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ORIGINAL REPORT

First-Line Single Agent Treatment With Gefitinib in Patients With Advanced Non–Small-Cell Lung Cancer: A Phase II Study

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ABSTRACT

Purpose
We conducted a phase II study of single agent treatment with gefitinib in chemotherapy-naïve patients with advanced non–small-cell lung cancer (NSCLC) to assess its efficacy and toxicity.

Patients and Methods
Patients received 250 mg doses of gefitinib daily. Administration of gefitinib was terminated if partial response (PR) was not achieved within 8 weeks or if tumor reduction was not observed within 4 weeks. In these cases, platinum-based doublet chemotherapy was given as a salvage treatment. We evaluated mutation status of the epidermal growth factor receptor (EGFR) gene in cases with available tumor samples.

Results
Forty-two patients were enrolled between March and November 2003, with 40 of these patients being eligible. The response rate was 30% (95% CI, 17% to 47%). The most common toxicity included grade 1 or 2 acne-like rash (50%) and grade 1 diarrhea (18%). Grade 2 or 3 hepatic toxicity was observed in 8% of patients. Four patients developed grade 5 interstitial lung disease (ILD). Thirty patients received second-line chemotherapy. Median survival time was 13.9 months (95% CI, 9.1 to 18.7 months), and the 1-year survival rate was 55%. Tumor samples were available in 13 patients, including four cases of PR, six cases of stable disease, and three cases of progressive disease. *EGFR* mutations (deletions in exon 19 or point mutations [L858R or E746V]) were detected in four tumor tissues. All four patients with *EGFR* mutation achieved PR with gefitinib treatment.

Conclusion
Single agent treatment with gefitinib is active in chemotherapy-naïve patients with advanced NSCLC, but produces unacceptably frequent ILD in the Japanese population.

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INTRODUCTION

Previous meta-analysis demonstrated that cisplatin-based chemotherapy yielded a modest but significant survival benefit over best supportive care in advanced non–small-cell lung cancer (NSCLC).[1-4] In the 1990s, new agents, including vinorelbine, gemcitabine, paclitaxel, docetaxel, and irinotecan became available for the treatment of NSCLC. Several phase III trials comparing doublet platinum-based chemotherapies demonstrated no significant difference with respect to response rate, survival, or quality of life.[5,6] Nonplatinum or triplet platinum-based combination chemotherapies have been investigated, but none of these produced longer survival than standard doublet platinum-based chemotherapy.[7-9]

Recently, molecular-targeted agents have been introduced for the treatment of NSCLC. Gefitinib is an orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which displays activity against recurrent NSCLC after platinum-based chemotherapy. Two international, randomized phase II trials in patients with advanced or metastatic NSCLC after platinum-based chemotherapy demonstrated response rates of 12% to 18% (28% in the Japanese population).[10,11] Two international, randomized, double-blinded, placebo-controlled phase III trials investigated the role of gefitinib combined with platinum-based chemotherapy regimens, including carboplatin and paclitaxel, or cisplatin and gemcitabine in chemotherapy-naïve patients with advanced NSCLC.[12,13] Surprisingly, there were no improvements in overall survival,

time to progression, or response rate. There are no data available regarding first-line treatment with single agent gefitinib against NSCLC in the Japanese population. Here, we conducted a phase II study of single agent treatment with gefitinib in chemotherapy-naïve patients with advanced NSCLC. If a failure with gefitinib treatment was perceived, standard platinum-based doublet chemotherapy was performed as salvage. The primary end point of this phase II trial was response rate, and the secondary end points were toxicity, survival, and response rate of salvage chemotherapy.

PATIENTS AND METHODS

Patient Population

Patients were required to have histologically or cytologically confirmed stage IIIB (malignant pleural or pericardial effusion and/or metastasis in the same lobe) or stage IV NSCLC. Recurrences after surgical resection were permitted. Other criteria included: (1) age 20 years or older, but younger than 75 years; (2) Eastern Cooperative Oncology Group performance status (PS) 0 or 1; (3) measurable disease; (4) PaO2 $\geq$ 60 mmHg; (5) adequate organ function (ie, total bilirubin $\leq$ 2.0, AST and ALT $\leq$ 100 U/L, serum creatinine $\leq$ 1.5 mg/dL, leukocyte count 4,000 to 12,000/mm^3, neutrophil count $\geq$ 2,000/mm^3, hemoglobin $\geq$ 9.5 g/dL, and platelets $\geq$ 100,000/mm^3); (6) no prior chemotherapy or thoracic radiotherapy; (7) no interstitial pneumonia or pulmonary fibrosis, as determined by chest x-ray; (8) no paralytic ileus or vomiting, (9) no symptomatic brain metastases, (10) no active infection; (11) no active concomitant malignancy; (12) no pregnancy or breast-feeding; (13) no severe allergy to drugs. Patients with PaO2 less than 60 mmHg were excluded, because those patients might have pulmonary fibrosis, which is a risk factor of interstitial lung disease (ILD).[14] All patients were required to provide written informed consent and the institutional review board at the National Cancer Center approved the protocol.

Treatment Plan

Treatment was started within a week after enrollment in the study. Patients received 250 mg of gefitinib orally daily. In the event of grade 3 or more and/or unacceptable toxicities, gefitinib was postponed until these toxicities were improved to grade 2 or less. Dose reduction was not performed. If treatment was postponed four times or more, the treatment was terminated. Therapy was continued unless the patient experienced unacceptable toxicity or progressive disease, partial response (PR) was not achieved within 8 weeks, or the sum of the longest diameters of the target lesions decreased less than 10% within 4 weeks. If the gefitinib treatment failed according to these criteria, platinum-based doublet chemotherapy was performed as a salvage regimen.

Previous trials of gefitinib for pretreated patients with NSCLC reported that most responding patients showed rapid tumor regression within 4 or 8 weeks.[11] Furthermore, most responses by gefitinib were extreme shrinkage of the tumor. Minor response, as frequently seen by the treatment with cytotoxic agents, was seldom experienced. Stable disease with gefitinib corresponded to no tumor reduction or slight progression. If patients with stable disease continued the treatment with gefitinib until progressive disease became obvious, those patients might not be able to receive platinum-based salvage chemotherapy because of poor PS due to progressive disease. Platinum-based combination chemotherapy is the standard care for patients with advanced NSCLC and good PS. Platinum-based chemotherapy was thought to be essential for patients with no response from the first-line single agent treatment with gefitinib. Therefore, we implemented these early stopping criteria for treatment with gefitinib.

Study Evaluations

Pretreatment evaluations consisted of a complete medical history, determination of performance status, physical examination, hematologic and biochemical profiles, arterial blood gas examination, ECG, chest x-ray, bone scan, and computed tomography (CT) scan of the chest, ultrasound or CT scan of the abdomen, and magnetic resonance imaging or CT scan of the whole brain. Evaluations performed included a weekly chest x-ray for 4 weeks, and once every 2 weeks for biochemistry, complete blood cell, platelet, leukocyte differential counts, physical examination, determination of performance status, and toxicity assessment. Imaging studies were scheduled to assess objective response every month.

Response and Toxicity Criteria

Response evaluation criteria in solid tumors (RECIST) guidelines were used for evaluation of antitumor activity.[15] The target lesions were defined as $\geq$ 2 cm in the longest diameter on CT scans. A complete response (CR) was defined as the complete disappearance of all clinically detectable tumors for at least 4 weeks. A PR was defined as an at least 30% decrease in the sum of the longest diameters of the target lesions for more than 4 weeks with no new area of malignant disease. Progressive disease (PD) indicated at least a 20% increase in the sum of the longest diameter of the target lesions or a new malignant lesion. Stable disease was defined as insufficient shrinkage to qualify for PR and insufficient increase to qualify for PD. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.

