regimen was applied to previously untreated patients with NSCLC, the response rate was high, at 29–46% [1, 3, 4, 8, 15, 18]. When a combination of PTX (administered every 3 weeks) and GEM was used for second-line chemotherapy, the response rate was either 18 or 39% [2, 14].

Weekly chemotherapy for lung cancer has recently been attempted at several facilities [3, 9]. Favorable results of weekly chemotherapy have also been reported for recurrent NSCLC [5, 16, 26, 28]. Compared to standard regimens of chemotherapy, with administration of drugs at intervals of 3–4 weeks, weekly chemotherapy has certain advantages. For example, the single dose level of anti-cancer drugs can be reduced with weekly chemotherapy, and the dose level can be adjusted after the start of treatment depending on signs of hematological toxicity of the drugs or the general condition of individual patients. In comparison with treatment at intervals of 3–4 weeks, weekly chemotherapy was of equal efficacy but had fewer side effects [3]. Weekly chemotherapy is thus a promising means of treating cases of recurrent NSCLC in which bone marrow function has been compromised by first-line chemotherapy.

The present study was undertaken to evaluate the effectiveness and safety of weekly chemotherapy using a combination of PTX and GEM in cases of advanced NSCLC in which tumor had recurred or relapsed after platinum-based first-line chemotherapy or platinum-based first-line chemotherapy had failed to exert efficacy.

Patients and methods

Patient selection

Patients were required to have histologically or cytologically confirmed non-resectable or metastatic NSCLC that had progressed during or after one or more chemotherapy regimens. The trial was initiated after a rest period of at least 4 weeks following previous chemotherapy (2 weeks in the case of radiotherapy). Patients were required to have recovered completely from prior therapy, and to have no ongoing toxicity greater than grade 1. Other eligibility criteria were as follows: measurable lesions; life expectancy of at least 12 weeks; Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2; adequate bone marrow reserve (defined as absolute granulocyte count $\geq 2{,}000$/ml and platelet count $\geq 100{,}000$/ml); adequate hepatic and renal function (defined as serum creatinine level ≤ 2 mg/dl, AST and ALT ≤ 1.5 times the upper limit of normal, and bilirubin ≤ 1.5 mg/dl). Exclusion criteria included pre-existing motor or sensory neurological signs or symptoms $\geq$ grade 2 (Common Terminology Criteria for Adverse Events version 3.0) and active infections. Asymptomatic treated or untreated patients with brain metastases were not excluded from the study. The Ethics Committee of the Tochigi Cancer Center approved the study protocols. Written informed consent was obtained from every patient stating that the patient was aware of the investigational nature of this treatment regimen.

Treatment

Paclitaxel was administered at a dose of 100 mg/m^2 intravenously during a 1-h infusion on days 1 and 8 of the treatment cycle. Gemcitabine was administered at a dose of 1,000 mg/m^2 intravenously during a 30-min infusion on days 1 and 8 of the treatment cycle. Prior to each treatment, patients were given diphenhydramine 50 mg orally, and an H2 blocker intravenously along with dexamethasone 16 mg 30 min before PTX administration. Granisetron 3 mg was administered intravenously as an antiemetic. The length of each chemotherapy cycle was 21 days. Patients who experienced grade 4 leukopenia or neutropenia that lasted for 3 or more days, or who experienced grade 4 thrombocytopenia or reversible grade 2 neurotoxicity or liver dysfunction, received reduced doses of both PTX and GEM (PTX 80 mg/m^2, GEM 800 mg/m^2) for the next cycle. If non-hematological toxicities of grade 3 or higher occurred, treatment was stopped. Subsequent courses of chemotherapy were started after 3 weeks when the leukocyte count was 3,000/mm^3 or more, the neutrophil count was 1,500/mm^3 or more, the platelet count was 75,000/mm^3 or more, serum creatinine were less than 1.5 mg/dl, GOT and GPT were less than twice the upper limit of the normal range, and neurotoxicity was grade 1 or less. If these variables did not return to adequate levels by the first day of the next course of chemotherapy, treatment was withheld until full recovery. If more than 6 weeks passed from the time of the last treatment before these criteria were met or if change in treatment more significant than reduction of dose was indicated, the patient was removed from the study at that time, but still included in the analysis of its results.

Evaluation of responses and toxicity

Pretreatment evaluation included medical history, physical examination, complete blood count, bone marrow examination, serum biochemical analyses,

chest roentgenogram, electrocardiogram, and urinalysis. All patients underwent radionuclide bone scan, magnetic resonance or computerized tomography (CT) of the brain, and CT of the thorax and abdomen. Complete blood count, biochemical tests, serum electrolytes, urinalysis, and chest roentgenograms were obtained before patients received chemotherapy.

