Case 1 had an exon 19 deletion of E746-A750, and case 2 had an exon 21-point mutation. These patients underwent surgery and were treated with platinum-based chemotherapy and EGFR tyrosine kinase inhibitors. The treatment with EKB-569 was effective in these two patients after resistance to gefitinib and cytotoxic chemotherapy. These cases suggest that EKB-569 is effective in patients with *EGFR* mutations as has been reported for gefitinib and erlotinib. Despite initial responses to these EGFR inhibitors, patients eventually progress by unknown mechanisms of "acquired" resistance.

Recently, a second mutation in the *EGFR* kinase domain, which is associated with acquired resistance of non-small cell lung cancer to gefitinib or erlotinib, was reported [16,17]. Pao et al. showed that in two of five patients with acquired resistance

to gefitinib or erlotinib, progressing tumors contained, in addition to a primary drug-sensitive mutation in EGFR, a secondary mutation in exon 20. This mutation leads to a substitution of methionine for threonine at position 790 (T790M) in the kinase domain [16]. Kobayashi et al. reported the case of a patient with EGFR-mutant, gefitinib-responsive, advanced non-small cell lung cancer who relapsed after two years of complete remission during treatment with gefitinib. The DNA sequence of the EGFR gene in his tumor biopsy specimen at relapse also revealed the presence of the secondary point mutation, T790M [17]. Kurata et al. reported an interesting case in which acquired resistance to gefitinib could be overcome [18]. In this case, the patient received gefitinib, then a combination of nedaplatin and gemcitabine, and then gefitinib again. The cytotoxic agents may have altered the *EGFR* gene or associated genes to produce acquired sensitivity to gefitinib.

Kobayashi et al. also found that CL-387,785, a specific and irreversible, anilinoquinoline EGFR inhibitor [19], strongly inhibited the EGFR kinase in cells transfected with DNA containing the L747-S752 deletion in the *EGFR* gene or a double mutation with the L747-S753 deletion and the T790M point mutation. They speculated that CL-387,785 inhibited the EGFR kinase of the double mutant because of its altered binding to the kinase domain or its covalent binding to EGFR [17]. Kwak et al. used a bronchoalveolar cancer cell line with an L746-A750 deletion in the *EGFR* gene to isolate gefitinib-resistant clones. These clones had not acquired secondary EGFR mutations but were sensitive to the irreversible, anilinoquinoline EGFR inhibitor EKB-569 [20].

We have shown that EKB-569 had clinical activity in two patients with advanced non-small cell lung cancer with EGFR mutations and acquired gefitinib resistance. Thus, irreversible EGFR inhibitors may be an effective therapy for patients with EGFR-mutant advanced non-small cell lung cancer who have relapsed after treatment with gefitinib.

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Small interfering RNA targeting survivin sensitizes lung cancer cell with mutant p53 to adriamycin

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Survivin is a member of the inhibitor of apoptosis protein (IAP) family that is specifically overexpressed in cancer tissues. p53 is one of the tumor suppressor genes; its induction in response to DNA damage causes apoptosis and correlates with drug sensitivity. To investigate the possible regulation of survivin by p53, we examined the level of survivin expression in lung cancer cell lines in response to adriamycin. Levels of survivin mRNA and protein in cell lines with wild-type p53 decreased dramatically after p53 induction, but no such reduction of survivin was observed in cell lines with mutated or null p53. Inhibition of wild-type p53 in A549 cells by small interfering (si) RNA significantly upregulated the expression of survivin. Survivin inhibition by siRNA in PC9 cells with mutated p53 significantly depressed cell proliferation. To investigate the sensitivity of cancer cells to adriamycin after inhibition of survivin, we depressed survivin expression using siRNA, and then added adriamycin at an IC_{50} dose. After a further 48 hr incubation with adriamycin, proliferation was significantly depressed in the cells treated with siRNA targeting survivin, in comparison with siRNA targeting scramble. Furthermore, both TUNEL and pro-caspase3 expression assay showed a significant increase in apoptosis after combined treatment with adriamycin and siRNA targeting survivin. Our results demonstrate that survivin is downregulated by p53, and that siRNA targeting of survivin increases cell sensitivity to adriamycin and promotes apoptosis. siRNA targeting of survivin could be potentially useful for increasing sensitivity to anticancer drugs, especially in drug-resistant cells with mutated p53.