Mutation Analysis of the EGFR Gene

Tumor specimens were obtained during diagnostic or surgical procedures. Biopsied or surgically resected specimens were fixed with formalin or 100% methanol, respectively. Tumor genomic DNA was prepared from paraffin-embedded sections using laser capture microdissection in biopsied specimens or macrodissection in surgically resected specimens at Mitsubishi Chemical Safety Institute LTD. Exons 18, 19, and 21 of the *EGFR* gene were amplified and sequenced as previously described.[16]

Statistical Analysis

In accordance with the minimax two-stage phase II study design by Simon,[17] the treatment program was designed to refuse response rates of 10% (P_0) and to provide a significance level of .05 with a statistical power of 80% in assessing the activity of the regimen as a 25% response rate (P_1). The upper limit for first-stage drug rejection was two responses in the 22 assessable patients; the upper limit of second-stage rejection was seven responses within the cohort of 40 assessable patients. Overall survival was defined as the interval between enrollment in this study and death or the final follow-up visit. Median overall survival was estimated by the Kaplan-Meier analysis method.[18] Fisher's exact test was used in a contingency table.

RESULTS

Patient Population

A total of 42 patients were enrolled in this study between March and November, 2003, with 40 of these patients being eligible. One patient was found ineligible due to anemia, the other because spinal magnetic resonance imaging could not confirm a positive bone scan. Patient characteristics are listed in Table 1. Sixty percent of patients were male; median age was 61 years. The most common histologic subtype was adenocarcinoma (75%). Most patients (93%) had stage IV disease or recurrence after surgical resection. Eighty percent of patients were current or former smokers.

Efficacy

One patient (3%) has been receiving gefitinib after 22 months. Four patients suspended gefitinib for 11, 14, 27, or 29 days, because of liver dysfunction (n = 3) and fever due to urinary tract infection (n = 1). Thirty-nine patients terminated gefitinib because of progressive disease (n = 20), no tumor reduction within 4 weeks (n = 12), not achieving PR within 8 weeks (n = 1), toxicities including pulmonary (n = 3), nausea and vomiting (n = 1), rash (n = 1), or hepatic dysfunction (n = 1).

There were 12 PRs in 40 eligible patients, and the objective response rate was 30% (95% CI, 17% to 47%; Table 2). All but one

Table 1. Patient Characteristics

Characteristic	No. of Patients
Patients enrolled	42
Patients eligible	40
Sex	
Male	24
Female	16
Age, years	
Median	61
Range	44-74
Performance status	
0	14
1	26
Stage	
IIIB	3
IV	34
Recurrence after surgery	3
Histologic type	
Adenocarcinoma	30
Squamous cell carcinoma	3
Large cell carcinoma	7
Smoking history	
Current	27
Former	5
Never	8

patient from this subgroup achieved PR within 4 weeks, with the remaining patient achieving PR within 8 weeks. The background of the 12 responding patients was as follows: nine females, three males; 11 adenocarcinomas, one large-cell carcinoma; six individuals who never smoked, five current smokers, and one former smoker. Response rates based on patient characteristics were as follows: three of 24 (13%) males, nine of 16 (56%) females ($P = .0050$); 11 of 30 (37%) individuals with adenocarcinoma, one of 10 (10%) individuals with squamous or large-cell carcinoma ($P = .0048$); six of 32 (19%) current or former smokers, and six of eight (75%) individuals who never smoked ($P = .0048$).

The median follow-up time was 23 months, and nine patients were still alive at the most recent follow-up. The median survival time was 13.9 months (95% CI, 9.1 to 18.7 months), and the 1-year survival rate was 55% (Fig 1).

Safety and Toxicity

Toxicity was evaluated in all eligible patients. The most common toxicity was rash (Table 3). Thirty-eight percent and 13% of patients experienced grade 1 or 2 rash, respectively. One patient experienced grade 3 nausea and vomiting, leading to gefitinib treatment being terminated. Grade 3 hepatic toxicity was observed in one patient, also causing termination of gefitinib treatment.

The most problematic toxicity was ILD. We reviewed the medical records, chest x-rays, and CT films of all the cases, which were suspected as ILD by the physician in charge. ILD was diagnosed on the basis of standard or high-resolution CT findings of the chest (diffuse ground-glass opacity, consolidation, or infiltrate) and no response to antibiotics. We diagnosed that four patients experienced grade 5 ILD during or after first-line treatment with gefitinib. The first patient was a 61-year-old man. He developed dyspnea and fever elevation (38.1°C) on day 23 of the treatment with gefitinib and administration of gefitinib was terminated. Chest CT demonstrated bilateral diffuse ground-glass opacity, and PaO2 was 43.7mmHg in the room air. KL-6 antigen, a serum marker of interstitial pneumonia, was not elevated

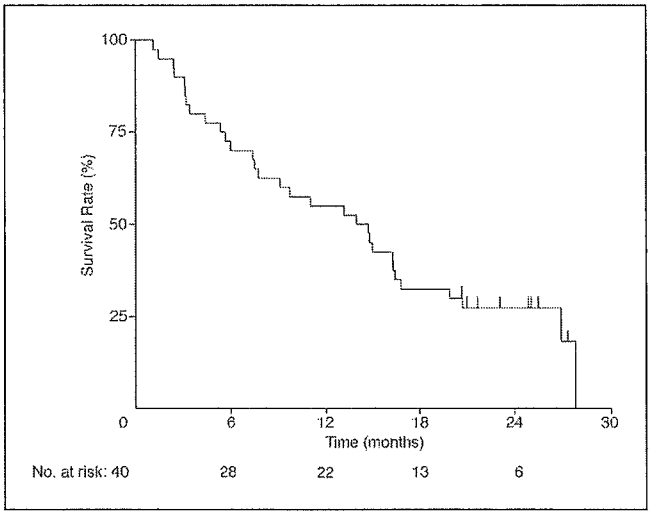

Fig 1. Overall survival of all eligible patients (n = 40) was calculated according to the Kaplan-Meier method. The median survival time was 13.9 months (95% CI, 9.1 to 18.7 months), and the 1-year survival rate was 55%.

Table 2. Efficacy of Single Agent Treatment With Gefitinib in Patients With Stage IIIb or IV Non–Small-Cell Lung Cancer

Type of Response	No. of Patients	% of Patients
Complete	0	0
Partial	12	30
CR + PR	12	30
95% CI	17 to 47	
Stable disease	16	40
Progression	12	30

Abbreviations: CR, complete response; PR, partial response.

Table 3. Maximum Toxicity Grades Associated With Single Agent Treatment With Gefitinib in 40 Patients With Non–Small-Cell Lung Cancer

	Toxicity Grade									
	1		2		3		4		5	
Toxicity	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
Rash	15	38	5	13	0	0	0	0	0	0
Dry skin	4	10	0	0	0	0	0	0	0	0
Diarrhea	7	18	0	0	0	0	0	0	0	0
Nausea	3	8	0	0	1	3	0	0	0	0
Mucositis	6	15	0	0	0	0	0	0	0	0
Alopecia	4	10	0	0	0	0	0	0	0	0
Hyponatremia	24	60	0	0	3	8	0	0	0	0
Hypokalemia	12	30	0	0	0	0	0	0	0	0
Hepatic	11	28	2	5	1	3	0	0	0	0
Renal	4	10	1	3	0	0	0	0	0	0
ILD	0	0	0	0	0	0	0	0	4	10

Abbreviation: ILD; interstitial lung disease.