Responses and toxicity were evaluated on the basis of tumor images obtained by CT and other techniques, laboratory data, and subjective/objective symptoms and signs before, during, and after administration of the study drugs and during the period from completion of treatment to final analysis. Measurable disease parameters were determined every 4 weeks by various means such as computerized tomography. Evaluation was performed in compliance with the Response Evaluation Criteria in Solid Tumors (RECIST) Guidelines for antitumor activity and with Common Terminology Criteria for Adverse Events version 3.0 for safety. Patients were withdrawn from the study if evidence of tumor progression was obtained. The Institutional Ethical Review Committee gave approval to the study.

The primary endpoint of the study was the response rate. Simon's two-stage optimum design was used to determine sample size and decision criteria. It was assumed that a response rate of 30% among eligible patients would indicate potential usefulness while a rate of 10% would be the lower limit of interest, with $\alpha = 0.05$ and $\beta = 0.10$. Using these design parameters, the fist stage of the study was initially to enroll 18 patients, and this regimen was to be rejected if fewer than two patients had an objective response. If two or more patients responded, accrual was to be continued to 36 patients. Considering the percentage of probable dropout cases, 40 patients were required. Secondary endpoints were toxicity and overall survival. Response and survival rates were both calculated on an intent-to-treat basis. Overall survival and time to progression were measured from the start of this treatment up to the time of death or up to the date of the last follow-up clinical assessment. Survival curves were constructed using the Kaplan–Meier method.

Results

Patient characteristics

Forty patients were enrolled in this study from October 2000 to July 2003. All patients were assessable for toxicity, response, and survival. Characteristics of the 40 patients are listed in Table 1. All 40 patients had received a prior platinum-based chemotherapy regimen (Table 1). Two of these patients had received more than one chemotherapy regimen. All 40 patients were eligible for toxicity assessment. Four patients had received prior chemotherapy in the neoadjuvant setting. Of the 40 patients, 15 had initially responded to platinum-based therapy, 24 patients had achieved stable disease (SD), and one had progressive disease (PD).

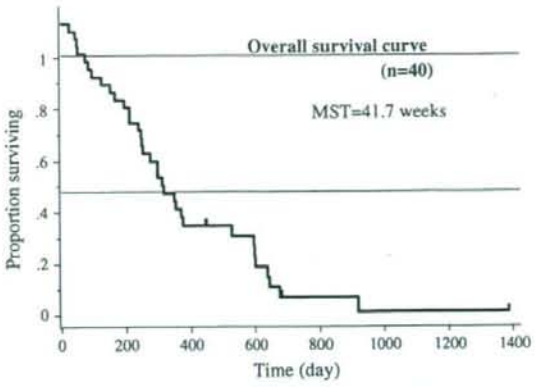

Fig. 1 Kaplan–Meier estimated overall survival curves. Median survival time, 41.7 weeks; 1-year survival rate, 38%

Efficacy of treatment

The mean number of cycles administered per patient was 4, and number of cycles ranged from one to twelve. Three patients required reduction of dose due to neutropenia and thrombocytopenia. Thirteen patients exhibited partial response (PR). Overall response rate was 32.5% (13/40) [95% confidence interval (CI): 18–47%]. SD was achieved in 26 patients (65%), and one (2%) achieved PD. All 40 patients were included in the survival analysis, with a median follow-up time of 82.9 weeks (range 56–263 weeks). The overall median survival time was 41.7 weeks (95% CI: 28.5–54.7 weeks). The 1-year survival rate was 37.5% (15/40) (Fig. 1). The median time to disease progression was 19 weeks.

Toxicities (Table 2)

Table 2 lists toxicities observed during this study. Hematological toxicities included high incidences of leukopenia and neutropenia, with leukopenia and neutropenia of grade 3 or higher occurring in 45 and 60% of patients, respectively. Anemia and thrombocytopenia of grade 3 or higher occurred in 15 and 12.5% of patients, respectively. Non-hematological toxicities