Key words: Survivin; siRNA; p53; lung cancer; Adriamycin

The success of cancer treatment depends on the response to chemotherapeutic agents. However, malignancies often acquire resistance to drugs if they are used frequently. Inhibition of the apoptosis pathway is one of the factors that may be responsible for such drug resistance.[1] Survivin is a member of the inhibitor of apoptosis protein (IAP) family that is specifically overexpressed in various cancers but not in normal adult tissues.[2] Overexpression of survivin is correlated with poor prognosis in a number of tumor types, including lung cancer,[3] colorectal cancer[4] and gastric cancer.[5] Like other mammalian IAPs (*e.g.*, XIAP, c-IAP-1, c-IAP-2 and livin), survivin binds to caspase-3 and caspase-7.[6] It has been suggested that survivin expression is regulated in a cell cycle-dependent manner.[7] Survivin is maximally expressed in the G2/M phase and physically associates with mitotic spindle microtubules that regulate progression through mitosis. In contrast, survivin is definitively depressed in the G1 phase. p53 is one of the tumor suppressor genes, and it is frequently mutated in cancer tissue/cells.[8] The crucial role of p53 is to maintain genetic stability through its participation in cell cycle checkpoints. After DNA damage induced by various cytotoxic agents, cells with wild-type p53 become preferentially arrested in the G0/G1 phase, after which they choose a path that results in either DNA repair or apoptosis. Apoptosis is closely linked to transcripts that are downregulated by p53. In contrast, mutation or deletion of p53 leads cells away from the apoptosis pathway, causing drug resistance.[9] It is generally accepted that p53 functions as a transcriptional factor and transactivates some genes, resulting in cell growth modulation or death. For example, an elevated level of p21, the first product of p53 transactivation, results in underphosphorylation of the retinoblastoma (Rb) protein, which in turn sequesters the E2F transcription factor; as a result, the cell cycle is blocked in the G1 phase.[10,11] Additionally, some genes, such as stathmin or cdc2, could be negatively regulated by p53.[12,13] Previous reports suggest that p53 also downregulates the expression of survivin in some cell models and cancer cell lines.[14,15] More recent reports have shown that inhibition of survivin by anti-sense oligonucleotide blocks the cell proliferation of myeloid leukemic cells[16] or lung cancer cells,[17] although the mechanism of this transcriptional regulation is not fully understood and requires additional research.

From another viewpoint, inhibition of survivin might play a role in overcoming acquired drug resistance. It has not been clarified how DNA-damaging agents influence survivin expression and cause cell cycle arrest and apoptosis. One report has suggested that anti-sense targeting of survivin sensitizes lung cancer cells to chemotherapy.[17] However, that study employed only 1 lung cancer cell line containing wild-type p53 and did not address the outcome that would be expected with mutated or deleted p53.

RNA interference (RNAi) is a mechanism whereby double-stranded RNA post-transcriptionally silences a specific gene. It has been reported that synthetic, double-stranded small-interfering RNA (siRNA) can effectively silence a gene through the RNAi mechanism.[18] siRNA can be a novel tool for clarifying gene function in mammalian cells and may be applicable to gene-specific therapeutics.[1] In our study, using siRNA, we aimed to sensitize lung cancer cell line to adriamycin. Our results suggest that siRNA targeting of survivin can inhibit cell growth and produce a combined anti-proliferative effect and apoptosis when combined with adriamycin, especially in cell lines containing mutated p53.