(351 U/mL) on day 24, but elevated on day 31 (1,400 U/mL). Beta-D-glucan, a serum marker of fungal infection and *Pneumocystis carinii* pneumonia, was also negative. Methylprednisolone and antibiotics were administered, with temporal improvement of ILD. However, subsequently, pulmonary function gradually deteriorated, leading to death. Autopsy revealed alveolar damage with organization around the bronchus and vessels in both neoplastic and non-neoplastic lesions, compatible with drug-induced ILD. The second patient was a 64-year-old man. Chest CT on day 27 showed stable disease, but administration of gefitinib was continued (protocol violation). Periodic chest x-ray film on day 45 showed abnormal shadow in the left lung field. High-resolution CT of the chest on the same day revealed reticular shadow on bilateral upper lobe. The treatment with gefitinib was terminated on day 45. KL-6 antigen was not elevated on day 49 (276 U/mL). Methylprednisolone and antibiotics were administered, but were not effective, leading to death. The third patient was a 67-year-old man. Chest CT on day 30 demonstrated enlargement of primary lesion and bilateral reticular shadow in subpleural lesions. Gefitinib was terminated on day 30. The patient developed dyspnea without fever elevation on day 37. Pao2 in the room air fell to 61.0 mmHg from 82.4 mmHg at pretreatment. Chest x-ray showed that the bilateral diffuse reticular shadow deteriorated. Methylprednisolone and antibiotics were administered, but were not effective, leading to death. Autopsy revealed severe fibrotic thickness of alveolar septum, compatible with severe interstitial pneumonia. There was no pathological evidence of carcinomatous lymphangiosis. The fourth patient was a 59-year-old woman. Chest x-ray showed consolidation in the left lung on day 21. Slight fever (37.9°C) developed on day 22. Blood culture was negative. Antibiotics were administered, but consolidation deteriorated and spread to both lungs on day 25. Gefitinib was terminated on day 25. KL-6 antigen was elevated to 3,590 U/mL. Methylprednisolone was administered, but was not effective, leading to death (Table 4). Four other patients experienced ILD after second-line or third-line chemotherapy. Two patients received second-line treatment with cisplatin plus vinorelbine (one and four courses), one patient received treatment with cisplatin plus gemcitabine (one course), and one patient received third-line treatment with docetaxel (four courses). Three of four patients received steroids, with temporal improvement of ILD being observed in two patients. However, ILD deteriorated during tapering of steroid treatment, with three patients subsequently dying. One patient stopped the third-line treatment with docetaxel, with the associated ILD showing improvement in this case without steroid treatment (Table 4).

We retrospectively reviewed the pretreatment chest x-rays and CT films of all patients. Interstitial shadow was not detected on pretreatment chest x-ray films in any patients. However, six patients showed evidence of interstitial shadow on pretreatment chest CT films. Three of the six patients with interstitial shadow, as determined by pretreatment chest CT, experienced ILD either during or following administration of gefitinib or second-line chemotherapy. None of the six patients responded to gefitinib treatment. On the other hand, four of 34 patients who showed no interstitial shadow on pretreatment chest CT films experienced ILD. Interstitial shadow as determined by pretreatment chest CT was not a statistically significant risk factor of ILD ($P = .0819$; Table 5).

Second-Line Chemotherapy

A total of 30 patients received second-line chemotherapy. Twenty-seven patients received platinum-based chemotherapy (cisplatin plus vinorelbine; n = 17), carboplatin plus paclitaxel (n = 5), cisplatin plus gemcitabine (n = 3), cisplatin plus docetaxel (n = 1), and cisplatin plus irinotecan (n = 1). The remaining three patients received vinorelbine plus gemcitabine or vinorelbine alone. Nine of 30 patients achieved PR with these second-line chemotherapies. The objective response rate of second-line chemotherapy was 30% (95% CI, 15% to 50%).

Mutation Status of the EGFR Gene

Out of 42 enrolled patients, 16 patients were diagnosed pathologically, 22 were diagnosed cytologically, and four patients recurred after surgical resection. Biopsied specimens were available in nine patients. Therefore, tissue samples were available in a total of 13 patients. These 13 patients included four PRs, six with stable disease, and three PDs. *EGFR* mutations were detected in four tumor tissues, including the in-frame nucleotide deletions in exon 19 (n = 3) and an L858R mutation in exon 21 (n = 1). One tumor had an in-frame deletion and

Table 4. Four Patients Developed Interstitial Lung Disease During First-Line Chemotherapy With Gefitinib, With Another Four Patients Showing ILD During Either Second- or Third-Line Chemotherapy

Age (years)	Sex	Smoking Index	Pathology	Onset of ILD	Response to Gefitinib	Death From Chemotherapy
61	M	1,520	AD	Day 23*	PD	Day 74
64	M	880	AD	Day 45*	SD	Day 51
67	M	1,880	SQ	Day 37†	PD	Day 45
59	F	0	AD	Day 21*	PD	Day 35
61	M	820	AD	Day 131‡	SD	Day 154
68	M	2,000	LA	Day 37‡	PD	Day 106
68	M	705	AD	Day 22§	PR	Day 87
59	M	1,170	AD	Day 108‖	SD	Alive

Abbreviations: ILD, interstitial lung disease; M, male; F, female; AD, adenocarcinoma; SQ, squamous cell carcinoma; LA, large-cell carcinoma; PD, progressive disease; SD, stable disease; PR, partial response.
*During gefitinib administration.
†One week after discontinuation of gefitinib.
‡ After 2nd-line chemotherapy of cisplatin and vinorelbine.
§ After 2nd-line chemotherapy of cisplatin and gemcitabine.
‖ After 3rd-line chemotherapy of docetaxel.

Table 5. Interstitial Shadow on Pretreatment Chest Computed Tomography Films and ILD

Interstitial Shadow on Pretreatment Chest Computed Tomography Scans	No ILD	ILD
No existence	29	5
Existence	3	3

NOTE. $P = .0819$.
Abbreviation: ILD interstitial lung disease.

an E746V mutation in exon 19. All four PR patients had *EGFR* mutations (Table 6).

DISCUSSION

This phase II study was designed to evaluate the efficacy and safety of first-line single agent treatment with gefitinib in patients with advanced NSCLC. There is no other paper that evaluates single agent treatment with gefitinib prospectively in patients with advanced NSCLC. The observed response rate of 30% (95% CI, 17% to 47%), median survival of 13.9 months and 1-year survival of 55% are promising. However, grade 5 ILD occurred in 10% (95% CI, 3% to 24%) of patients. This high rate of ILD was not acceptable. The incidence of ILD was seen to be less than 1% in two randomized controlled studies comparing gefitinib with placebo in combination with gemcitabine and cisplatin or paclitaxel and carboplatin.[12,13] The reason for the high incidence of ILD observed in our study is unknown. The West Japan Thoracic Oncology Group analyzed 1,976 patients receiving gefitinib retrospectively. In this case, the incidence of ILD was 3.2% (95% CI, 2.5% to 4.6%) and the death rate due to ILD was 1.3% (95% CI, 0.8% to1.9%). Multivariate analyses found that risk factors included being male, individuals who smoked, and complication of interstitial pneumonia.[14] Our retrospective analyses revealed that three of six patients with interstitial shadow on pretreatment chest CT films, but not detected on chest x-ray films developed ILD; on the other hand, five of 34 patients without interstitial shadow developed ILD. Interstitial shadow on pretreatment chest CT was a marginally significant risk factor of ILD ($P = .0819$). It might be suggested that patients with interstitial shadow on pretreatment chest CT films be excluded from administration of gefitinib; however, our analyses were biased because we analyzed retrospectively and did not blind patient clinical information. Prospective analysis is needed to evaluate interstitial shadow by chest CT before treatment with gefitinib.

The Southwest Oncology Group conducted a phase II trial to evaluate gefitinib in patients with advanced bronchioloalveolar carcinoma (SWOG 0126). Previously untreated (n = 102) and treated (n = 36) patients were entered and eligible in SWOG 0126. The response rate was 19% and the median survival time was 12 months in the untreated population.[19] These subset analyses were comparable to our results.