Table 1 Patient characteristics

Eligible patients	40
Gender	
Male	27
Female	13
Age (years)	
Median	59
Range	33–75
Performance status	
0	7
1	27
2	6
Histology	
Adenocarcinoma	30
Squamous cell	8
Large cell	2
Stage III	10
Stage IV	30
Number of metastatic sites	
Median	2
Range	0–3
Location of metastases	
Bone	13
Lung nodules	12
Brain	10
Lymph nodes	7
Liver	5
Adrenals	3
Subcutaneous	1
Prior surgery	4
Prior irradiation	15
Lung only	9
Brain only	4
Lung and bone	2
Prior chemotherapy	40
Cisplatin/vinorelbine	32
Cisplatin/docetaxel	5
Cisplatin/irinotecan	3
Response to prior chemotherapy	
Partial response	15
Stable disease	24
Progressive disease	1

observed included grade 3 pneumonitis in one patient, who exhibited rapid recovery following administration of steroids, grade 3 diarrhea in one, and grade 3 rash in one. Other non-hematological toxicities observed were of grade 2 or less and included nausea in 47.5%, vomiting in 20%, alopecia in 45%, sensory neuropathy in 35%, and fatigue in 32.5% of patients. All of these toxicities disappeared or were improved by symptomatic treatment. There were no deaths due to toxicity.

Discussion

Although a standard regimen of chemotherapy for recurrent NSCLC is being established, it is still important to determine how the outcome of treatment of this cancer can be improved [13, 23, 24]. At this point, the results of large-scale phase III clinical trials indicate single-agent chemotherapy with docetaxel, erlotinib, or pemetrexed as the standard chemotherapy regimen for recurrent NSCLC. In recent years, however, many reports have been published investigating two-drug combined therapy rather than single-agent therapy for recurrent NSCLC, with the objective of further improving therapeutic outcomes [2, 5, 7, 11–14, 20–26, 28].

A large number of reports have been published concerning salvage chemotherapy for recurrent NSCLC. Platinum-based chemotherapy is now used as the first-line chemotherapy at most medical facilities. Reports on second-line chemotherapy for NSCLC published to date have principally concerned uncombined drug therapy or two-drug combined therapy using non-platinum preparations [2, 5, 7, 11, 12, 14, 16, 17, 20–22, 25, 26, 28]. At several facilities, weekly administration chemotherapy has been adopted [5, 16, 26, 28]. Weekly-administration chemotherapy allows single dose levels to be reduced, thus making it possible to adjust the dose levels of anti-cancer agents after the start of treatment depending on adverse reactions or the general condition of individual patients.

Table 3 summarizes the results of two-drug combined therapy for recurrent NSCLC using non-platinum preparations [2, 6, 9, 10, 14, 19, 27]. The studies shown in this table were phase I-II in the case of that reported by Iaffaioli [14], phase III in that by Fossella [9], and phase II in the other studies. The overall response rate varied widely among studies, from 0.8 to 39%. The overall median survival time was 24–47 weeks and the one-year survival rate was 19–46%. Major adverse reactions observed in these studies were signs of hematological toxicity (particularly neutropenia), excluding the studies involving prophylactic G-CSF treatment reported by Androulakis [2] and Wachters [27]. Signs of non-hematological toxicity varied depending on the drugs used, and symptoms and signs unique to each drug were noted.

For combined PTX and GEM therapy for recurrent NSCLC, Androulakis [2] reported an overall response rate of 18%, an overall median survival time of 47 weeks, and a median time to disease progression of 34 weeks. Compared to the present study, the overall response rate reported by Androulakis was lower, while the overall median survival time and median time to disease progression were more favorable in the study by Androulakis. The dosing regimen used by Androulakis involved administration of PTX (175 mg/m^2; day 8), GEM (900 mg/m^2; days 1 and 8), and granulocyte colony-stimulating factor (G-CSF; days

Table 2 Maximum toxicity over 152 cycles (40 patients)

	CTCAE v 3.0 grade (number of patients)					Grade 3 ≤ (%)
	0	1	2	3	4	
Leukopenia	7	4	11	15	3	18 (45)
Neutropenia	6	5	5	17	7	24 (60)
Febrile neutropenia	–	–	–	2	–	2 (5)
Anemia	4	8	22	5	1	6 (15)
Thrombocytopenia	9	21	5	3	2	5 (12.5)
Pneumonitis	36	1	0	1	0	1 (2.5)
Diarrhea	27	9	3	1	0	1 (2.5)
Rash	22	15	2	1	0	1 (2.5)
Nausea	21	19	0	0	0	
Vomiting	32	3	5	0	0	
Fatigue	27	11	2	0	0	
Alopecia	22	17	1	0	0	
Neuropathy-sensory	26	14	0	0	0	
Edema	32	8	0	0	0	
Arthralgia	33	7	0	0	0	

CTCAE v 3.0 Common terminology criteria for adverse events version 3.0

Table 3 Non-platinum regimens used as second-line treatment of non-small cell lung cancer