Material and methods

Drugs and cell lines

Adriamycin, obtained from Kyowa Hakko Kogyo Co. (Tokyo, Japan), was dissolved in distilled water and stored at −30°C until use. All cell lines used in our study were derived from patients with lung cancer. Lines NCI H226, H292, H358, H460, H522 and H1299 were obtained from the American Type Culture Collection (Manassas, VA). Lines A549, EBC-1, LK-2, Lu99, Lu99B, OBA-LK-1 and Sq-1 were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). SBC3, Lu65 and RERF-LC-KJ were obtained from the Japan Health Sciences Foundation (Tokyo, Japan). Lines PC9 and PC14 were kindly donated by Prof. Hayata, Tokyo Medical University (Tokyo, Japan). SBC3/ADM,[20]

Abbreviations: dH_2O, distilled H_2O; DW, distilled water; FBS, Fetal Bovine Serum; GAPDH, glyceraldehyde-3- phosphate; IAP, inhibitor of apoptosis protein; IC_{50}, 50% inhibitory concentration; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSCLC, non-small cell lung cancer; si RNA, small interfering RNA; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; SD, standard deviation; SE, standard error, TUNEL, TdT mediated dUTP nick end labeling.

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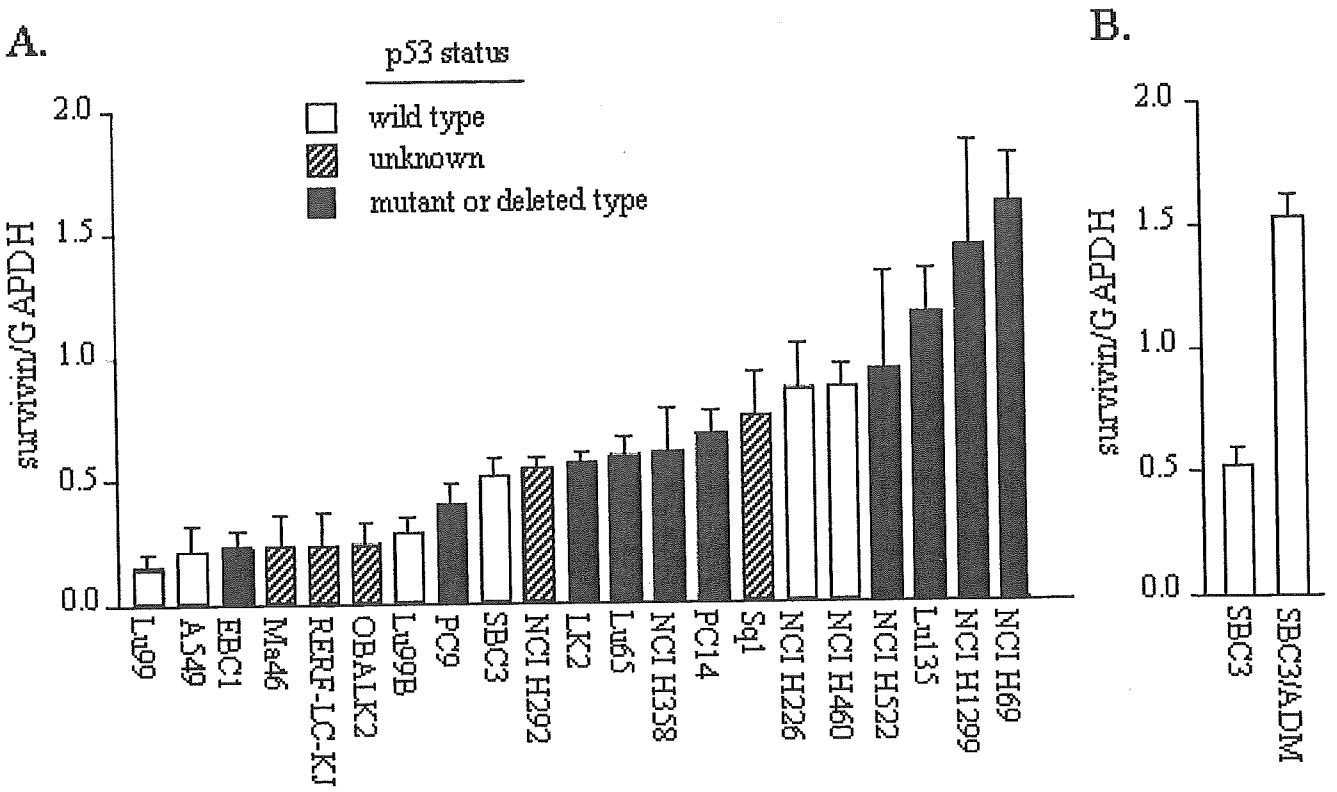