Recently, mutations in the tyrosine kinase domain of EGFR were found to be associated with gefitinib sensitivity in patients with NSCLC.[16,20,21] Our retrospective analyses demonstrated that *EGFR* mutations were detected in four of 13 patients, and those four patients achieved PR in the single agent treatment of gefitinib. These results were compatible with previous reports.[16,20,21]

Thirty patients received second-line chemotherapy, including platinum-based (n = 27) and nonplatinum-based (n = 3) regimens; the response rate was 30%. Pretreatment with gefitinib does not seem to adversely affect the response of second-line chemotherapy. However, our small-scale study does not suggest the best second-line regimen. Platinum combined with any third-generation agents including paclitaxel, docetaxel, vinorelbine,

Table 6. Mutation Status of the *EGFR* Gene

Sex	Age (years)	Pathologic Type	Smoking Status	Overall Survival (months)	*EGFR* Gene	Effect of Mutation	Response to Gefitinib	Response to Second Line Chemotherapy
M	68	AD	Current	14.9	Deletion of 15 nucleotides (2236-2250)	In-frame deletion (E746-A750)	PR	PD
F	67	AD	Current	16.2	Deletion of 15 nucleotides (2236-2250)	In-frame deletion (E746-A750)	PR	PD
F	54	AD	Current	5.6	Deletion of 18 nucleotides (2238-2255) and substitution of T for A at nucleotides 2237	In-frame deletion (L747-S752) and amino acid substitution (F746V)	PR	NR
F	57	AD	Never	25.4	Substitution of G for T at nucleotide 2573	Amino acid substitution (L858R)	PR	SD
M	61	AD	Current	7.5	Wild	—	SD	SD
M	54	AD	Current	9.7	Wild	—	SD	SD
M	45	AD	Current	16.2	Wild	—	SD	PR
M	59	AD	Current	14.7	Wild	—	SD	PR
M	67	SQ	Current	2.4	Wild	—	SD	NR
M	59	AD	Current	24.9	Wild	—	SD	PR
M	61	AD	Current	2.4	Wild	—	PD	NR
F	61	SQ	Current	3.4	Wild	—	PD	PD
F	61	AD	Current	16.3	Wild	—	PD	PR

Abbreviations: EGFR, epidermal growth factor receptor; M, male; F, female; AD, adenocarcinoma; SQ, squamous cell carcinoma; PR, partial response; SD, stable disease; PD, progressive disease; NR, not received.

gemcitabine, or irinotecan is probably acceptable as the current standard first-line chemotherapy.

First-line single agent with gefitinib is active, but produces unacceptably frequent ILD in the Japanese population. Being female, as well as adenocarcinoma, those who never smoked, and *EGFR* mutation were associated with response to gefitinib. Patients who responded to gefitinib did not experience ILD during gefitinib chemotherapy. Further research via genetics and image analysis is needed to avoid ILD and identify a subgroup of patients that benefit from gefitinib treatment. If this is realized, single agent treatment with gefitinib could be an option as first-line chemotherapy in selected patients with advanced NSCLC. Furthermore, randomized trials are warranted to compare first-line single agent treatment with gefitinib followed by second-line platinum-based chemotherapy with first-line platinum-based chemotherapy followed by second- or third-line gefitinib treatment.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

Author Contributions

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A Phase I/II Study Comparing Regimen Schedules of Gemcitabine and Docetaxel in Japanese Patients with Stage IIIB/IV Non-small Cell Lung Cancer

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Objective: Gemcitabine and docetaxel are non-platinum agents with activity in non-small cell lung cancer (NSCLC). This study was conducted to determine and evaluate the recommended regimen of gemcitabine–docetaxel and evaluated its efficacy and safety in chemonaive Japanese NSCLC patients.

Methods: In phase I, patients with stage IIIB/IV NSCLC were randomized and received either gemcitabine on days 1 and 8 plus docetaxel on day 1 or gemcitabine on days 1 and 8 plus docetaxel on day 8. The recommended regimen was the dose level preceding the maximum tolerated dose; once determined, patients were enrolled in phase II. Efficacy and toxicity were evaluated in all patients.

Results: Twenty-five patients were enrolled in phase I and six patients were given the recommended regimen; gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8. An additional 34 patients were enrolled into phase II and administered with the recommended regimen. The response rate was 32.2% [95% confidence interval (CI) 20.6–45.6%] overall and 30.0% (95% CI 16.6–46.5%) in patients with the recommended regimen (40 patients). Although grade 3 interstitial pneumonia was observed in two patients (5.0%) who received the recommended regimen, both recovered shortly after steroid treatment. No unexpected events were observed throughout this study.

Conclusions: Gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 has comparable efficacy and more tolerable toxicities than previously reported platinum-based regimens. These results should be verified by a phase III study.

Key words: docetaxel – gemcitabine – non-small cell lung cancer

INTRODUCTION

Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors, progresses in a short time period, has a bleak prognosis, and represents the leading cause of cancer death in the world. The number of patients with NSCLC is increasing, and most tumors are inoperable. Despite improvements in the detection and treatment of NSCLC, long-term survival is rare. Therefore, the development of new chemotherapy treatments is essential.

The use of single-agent and combination chemotherapy against NSCLC has been studied. Platinum-based regimens have shown high efficacy but at the cost of severe toxicities (1,2). Therefore, non-platinum agents such as gemcitabine, docetaxel, paclitaxel, irinotecan and vinorelbine have been developed and have proven their efficacies. Among the new agents, the combination of gemcitabine and docetaxel has emerged as one of the most promising, showing equivalent efficacy with, and less toxicity than, cisplatin-based chemotherapies (3).

Gemcitabine (2′-deoxy-2′,2′-difluorocytidine monohydrochloride) is a nucleoside antimetabolite against deoxycytidine. It is intracellularly metabolized to gemcitabine triphosphate,

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which inhibits DNA synthesis, and has shown potent cytocidal activity against solid tumors (4–8).

Docetaxel, an antineoplastic agent that acts on microtubules to promote formation of abnormal microtubule bundles, has also shown cytotoxicity (9–11). Gemcitabine and docetaxel have different mechanisms of action, but by combining them, there is the potential of synergistic antitumor activity (12).

Several studies have been conducted to evaluate the therapeutic benefits of gemcitabine and docetaxel (13–15). The efficacy of gemcitabine–docetaxel is similar to platinum-based regimens, but due to each drug's non-overlapping toxicities, their combination produces toxicities more tolerable than platinum-based regimens. Georgoulias et al. (16) compared gemcitabine 1100 mg/m^2 on days 1 and 8 plus docetaxel 100 mg/m^2 on day 8 with cisplatin 80 mg/m^2 on day 2 plus docetaxel 100 mg/m^2 on day 1 in 441 patients with NSCLC. They reported that the two regimens were equivalent in efficacy, but toxicities were more severe for the combination of docetaxel and cisplatin.

There has been no published report considering both administering dose and schedule for the combination of gemcitabine and docetaxel. Therefore, we conducted a phase I/II study to compare two schedules of gemcitabine–docetaxel in patients with NSCLC and determine the recommended regimen in phase II. We assessed the efficacy and safety in all 59 patients: the efficacy and detailed safety profile were also evaluated in 40 patients who were given the recommended regimen.

SUBJECTS AND METHODS

Eligibility Criteria

Japanese patients with histologically or cytologically confirmed unresectable TNM stage IIIB or IV NSCLC who met the following criteria were eligible for the study: suitable for first-line chemotherapy with no prior chemotherapy; measurable lesions that can be accurately measured in at least one dimension; aged 20–74 years; Eastern Cooperative Oncology Group (ECOG) performance status of 0–1; a life expectancy of at least 3 months; and adequate organ functions as indicated by white blood cell count $\geq 4.0 \times 10^9$/l, absolute neutrophil count $\geq 2.0 \times 10^9$/l, platelets $\geq 100 \times 10^9$/l, hemoglobin ≥ 9.5 g/dl, aspartate aminotransferase/alanine aminotransferase ≤ 2.5 times the upper limit of normal, total bilirubin ≤ 1.5 times the upper limit of normal, serum creatinine $\leq$ the upper limit of normal, PaO_2 in arterial blood ≥ 60 torr. If a patient had received radiotherapy during the 3 weeks before enrollment, the measurable disease had to be outside of the radiation port.