First author (Ref.)	No. of patients	Regimen and schedule			Response rate (%)	Survival	
						Median (weeks)	1-year (%)
Androulakis [2]	49	P	175 mg/m^2	d 8 q 3w	18	47	37
		G	900 mg/m^2	d 1,8 q 3w			
		G-CSF	150 μg/m^2	d 9–15			
Iaffaioli [14]	37	P	90–240 mg/m^2	d 1 q 3w	39	40	46
		G	1,000 mg/m^2	d 1,8 q 3w			
Fossella [9]	123	FO	2 g/m^2/day	d 1–3 q 3w	0.8	24	19
		V	30 mg/m^2	d 1,8,15 q 3w			
Kosmas [19]	43	D	100 mg/m^2	d 8 q 3w	33	36	28
		G	1,000 mg/m^2	d 1,8 q 3w			
Cao [6]	33	CPT11	300 mg/m^2	d 1 q 4w	9	25	23
		V	30 mg/m^2	d 1,14 q 4w			
Georgoulias [10]	76	CPT11	300 mg/m^2	d 8 q 3w	18.4	38	24.5
		G	1,000 mg/m^2	d 1,8 q 3w			
Wachters [27]	52	CPT11	200 mg/m^2	d 1 q 3w	10	27	30
		D	60 mg/m^2	d 1 q 3w			
		G-CSF	150 μg/m^2	d 2–12			
Present study	40	P	100 mg/m^2	d 1,8 q 3w	32.5	42	38
		G	1,000 mg/m^2	d 1,8 q 3w			

P paclitaxel, *G* gem citabine, *F0* infostamide, *V* vinorebine, *D* docetaxel, *CPT-11* irinotecan, *G-CSF* granulocyte colony-stimurating factor, *d* day, *q* every

9–15), with each cycle of treatment lasting for 3 weeks. Because their regimen involved prophylactic administration of G-CSF, the incidence of grade 3 or worse neutropenia was lower than that in the present study (12 vs. 60%). However, the incidence of grade 2 or worse fatigue (a sign of non-hematological toxicity) was lower in the present study (4%) than in that reported by Androulakis (51%).

Belani [19] reported the results obtained with combined use of PTX and GEM as first-line chemotherapy for NSCLC. In their study, PTX was administered using two regimens and a comparison was made between treatment with PTX on day 1 (200 mg/m^2) and weekly treatment with PTX on days 1 and 8 (100 mg/m^2/dose; identical to the regimen used in the present study). According to their report, the response rate was 45% for the first regimen and 46% for the second regimen, the median survival time was 42 and 39 weeks and the 1-year survival rate 46 and 41% for the first and second regimens, respectively. Efficacy thus did not differ

significantly between the two regimens. Signs of hematological toxicity were the major adverse reactions observed following treatment with both regimens. The incidences of neutropenia and alopecia were lower with the weekly regimen. On the basis of these results, Belani concluded that weekly PTX treatment combined with GEM is also useful as first-line chemotherapy for NSCLC.

In conclusion, weekly chemotherapy with PTX and GEM is a tolerable and active regimen for patients with advanced NSCLC previously treated with platinum-containing chemotherapy regimens. It should be recommended as a candidate regimen in planning a phase III clinical study of NSCLC previously treated with platinum-containing chemotherapy, and will ultimately be evaluated in a phase III clinical study.

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PD4-3-5 Cytotoxic Chemotherapy II, Tue, 16:00 - 17:30

Phase I/II study of oral TS-1 and gemcitabine in elderly patients with advanced non-small-cell-lung cancer (NSCLC): Thoracic Oncology Research Group Study 0502

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Background: Optimal treatment for elderly patients with NSCLC has been under active investigation. This study evaluated the safety and initial efficacy of a novel combination regimen of oral fluoropyrimidine TS-1 plus gemcitabine (GEM) for elderly patients (pts) with advanced NSCLC.

Methods: A phase I/II trial in 11 centers examined TS-1 and GEM in pts with age ≥ 70, stage IIIB/IV previously untreated NSCLC. The starting dose was 60 mg/day (day 1-14) for TS-1 and 800 mg/m^2 for GEM (day 8, 15). GEM was increased to 1000 mg/m^2 at dose level 2 and TS-1 was increased to 80 mg/day at dose level 3. Phase II portion of the study assessed the efficacy and tolerability of the combination regimen at the dose determined in the phase I portion. The primary endpoint was objective response rate.