FIGURE 1 – Level of survivin mRNA in 22 lung cancer cell lines. (*a*) Cells were incubated in a 75 cm^2 flask, harvested and analyzed using real-time PCR as described in Material and methods. All data were normalized relative to the concentration of mRNA for the housekeeping gene GAPDH and are presented as the mean ± SD for at least 3 independent experiments. p53 status is presented. (*b*) Comparison between SBC3 and SBC3/ADM, the adriamycin-resistant subline, is shown.

a subline of SBC3 with approximately 8-fold stronger resistance to the growth-inhibitory effect of adriamycin, as determined by the MTT assay, was provided by Dr. Kiura, Okayama University (Okayama, Japan). Lu135 was provided by Riken Cell Bank (Tokyo, Japan). Ma46 was established in our laboratory from malignant effusion of an NSCLC patient. The cells were cultured in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum under a humidified atmosphere of 5% CO_2 and air at 37°C. All cell lines were discarded after 20 generations, and new lines were obtained from frozen stocks. Some cell lines were analyzed for their IC_{50} values using the MTT assay by incubating them with adriamycin for 72 hr.[21] With regard to p53 status, NCI H226, H460, A549, SBC3, SBC3/ADM, Lu99 and Lu99B possess wild-type p53. EBC-1, PC9, LK2, Lu65, NCI H358, H522, H69, PC14, Lu135 and Lu65 possess mutated p53. NCI H1299 has deleted p53.[22–26]

Real-time RT-PCR

Total RNA was extracted from cells treated with adriamycin, siRNA or water using an RNeasy Mini Kit (Qiagen, Inc., Tokyo, Japan). For first-strand cDNA synthesis, 1 μg total RNA from a sample was added to components of the Super Script Preamplification System (Life Technologies, Inc., Gaithersburg, MD), as described in the user's manual. Real-Time PCR was performed using the Gene Amp 5700 Sequence Detection System (Perkin-Elmer), and mRNA expression was quantified. For this purpose, 1 μl cDNA was mixed with commercial reagents (TaqMan PCR Reagent Kit, Perkin-Elmer Biosystems), following the manufacturer's protocol. Survivin cDNA was amplified using a forward primer consisting of 5′-ATGGGTGCCCCGACGT-3′ and a reverse primer consisting of 5′-AATGTAGAGATGCGGTGG-TCCTT-3′ and detected by a Taqman probe consisting of 5′-CCCCTGCCTGGCAGCCCTTTC-3′, each nucleotide corresponding to positions 50–65, 92–114 and 69–89 of the 1,619 bp survivin mRNA (GenBank NM001168). Relative quantification of gene expression was performed as described previously,[27] using the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) as an internal standard.

Western-blotting analysis

Cells treated with adriamycin, siRNA or water were harvested with trypsin/EDTA, and PBS-washed cell pellets were treated with HEPES lysate buffer (30 mM HEPES, 1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA and 10 mM NaCl). Equal amounts of protein extracts were loaded onto sodium dodecyl sulfate-polyacrylamide gels and ran at 200 V for 45 min followed by transfer to nitrocellulose membranes at 100 V for 30 min. at room temperature. The membranes were probed with the following primary antibodies: affinity-purified rabbit anti-survivin antibody (R&D Systems, Inc., Minneapolis, MN), mouse monoclonal anti-p53 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-actin affinity isolated antibody (Sigma-Aldrich Co., St. Louis, MO) and mouse monoclonal anti-caspase3 antibody (Santa Cruz Biotechnology) at room temperature for 120 min. As secondary antibodies, goat anti-rabbit labeled with horseradish peroxidase (Amersham Biosciences, England) and sheep anti-mouse labeled with horseradish peroxidase (Santa Cruz Biotechnology) were used. Blots were developed using a chemiluminescence detection system (Perkin Elmer Life Sciences, Boston, MA).[28]

Flow cytometry

Cells were treated with adriamycin, harvested, washed with PBS, fixed with 70% methanol, washed with PBS and stained with propidium iodide solution (0.05 mg/ml propidium iodide, 0.1% Triton X-100, 0.1 mM EDTA and 0.05 mg/ml RNase A). Approxi-