Patients were excluded from the study if they had radiologically and clinically apparent interstitial pneumonia or pulmonary fibrosis, intracavitary fluid retention requiring treatment, or grade 2–4 peripheral neuropathy or edema. Additional exclusion criteria included: superior vena cava syndrome; symptomatic brain metastasis; pregnancy or breast-feeding; active concurrent malignancy; any serious concurrent illness (e.g. uncontrolled diabetes mellitus, hepatopathy, angina pectoris, myocardial infarction within 3 months after onset, severe infection, or fever suggestive of severe infection); history of serious drug allergy; or any condition that, in the opinion of the investigator, disqualified the patient based on safety.

This study was conducted in accordance with the Declaration of Helsinki, Japanese Guidelines for Clinical Evaluation of Antineoplastic Agents (promulgated in February 1991) and good clinical practice. All patients who entered into this study were required to give written informed consent.

Study Design And Treatment

This was a multicenter, open-label, phase I/II study of gemcitabine and docetaxel in Japanese patients with advanced NSCLC.

In the phase I portion of this study, patients were randomized into two arms, each with a different treatment schedule. In both arms (Arm 1 and Arm 2), gemcitabine was administered in a 30-min infusion on days 1 and 8, every 21 days. In Arm 1, docetaxel was administered intravenously over at least 1 h on day 1; in Arm 2, docetaxel was given on day 8. The administration of docetaxel followed an intravenous infusion of dexamethasone 4 mg, and gemcitabine was given immediately after the docetaxel infusion.

Patients were discontinued from the study due to progressive disease; inability to initiate a treatment cycle even at 6 weeks after the start of the previous cycle; recurrence of a dose-limiting toxicity (DLT) after resumption of the study treatment at a reduced dose; occurrence of a serious adverse event or aggravation of a concomitant illness (e.g. interstitial pneumonia, pulmonary fibrosis, or severe infection) which caused rapid aggravation of disease and precluded continuation of the study treatment; patient's request to withdraw from the study; or any event that required discontinuation in the opinion of the investigator.

During study enrollment, the current approved maximum dosage of gemcitabine and docetaxel as single agents in Japan was 1000 mg/m^2 and 60 mg/m^2, respectively. In phase I, the sample size was determined to be six per cohort based on the conventional design of phase I clinical studies of antineoplastic agents. In this study, both arms were randomized according to a predetermined schedule, enrolled patients in cohorts of six, and were initially treated at dose level 1 (gemcitabine 1000 mg/m^2 and docetaxel 50 mg/m^2). For the first cycle of treatment, patients were treated on an inpatient basis; if their condition permitted, patients were treated on an outpatient basis thereafter. If fewer than 50% of the patients in dose level 1 experienced DLTs, patients were enrolled at dose level 2 (gemcitabine 1000 mg/m^2 and docetaxel 60 mg/m^2). If 50% or more of the patients in dose level 1 experienced DLTs, patients were enrolled at dose level 0 (gemcitabine 800 mg/m^2 and docetaxel 50 mg/m^2) (Fig. 1). The maximum tolerated dose (MTD) was defined as the dose level that produced any of the following DLTs (per the National Cancer Institute–Common

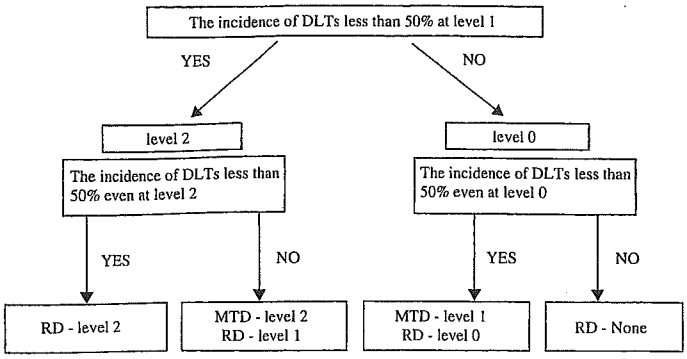

Figure 1. Recommended dosages in each arm. DLT, dose-limiting toxicity; RD, recommended dosage; MTD, maximum tolerated dose.

Toxicity Criteria scale) in 50% or more of patients during the first treatment cycle: grade 4 leukopenia or neutropenia persisting for at least 4 days; grade 3/4 neutropenia associated with a fever ≥38.0°C or infection; thrombocytopenia (<20 × 10^9/l) or need of a platelet transfusion; or grade 3/4 non-hematological toxicities (excluding nausea/vomiting, anorexia, fatigue and hypersensitivity). G-CSFs were administrated for the treatment of grade 4 neutropenia or grade 3 neutropenic fever. A DLT was also reported if any day-8 doses were omitted and dosing requirements were not satisfied until after day 15, or if the second cycle was delayed until after day 29 because the dosing requirements were not satisfied.

The recommended dose for phase II had to be determined from the arm that reached the highest dose level. If at dose level 2 the incidence of DLTs was less than 50%, the recommended dose was defined as dose level 2. The arm that reached the higher dose level reflected the recommended regimen for phase II. If the recommended dose level for the two arms was identical, the recommended regimen would be decided according to the following steps: (i) if frequency of DLTs was 0% in one arm and 33.3% or more in the other arm, the former was selected. If this did not occur, then (ii) if the dose intensity for evaluable patients in one arm was higher by 10% or more than the other arm, the arm with the higher dose intensity was selected. If this did not occur, then (iii) the arm with the fewer day-8 dose omissions in first and second cycles was selected. If the recommended dosage regimen still could not be decided, the sponsor (Aventis Pharma Japan and Eli Lilly Japan K.K.) and the coordinating investigator determined the recommended phase II regimen. If the MTD was dose level 0 in both arms, the study was terminated (Fig. 1).

The sample size for the recommended regimen was determined as follows. The response rate of this regimen and gemcitabine single agent was assumed to be 35 and 20%, respectively, in view of the response rates previously achieved (9,10,17,18). If the sample size of the recommended regimen was set as 40 patients, the probability for the one-sided 90% lower limit of response rate to exceed 20% was 82%. Thus, the target sample size in the recommended regimen including six patients in phase I was set at 40 patients.

The phase II study was conducted with 34 patients. Forty patients who were given the recommended regimen were evaluated for the efficacy and detailed safety profile: these patients consisted of six and 34 patients who entered into the study at phase I and II, respectively.

In this phase I/II study, patients received a minimum of two cycles of gemcitabine–docetaxel and up to four additional cycles.

Dose Modifications

During a cycle, dose modifications were not allowed. If not all of the following requirements were satisfied on either the day of treatment or the previous day, administrations of gemcitabine and docetaxel were delayed until the patient completely recovered. For gemcitabine and docetaxel doses administered on day 1 of Arm 1 or gemcitabine on day 1 of Arm 2, delays occurred for patients with an absolute neutrophil count <1.5 × 10^9/l, a platelet count <70 × 10^9/l, any grade 3/4 non-hematologic toxicities (except PaO_2), or PaO_2 <60 torr. When gemcitabine was given on day 8 of Arm 1, exceptions included leukopenia <2.0 × 10^9/l and an absolute neutrophil count <1.0 × 10^9/l, a platelet count <70 × 10^9/l, any grade 3/4 non-hematological toxicities. When gemcitabine was given on day 8 of Arm 2, exceptions included an absolute neutrophil count <1.5 × 10^9/l, a platelet count <70 × 10^9/l, any grade 3/4 non-hematological toxicities. If a patient developed a DLT, the subsequent doses were cancelled, and in the next cycle the patient could resume the study treatment at the next lower dose level. If a patient developed a DLT at dose level 0, gemcitabine 800 mg/m^2 and docetaxel 40 mg/m^2 were administered in the next cycle.