Results: Twenty two pts were enrolled in the phase I portion: 6 pts on dose level 1, 10 on dose level 2 and 6 on dose level 3. Median age of this group was 75 yrs (range 70-85). Dose limiting toxicities included Gr. 4 neutropenia (2 pts) and Gr.3 skin toxicity (4 pts). The recommended dose (RD) was TS-1 60 mg/day and GEM 1000 mg/m^2, with which 20 pts were subsequently treated in the phase II portion. The median age of 30 pts treated with the RD was 76 yrs (range 70-85). Grade (Gr) 3/4 toxicities include neutropenia (12 pts; 7 with Gr 4), thrombocytopenia (4 pts; 0 with Gr 4), skin toxicity (8 pts), thrombus (1 pt) and peumonitis (2 pts). Nine patients (30%, 95% confidence interval [CI] = 14 to 46%) had partial responses and 16 (53%, 95% CI = 35 to 71%) had stable disease.

Conclusion: Encouraging antitumor activity and safety of TS-1 plus gemcitabine support further development of this combination therapy for elderly patients with advanced NSCLC.

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Phase II Study of Paclitaxel and Irinotecan Chemotherapy in Patients With Advanced Nonsmall Cell Lung Cancer

Fumihiro Oshita, MD, Haruhiro Saito, MD, Kouzo Yamada, MD, and Kazumasa Noda, MD

Objectives: We conducted a phase II study of combination chemotherapy with paclitaxel (Pac) and irinotecan (CPT) to determine the qualitative and quantitative toxicities and efficacy of the combination against advanced nonsmall cell lung cancer (NSCLC).

Patients and Methods: Patients with stage IIIB or IV NSCLC were treated with CPT at 60 mg/m^2 and Pac at 160 mg/m^2 every 2 weeks.

Results: Between May 2002 and July 2004, 39 of registered 46 patients received 4 to 6 cycles of chemotherapy, and 7 patients discontinued treatment because of disease progression in 5 patients and grade 2 pneumonitis in 2 patients. Grade 3 anemia, leukopenia, neutropenia, and elevation of bilirubin occurred in 4.0%, 0.5%, 1.0%, and 0.5%, respectively. Twenty-one patients responded, and the overall response rate was 45.6%. The median survival time was 355 days and the 1-year survival rate was 47.8%.

Conclusion: Pac plus CPT was efficacious and safe in NSCLC.

Key Words: paclitaxel, irinotecan, nonsmall cell, lung cancer

(*Am J Clin Oncol* 2007;30: 358–360)

Current chemotherapy regimens for metastatic nonsmall cell lung cancer (NSCLC) are not particularly effective, and the disease cannot be cured even with the most effective chemotherapy. Current international guidelines recommend the use of platinum-based chemotherapy for patients with advanced NSCLC,[1] and the use of doublets including platinum plus a third-generation agent has been widely accepted for patients with a good performance status. A meta-analysis of the published literature clearly showed the superiority of platinum-containing regimens in terms of objective response rate, and this superiority was found throughout the subgroup analyses performed.[2] The study results also confirmed that platinum-based therapy is generally associated with higher toxicity, particularly nausea and vomiting, hematologic toxicity, and nephrotoxicity. Nevertheless, platinum-based regimens can be administered as safely as nonplatinum therapies when patients are selected correctly. However, this study did not include every combination of nonplatinum drugs, and it is necessary to examine every such new combination for efficacy and toxicity.

Combined analysis of two randomized phase III studies demonstrated that irinotecan (CPT) combined with cisplatin significantly improves survival compared with vindesine and cisplatin in patients with advanced NSCLC.[3] In Japan, CPT is considered a key drug against NSCLC. Preclinical studies that have evaluated combinations of a camptothecin with a taxane have yielded promising results, and several studies have demonstrated an additive or synergistic interaction between camptothecin and taxanes.[4] Our previous phase I study of a Pac and CPT combination showed that pneumonitis was the dose-limiting toxicity and led to a recommendation of Pac 160 mg/m^2 and CPT 60 mg/m^2 every 2 weeks for further study.[5] This study also demonstrated an objective response rate of 58.3% and a 1-year survival rate of 54.2%. Accordingly, we expected the combination of Pac and CPT to display high activity against NSCLC and designed a phase II study to determine the efficacy and toxicities.

PATIENTS AND METHODS

The Institutional Review Board of Kanagawa Cancer Center reviewed and approved this study prior to commencement.

Patients

Patients with histologically or cytologically confirmed NSCLC were registered. Eligibility criteria were: clinical stage IIIB or IV, an expected survival of at least 12 weeks, age <70 years, Eastern Cooperative Oncology Group PS score ≤1, leukocyte count ≥4000/μL, hemoglobin ≥10 g/dL, platelet count ≥100,000/μL, total serum bilirubin ≤1.5 mg/dL, aspartate aminotransferase and alanine aminotransferase ≤90 IU/L, and serum creatinine ≤1.5 mg/dL. Patients who had experienced postoperative recurrence were eligible for this study, but a 4 or more week rest period was required after surgery. Patients who had received chemotherapy or radiotherapy were excluded from this study. Written informed consent was obtained from every patient.