Baseline and Treatment Assessment

Assessments at baseline included tumor measurements by X-ray and computed tomography (CT) scan within 4 weeks before the day of starting the study treatment. Equally, grading performance status and physical examination were performed within a week; hematology, blood chemistries, urinalysis, arterial blood gas analysis and electrocardiogram were observed within 2 weeks.

After the start of treatment, tumor measurements were obtained every 2 weeks via X-ray and 4 weeks via CT scan. Tumor response was assessed with the World Health Organization (WHO) criteria. Safety assessments, including performance status, hematology, blood chemistries and urinalysis, were obtained weekly. Physical examination, arterial blood gas analysis and electrocardiogram were performed at any time. Adverse events were estimated according to National Cancer Institute–Common Toxicity Criteria version 2.0. All patients were assessed for efficacy and safety. An additional response rate was recorded for patients who received the recommended regimen in phase I and all phase II patients.

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.

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 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.

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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Phase II Study of Weekly Paclitaxel for Relapsed and Refractory Small Cell Lung Cancer

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Abstract. *The purpose of this study was to evaluate the efficacy and toxicity of single-agent paclitaxel given weekly to patients with relapsed and refractory small cell lung cancer (SCLC). Patients were treated with 80 mg/m^2 paclitaxel administered weekly for 1 h for 6 weeks in an 8-week cycle. Twenty-two patients were enrolled, 21 of whom were eligible. The patient characteristics included: 20 males, 1 female; median age 66 years (range 48 - 75); performance status 0/1 in 19 and 2 in 5 patients. Grade 3/4 leukopenia and neutropenia occurred in 47.5% and 64%, respectively. Other grade 3/4 toxicities included infection, skin rash, neuropathy and pulmonary toxicity. There were 5 partial responses in 3 out of the 11 sensitive cases and 2 out of the 10 refractory cases, respectively. Paclitaxel, administered as a weekly infusion at a dose of 80 mg/m^2, was effective in treating relapsed and refractory SCLC.*

More than 95% of patients with small cell lung cancer (SCLC), who are initially treated with paclitaxel 80 mg/m^2, present a relapse and their response to a second-line therapy is poor. The responses obtained are usually brief, and the median survival is generally less than 4 months (1). Nevertheless, second-line chemotherapy may provide a significant palliation of symptoms and does result in a prolongation of survival in many patients.

The activity of paclitaxel as a single agent has been investigated in both previously-untreated and -treated SCLC patients. Two phase II trials were conducted to investigate its efficacy as a first-line treatment for SCLC. In a trial conducted by the Eastern Cooperative Oncology Group (ECOG), Ettinger *et al.* administered 250 mg/m^2 paclitaxel as a 24-h infusion to 36 patients (2), among whom 11 partial responses were observed. Kirschling *et al.* obtained a similar response rate, 41%, in a group of 37 patients on an identical paclitaxel dose-schedule (3). The results of a phase II study in previously treated patients were reported by Smit *et al.* (4). All 24 patients in that trial developed progressive disease within 3 months of receiving at least one previous chemotherapy regimen. Seven patients (29%) had a partial response to 175 mg/m^2 paclitaxel as a 3-h infusion. These data show that paclitaxel exhibits single-agent efficacy in SCLC comparable to that of the best agents. The results of Smit *et al.'s* study in patients with refractory SCLC are particularly impressive, since most response rates reported with single-agent or combination regimens in this population have been less than 15%. However, life-threatening toxicity occurred in 4 of these patients, 2 of whom experienced hematological toxicity.

Recent reports of the activity and tolerability of weekly doses of paclitaxel have generated a great deal of clinical interest. Weekly paclitaxel therapy has generally been quite well tolerated, causing minimal toxicity and no apparent cumulative myelosuppression. Substantial evidence from clinical trials indicates that weekly paclitaxel is effective and generally well tolerated as both a first- and second-line treatment for advanced NSCLC. A phase I/II trial by Koumakis *et al.* in a second-line setting tested weekly paclitaxel infused for the first 6 weeks of each 8-week cycle, and demonstrated that a paclitaxel dose escalation from 60 mg/m^2 to 90 mg/m^2 was tolerated (5).

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Fennelly *et al.* reported a recommended dose of 80 mg/m^2 administered weekly for 6 weeks of an 8-week cycle in patients with recurrent ovarian cancer (6).

Based on this evidence, a phase II trial of 80 mg/m^2 weekly paclitaxel as a 1-h infusion for 6 consecutive weeks followed by 2 weeks without treatment (8-week cycle) was conducted in patients with relapsed SCLC. The objective of this study was to evaluate the efficacy and safety of weekly paclitaxel in patients with relapsed and refractory SCLC. The primary end-point was the response rate, while the secondary end-points were the toxicity profile and survival rate.

Patients and Methods

Patient selection. Patients who met all of the following criteria were considered eligible: a) histological or cytological proof of SCLC with no response to prior chemotherapy or progression after chemotherapy, b) measurable disease, c) most recent cytotoxic treatment less than 4 weeks before entry, d) ECOG performance status 0-2, e) age ≤75 years, f) adequate bone marrow function (leukocyte count ≥4,000/μl, hemoglobin level ≥9.0 g/dl and platelet count ≥100,000/μl), hepatic function (transaminases ≤2.5 times the upper limit of normal, bilirubin level ≤1.5 mg/dl), and renal function (creatinine ≤1.5 times upper limit of normal) and g) arterial oxygen partial pressure ≥60 torr. Excluded patients were those with any active concomitant malignancy, symptomatic brain metastases, a past history of drug allergy reactions, complication by interstitial pneumonia, treatment with non-steroidal anti-inflammatory drugs or steroids or other serious complications such as uncontrolled angina pectoris, myocardial infarction within 3 months, heart failure, uncontrolled diabetes mellitus or hypertension, massive pleural effusion or ascites or serious active infection. All patients gave written informed consent and our institutional review board for human experimentation approved the protocol.

Treatment schedule. Paclitaxel was infused intravenously (*i.v.*). over a 1-h period at a dose of 80 mg/m^2 each week for 6 consecutive weeks followed by a 2-week break. This 8-week period comprised one treatment cycle. Premedication consisted of 20 mg dexamethasone, 50 mg ranitidine and 50 mg diphenhydramine given *i.v.* 30 min prior to paclitaxel.

If the leukocyte count fell below 2,000/μl or the neutrophil count fell below 1,000/μl, recombinant granulocyte colony-stimulating factor (rhG-CSF) at a daily dose of 2 μg/kg was administered until the leukocyte count recovered to ≥10,000/μl, except on the days of paclitaxel administration. The toxicity assessment was based on the National Cancer Institute – Common Toxicity Criteria version 2.0. If grade 3 leukopenia, grade 4 neutropenia, grade 2 neuropathy or other grade 3 non-hematological toxicities occurred, the dose of paclitaxel in subsequent cycles was reduced by 10 mg/m^2 from the planned dose. Paclitaxel was not administered if the leukocyte count was <2,000/μl, the platelet count was <5,000/μl, or if there was grade 3 nausea/vomiting, infection with a fever of more than 38°C, or other grade 2 non-hematological toxicities except alopecia. The treatment was discontinued if there was disease progression, grade 3 neuropathy, other grade 4 non-hematological toxicities or a 2 consecutive weeks without paclitaxel administration.

Evaluation of response and survival. The tumor response was classified according to the WHO criteria (7). A complete response (CR) was defined as the total disappearance of all measurable and assessable disease for at least 4 weeks. Partial response (PR) was defined as a ≥50% decrease in the sum of the products of the 2 largest perpendicular diameters of all measurable tumors lasting for at least 4 weeks without the appearance of any new lesions. No change (NC) was defined as a decrease of <50% or an increase of <25% in tumor lesions for at least 4 weeks with no new lesions. Progressive disease (PD) was defined as the development of new lesions or an increase of 25% in the sum of the products of the 2 largest perpendicular diameters of all measurable tumors. The overall survival was measured from the time of study entry until death.

Statistical methods. The median probability of survival was estimated by the method of Kaplan and Meier (8). This study was designed as a phase II study, with the response rate as the main end-point. According to the Simons minimax design, with a sample size of 20 our study had a 90% power to accept the hypothesis that the true response rate was greater than 25%, while a 10% significance sufficed for rejection of the hypothesis that the true response rate was less than 5% (9).

Results

Patient characteristics. Between December 1999 and February 2002, a total of 22 patients were enrolled in the study, 1 of whom was deemed ineligible due to age (>75 years), leaving a total of 21 patients assessable for toxicity, response and survival. The main demographic characteristics of the cohort are summarized in Table I. The patient cohort consisted of 1 female and 20 males with a median age of 66 years (range, 48 to 75). Four patients exhibited limited disease and 19 exhibited extensive disease at the start of treatment. The majority of the patients had received no prior surgical treatment, while 67% had received prior radiation therapy. All patients had been treated with some form of cisplatin- or carboplatin-based combination chemotherapy regimen. Eighteen patients had received prior etoposide-containing chemotherapy and 10 prior irinotecan-containing chemotherapy. The median number of previous chemotherapy regimens administered was 1 (range, 1 to 2). Among the 10 patients who proved refractory to chemotherapy, 5 had NC or PD on first- or second-line treatment, 2 had PR but experienced disease progression during treatment and 3 had a relapse within a 90-day treatment-free interval after completing their treatments.

Toxicity. The toxicity of the regimen is summarized in Table II. Neutropenia was the main toxicity, with 6 out of the 21 patients experiencing grade 4 neutropenia during the entire study. Grade 3 anemia was observed in 2 patients. One patient experienced grade 4 anemia, secondary to digestive tract bleeding. Thrombocytopenia remained infrequent throughout the study. No cases of grade 3 or 4 thrombocytopenia were observed and there was no evidence of cumulative hematological toxicity.

Table I. *Baseline characteristics of all patients.*

Baseline characteristics		No. of patients
Sex	Male / Female	20 / 1
Age (years)	Median (Range)	66 (48-75)
ECOG PS	0/1/2	5 /12 /4
Disease extent	LD/ ED	4 / 17
Previous treatment	Chemotherapy only	4
	Chemotherapy + radiotherapy	14
	Chemotherapy + others	3
Previous chemotherapy	Platinum + etopoisde +/- others	18
	Including irinotecan HCl	10
	Others	1
No. of previous chemotherapy regimens	1 / 2 / 3	16 / 4 / 1
Response to prior chemotherapy	CR / PR / NC / PD / NE	2 / 13 / 5 / 0 / 1

No.: number
PS: performance status, LD: limited disease, ED: extensive disease.

Table II. *Toxicity of treatment for all cycles.*

Toxicity	No. of patients with event by grade				
	G0	G1	G2	G3	G4
Nausea	12	7	2	0	0
Vomiting	19	1	1	0	0
Diarrhea	17	3	1	0	0
Constipation	10	5	6	0	0
Mucositis	21	0	0	0	0
Gastric ulcer	20	0	1	0	0
Fever	16	3	2	0	0
Fatigue	13	0	8	0	0
Skin rash	20	0	0	1	0
Infection	18	0	0	3	0
Neuropathy	9	9	1	2	0
Myalgia	16	4	1	0	0
Dyspnea	17	0	1	2	1
Hemoglobin	1	9	9	1	1
WBC count	2	1	8	8	2
Neutrophil count	0	5	2	8	6
Platelet count	16	5	0	0	0
GOT	12	7	2	0	0
GPT	16	4	1	0	0
Total bilirubin	19	1	1	0	0

Other grade 3 and 4 toxicities included infection, skin rash, neuropathy and pulmonary toxicity. Grade 1 or 2 neuropathy was seen in 10 patients, and greater than grade 2 was observed in 2 individuals. No hypersensitivity reactions were encountered. Grade 3 or 4 pulmonary toxicity was reported in 3 patients and was characterized by dyspnea. Life-threatening complications of grade 4 infection and grade 4 dyspnea were encountered in 1 patient, who experienced febrile neutropenia and respiratory failure secondary to pneumonia after the third weekly dose. He was treated with antibiotics and supportive measures, but the respiratory distress worsened and he died on day 41. One of 2 grade 3 pulmonary toxicities was pneumonitis, probably induced by paclitaxel, but was resolved by steroid therapy.

Response to treatment and survival. The responses to therapy are shown in Table III according to whether the patient had primary refractory disease or primary sensitive cancer that subsequently relapsed. Although 1 out of the 21 patients was not assessable for response, having died during the first cycle, a ≥50% decrease in the sum of the products of the 2 largest perpendicular diameters of the tumor was achieved in this patient. Five of the 22 patients had a PR, but no CRs were observed and the overall response rate was 23.8% (95% confidence interval, 5.59 to 42.03). When only evaluable patients were included in the analysis, however, the response rate improved to 25% (95% confidence interval, 6.02 to 43.98). Two PRs (20%) occurred in refractory cases and 3 PRs (27%) were achieved in sensitive cases. Four patients showed no change, and 1 exhibited disease progression. The survival analysis was performed in January 2003, by which point 10 patients had died and 2 were still alive. The median survival time (MST) was 5.8 months and the 1-year survival rate was 13.4% (Figure 1).

Table III. *Response data.*

		No. of patients					Response rate (%)
		CR	PR	NC	PD	NE	
Total	21	0	5	4	11	1	23.8
Sensitive	11	0	3	3	5	0	27.3
Refractory	10	0	2	1	6	1	20.0

CI = confidence interval; CR = complete response; NE = not evaluable; PD = progressive disease; PR = partial response; NC = no change.

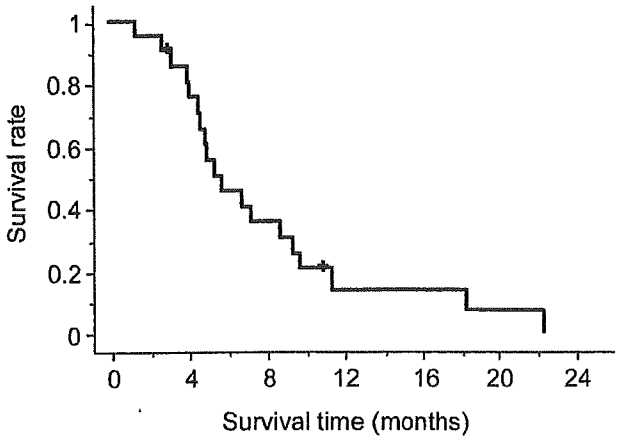

Figure 1. *Overall survival.*

Discussion

Since the outlook for SCLC patients who receive second-line therapy is poor, several new drugs, such as paclitaxel, docetaxel, gemcitabine, vinorelbine, topotecan and irinotecan, are currently under investigation. The new chemotherapy agents that have been most extensively evaluated in SCLC are the topoisomerase I inhibitors, including topotecan and irinotecan. Von Pawel *et al.* conducted a phase III study comparing single-agent topotecan with cyclophosphamide, doxorubicin and vincristine (CAV) in patients with progression at least 60 days after initial therapy and reported response rates of 24.3% for topotecan and 18.3% for CAV with a median survival time (MST) of 25.0 and 24.7 weeks, respectively, and found that topotecan was at least as effective as CAV in the treatment of patients with recurrent SCLC (10). Two studies of irinotecan in patients with refractory SCLC have been reported in Japan and the response rates in both studies were high, *i.e.*, 50% in 16 patients, and 47% in 15 patients, respectively (11, 12). We therefore consider that topoisomerase I inhibitors, such as topotecan and irinotecan, are key drugs in the second-line treatment of SCLC. However, the number of SCLC patients treated with an irinotecan-containing regimen as first-line chemotherapy has increased in Japan since, in a randomized phase III trial in Japan (13), a combination of irinotecan and cisplatin was shown to yield better survival than the standard etoposide and cisplatin regimen in patients with untreated extensive SCLC. Therefore, the search for effective drugs, other than topoisomerase I inhibitors, for previously treated SCLC, especially refractory SCLC, must be continued.