Chemotherapy

All patients without disease progression were treated every 2 weeks for a total of 4 courses of chemotherapy. CPT was administered at a dose of 60 mg/m^2 on day 1. Pac was administered at a dose of 160 mg/m^2 on day 1. Premedication

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consisting of 20 mg dexamethasone and 50 mg ranitidine was infused. A 50-mg oral dose of diphenhydramine was also administered. Prophylactic G-CSF, 50 $\mu g/m^2$ per day or 2 $\mu g/kg$ per day, was administered subcutaneously on days 6 to 10. Patients were given a 5-HT_3 antagonist intravenously. Subsequent courses of chemotherapy were started when patients satisfied the organ function criteria: leukocyte count $\geq 3000/\mu L$, neutrophil count $\geq 1500/\mu L$, platelet count $\geq 75{,}000/\mu L$, and less than grade 1 nonhematologic toxicities, except alopecia. Grade 3 nausea and vomiting did not preclude subsequent courses of chemotherapy. Chemotherapy was repeated for a maximum of 6 courses unless the disease progressed, but it was stopped if the tumor response was judged to be NC after 4 courses. Tumor response was evaluated according to RECIST criteria.[6] Toxicities were evaluated according to the NCI-CTC (version 2) criteria.[7]

Study Design

We chose a 50% response rate as a desirable target level and a 30% response rate as uninteresting. The study design had power in excess of 90% and less than 10% error; therefore, 22 assessable patients in the first step and 24 in the second step were required according to the optimal design of Simon.[8] We decided to stop the study if there were fewer than 8 responders in the first step. The regimen was defined as active if there were 18 or more responders out of the total of 46 patients. Overall survival was estimated by the method of Kaplan and Meier.

RESULTS

Between May 2002 and July 2004, 46 patients were registered in the phase II study (Table 1). A total of 22 patients were registered for assessment of response in the first stage. Nine of 22 patients in the first stage responded and 24 patients were registered in the second stage. A total of 198 cycles was administered to 46 patients. Thirty-nine patients received 4 to 6 cycles of chemotherapy, except for 7 patients who discontinued treatment in the first or second cycles because of disease progression in 5 patients and grade 2 pneumonitis with pulmonary infiltration in 2 patients. Adverse effects and events are summarized in Table 2. Grade 3 anemia, leukopenia, neutropenia, and elevation of bilirubin occurred in 4.0%, 0.5%, 1.0%, and 0.5%, respectively. There were no grade 4 toxicities.

Twenty-one of 46 patients achieved partial response, 18 no change, 6 progressive disease, and 1 not evaluated, and the overall response rate was 45.6% in phase II study. The median duration of partial response was 154 days (range, 76–380 days). The median survival time was 355 days and the 1-year survival rate was 47.8% (Table 3). The outcome in 70 patients including those from the phase I study (5) demonstrated that 1 patient achieved complete response, 34 PR, and the overall response rate was 50.0%. The median survival time was 361 days and the 1-year survival rate was 50.0%.

DISCUSSION

The objective response rate of 50.0% and 1-year survival rate of 50.0% with our nonplatinum Pac and CPT regimen in 70 patients in phase I and phase II studies are somewhat better than in a large phase III trial of 4 platinum-based chemotherapy regimens, which showed response rates of 17% to 22% and 1-year survival rates of 31% to 34%.[9] The

TABLE 1. Patient Characteristics

Characteristic	Value
Total	46
Age (years)	
Median	61
Range	43–69
Gender (no. patients)	
Male	29
Female	17
Performance status (ECOG) (no. patients)	
0	12
1	34
Clinical stage (no. patients)	
IIIB	6
IV	34
Postoperative recurrence	6
Histology (no. patients)	
Adenocarcinoma	36
Others	10
No. metastatic organs (no. patients)	
1	27
≥2	13
Brain metastasis (no. patients)	8

TABLE 2. Adverse Effects and Events

	NCI-CTC Grade (No. Cycles)					
Toxicity	0	1	2	3	4	% ≥Grade 3
Hemoglobin	29	137	24	8	0	4.0
Leukocyte	162	23	12	1	0	0.5
Neutrophil	167	19	10	2	0	1.0
Platelets	188	10	0	0	0	—
Bilirubin	165	22	10	1	0	0.5
Creatinine	192	6	0	0	0	—
SGOT	146	51	1	0	0	—
SGPT	135	55	8	0	0	—
Infection	194	3	1	0	0	—
Nausea/vomiting	143	51	4	0	0	—
Diarrhea	165	31	2	0	0	—
Myalgia	97	76	25	0	0	—
Arthralgia	110	64	24	0	0	—
Neuropathy	107	76	15	0	0	—
Fever	183	14	1	0	0	—
Allergic reaction	195	3	0	0	0	—
Alopecia	95	79	24	0	0	—
Pneumonitis	196	0	2	0	0	—
Hypotension	193	5	0	0	0	—
Arrhythmia	194	4	0	0	0	—

NCI-CTC, National Cancer Institute-Common Toxicity Criteria (version 2).