Single-agent paclitaxel, at a dose of 175 mg/m^2 as a 3-h infusion every 3 weeks in patients with previously treated SCLC, produced a response rate of 29% and an MST of 100 days (4). The results of our phase II study demonstrated that weekly paclitaxel at a dose of 80 mg/m^2 yielded a similar response rate of 23.8% and a much better MST of 5.8 months than that of paclitaxel given every 3 weeks. Because the antiproliferative activity of paclitaxel is cell-specific, prolonging patient exposure to a low dose of the drug beyond a threshold concentration is ultimately more efficacious than a short-term exposure to higher drug concentrations, a hypothesis supported by *in vitro* experiments with a variety of cell lines and suggested by the results of clinical studies. As clinical experience with paclitaxel treatment of various types of tumors has progressed, so has the use of weekly regimens at lower doses administered as 1-h infusions, as opposed to standard higher doses delivered once every 3 weeks as 3-h infusions.

A response rate of more than 10% is considered evidence of drug efficacy in previously-treated SCLC patients (14). Before newer drugs, such as topoisomerase I inhibitors, taxane, gemcitabine and vinorelbine were introduced, salvage chemotherapy did not usually prolong survival in SCLC and MSTs after relapse were 2.5 – 3.9 months (1). Single-agent phase II trials of gemcitabine, docetaxel and vinorelbine in patients with relapsed or refractory SCLC have been reported. Smyth *et al.* (15), using a 100 mg/m^2 dose of docetaxel, obtained a response rate of 25% in 28 assessable patients who had received prior chemotherapy. A trial of gemcitabine in 46 previously-treated patients yielded an 11.9% response rate (16) and vinorelbine provided response rates of 12% and 16% in second-line patients with sensitive disease (17,18). Thus, the MST of 5.8 months and response rate of 23.8% in this study compare favorably with those of published single-agent trials in relapsed or refractory SCLC.

The toxicity profile noted in this trial was predictable based on the toxicity profile previously described in weekly paclitaxel trials, neutropenia being the major toxic effect. All side-effects, except fatal neutropenic pneumonia in 1 case, were manageble. Grade 3 or 4 neutropenia occurred in 14 of the patients in our study but was immediately alleviated by treatment with G-CSF. Grade 3 or 4 anemia occurred in 1 patient, but there was no grade 3 or 4 thrombocytopenia in our study. The incidence of grade 3/4 myelosuppression was considered tolerable. There were 3 cases of grade 3 or 4 pulmonary toxicity, 2 of which occurred due to bacterial infection. This regimen required a dose of 20 mg of dexamethasone weekly as premedication. We believe that this occurrence of bacterial pneumonia might be related to the use of steroids.

Testing new drugs in previously-treated patients has the clear advantages of determining the degree of non-cross resistance with other drugs. Its greatest disadvantage is the risk of a considerable dose reduction (especially of myelotoxic drugs) to avoid extensive hematological side-

effects, perhaps resulting in doses that are too low to fairly evaluate the drug. Since a weekly administration of paclitaxel causes only mild myelosuppression and as there may be no cross resistance with platinum, etoposide, irinotecan, or topotecan, which are usually used to treat SCLC, we find this regimen suitable for previously-treated SCLC.

In summary, the weekly paclitaxel regimen is moderately effective in SCLC patients who have received prior chemotherapy. Based on the statistical design of this study, the 5 PR observed suggest that weekly paclitaxel warrants further evaluation in this patient population. Additional investigations will serve to clarify the role of this agent, either alone or in combination with other agents. Combining paclitaxel with other agents with proven non-cross resistance such as irinotecan, topotecan, or gemcitabine or new target-based agents is the next step needed to evaluate second-line situations, especially in patients with resistant disease.

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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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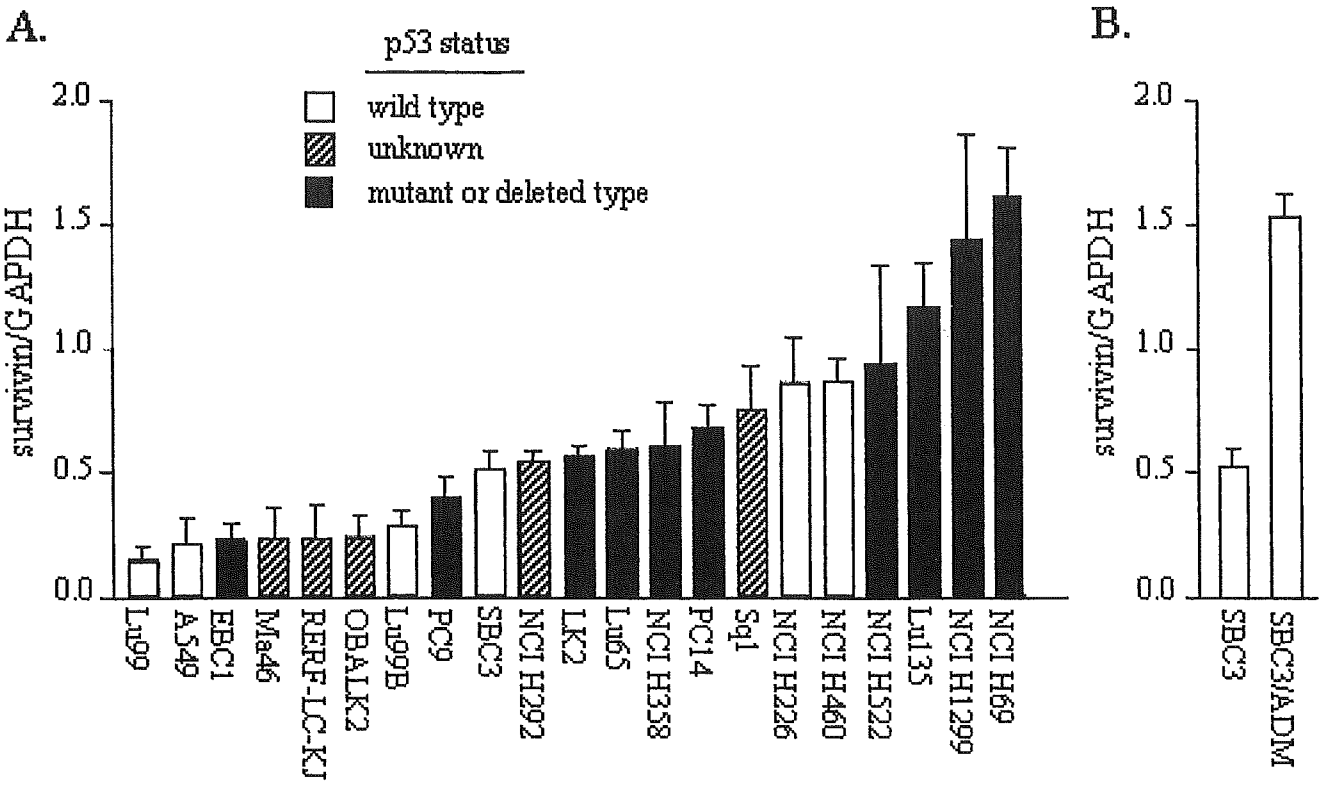

FIGURE 1 – Level of survivin mRNA in 22 lung cancer cell lines. (*a*) Cells were incubated in a 75 cm^2 flask, harvested and analyzed using real-time PCR as described in Material and methods. All data were normalized relative to the concentration of mRNA for the housekeeping gene GAPDH and are presented 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-