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TABLE 3. Therapeutic Outcome in Phase II Study

Response	No. Patients
Complete response	0
Partial response	21
No change	18
Progressive disease	6
Not evaluated	1
Response rate (%)	45.6
Median survival time (days)	355
% of 1-year survivor	47.8

antitumor activity of the Pac and CPT combination is thought to be attributable to a synergistic action between these drugs. A possible mechanism of the synergy is a drug-drug interaction, such as that shown in a pharmacokinetic study that demonstrated elevation of the AUC of CPT and SN-38 by Pac infusion.[10] Although we acknowledge the possibility that Pac and CPT might affect each other's pharmacokinetics, increasing their activity against NSCLC, we also considered that another possible mechanism for this high activity of the Pac and CPT combination might be related to influx and efflux in the cell system. The combination of Pac and SN-38 down-regulates the level of multidrug resistance-associated protein, which may be an efflux pump for cisplatin, in ovarian cancer cell lines, suggesting that this combination will overcome drug resistance.[11]

The combination of Pac and CPT also appears useful in that little toxicity was observed in this study. No patients experienced grade 4 toxicities. All patients, except the 5 patients who developed disease progression during treatment and the 2 patients who experienced grade 2 pneumonitis with pulmonary infiltration, were able to receive 4 to 6 cycles of this therapy. The pneumonitis was thought to be attributable to a booster effect of an allergic reaction when 180 mg/m^2 or higher of Pac was combined with CPT in the phase I study, but no patients experienced pneumonitis during cycles 2 to 6 of chemotherapy in this phase II study. Therefore, pneumonitis was seen at a frequency similar to that in other combination chemotherapies. Neutropenia was mild because of the prophylactic use of G-CSF in this study. We used G-CSF when monocytopenia less than 150/μL appeared in the phase I study,[5] and most patients received G-CSF for 5 days starting on days 5, 6, or 7. Consequently, G-CSF was given routinely for 5 days from day 5 to day 9 in every cycle in the present study. This less toxic regimen may be helpful in the treatment of high-risk patients, such as the elderly or those with poor performance status or moderately severe complications.

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Prospective Validation for Prediction of Gefitinib Sensitivity by Epidermal Growth Factor Receptor Gene Mutation in Patients with Non-Small Cell Lung Cancer

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Introduction: We evaluated the efficacy of gefitinib monotherapy prospectively in patients with advanced or pretreated non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (EGFR) mutations.

Methods: Patients with NSCLC were examined for EGFR exon 19 deletion mutations by fragment analysis and for EGFR L858R point mutations by the Cycleave polymerase chain reaction technique. EGFR mutation-positive patients with locally advanced, metastatic, or recurrent/refractory NSCLC that was not curable with surgery or thoracic radiotherapy were candidates for gefitinib treatment administered at 250 mg/day until disease progression.

Results: Mutations of the EGFR gene were detected in 27 (41%) of 66 patients. Ten had exon 19 deletion, and 17 had L858R. Twenty-one patients harboring EGFR mutations were treated with gefitinib and were considered assessable for responses and adverse events. Nineteen patients with EGFR mutations achieved objective responses (three complete responses and 16 partial responses), resulting in an overall response rate of 90.5% (95% confidence interval, 69.6%–98.8%). The median progression-free survival was 7.7 months (95% confidence interval, 6.0 mo to not reached). The median overall survival has not been reached. Common adverse events were skin toxicity, diarrhea, and elevated aminotransferases, but no pulmonary toxicity was observed.

Conclusions: Detection of common EGFR mutations seems to be useful for selecting patients with NSCLC who would likely benefit from gefitinib monotherapy.

Key Words: EGFR, Gefitinib, Lung cancer, Mutations, Drug sensitivity.

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Lung cancer remains the most common cause of cancer death in both men and women worldwide. Lung cancer frequently presents at an advanced and biologically aggressive stage, resulting in poor prognosis. Non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancers. Currently, platinum-based combination chemotherapy regimens, including several active new chemotherapeutic agents, comprise the standard option for patients with advanced NSCLC. However, various combinations of drugs have similar efficacy, producing objective response rates of 30 to 40%, median survival time of eight to 10 months, and 1-year survival rates of 30 to 40%.[1,2] These results remain unsatisfactory, and new modalities of treatment are urgently awaited. Recently, novel molecular targeted strategies that block cancer progression pathways have been suggested as the ideal treatment to control cancer and are considered an exciting therapeutic approach for treating NSCLC.[3]

The epidermal growth factor receptor (EGFR) is a 170 kDa receptor tyrosine kinase and a member of the erbB receptor family that plays a pivotal role in the signaling processes of tumor progression.[4–6] EGFR is overexpressed in several solid tumors, including NSCLC, and it is one of the leading therapeutic molecular targets.[7] Gefitinib is an orally bioavailable, selective EGFR tyrosine kinase inhibitor (TKI) and was the first targeted drug for NSCLC. Phase II and III monotherapy trials for patients pretreated for NSCLC demonstrated objective response rates of only 8 to 18%.[8–10] However, subset analyses of these trials and a retrospective study[11] showed a small group of clinical responders comprising women, patients with adenocarcinomas, nonsmokers, and Japanese or Asian patients. These results suggest that identifying predictive molecular or genetic biomarkers for gefitinib sensitivity may be useful for selecting patients who are most likely to benefit from treatment.

In 2004, three independent groups reported that somatic EGFR mutations correlated with sensitivity of NSCLC to gefitinib or erlotinib, another EGFR TKI.[12–14] Subsequently, several groups confirmed this striking correlation between EGFR mutations and gefitinib sensitivity, yielding a response rate of about 60 to 94% in retrospective analyses.[15–22] EGFR mutations are likely to be significantly associated with survival benefit attributed to gefitinib treatment.[17,18,21] In con-

trast to these results, recent reports concerning molecular analyses of large-scale phase II and III trials showed lower response rates than previously reported and no survival benefit in patients with mutations treated with TKIs.[23–26] Around the same time, the EGFR gene amplification/copy number was demonstrated as another useful predictive molecular marker of TKI efficacy.[23,26–28] However, these contradictory results were obtained through the retrospective collection of tumor samples, and prospective validation studies that predict TKI efficacy by EGFR mutations are needed.

Data from previous reports show that in-frame deletions in exon 19 and specific missense mutation of codon 858 in exon 21 (L858R) account for about 90% of all EGFR mutations, and about 80% of responders to gefitinib or erlotinib harbor either of these two hotspot mutations. Therefore, we developed a rapid, sensitive screening assay of two hotspot mutations[29] and conducted a prospective cohort study to explore the prediction of gefitinib sensitivity in EGFR mutation-positive patients.

MATERIALS AND METHODS

Study Design

This prospective cohort study was conducted to identify patients with NSCLC who would most likely benefit from gefitinib treatment according to their EGFR mutation. Patients with EGFR mutation were treated with oral administration of gefitinib at a dose of 250 mg once a day until disease progression or intolerable toxicity occurred, or until the patient refused to continue treatment. The primary endpoint was objective tumor response rate. Secondary endpoints included adverse effects, disease control rate (response + stable disease), progression-free survival (PFS), and overall survival (OS). This study was approved by the institutional review board of Aichi Cancer Center Hospital.

Patient Eligibility

Eligibility criteria for gefitinib treatment were adult (age ≥20 yr) with cytologic or histologic confirmation; locally advanced, metastatic, or recurrent/refractory NSCLC that was not curable by surgery or radiotherapy; harboring EGFR mutation; and one or more measurable or assessable lesions. All patients were admitted to the study regardless of prior treatment, extent of performance status (PS), or main organ functions. The exclusion criteria were pulmonary fibrosis, interstitial pneumonia, or prior treatment with an EGFR TKI or antibody. All patients gave written informed consent in accordance with institutional regulations before entering the study.

Efficacy and Toxicity Evaluation

Tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors[30] and were confirmed by repeated imaging studies after 4 to 8 weeks of gefitinib treatment. During the treatment and for 30 days after the last dose of gefitinib, patients were monitored for adverse events, which were graded using Common Terminology Criteria for Adverse Events, version 3.0. PFS was assessed from the date of gefitinib treatment until the date of objective disease progression, death from any cause, or the last follow-up. OS was assessed from the date of gefitinib treatment until the date of death from any cause, or the last follow-up.

Detection of EGFR Mutations

Genomic DNA was extracted from tumors embedded in paraffin blocks or from aspirated tumors obtained in pleural effusions, superficial lymph nodes, or subcutaneous metastasis. All specimens were reviewed by a single reference pathologist (Y.Y.) and marked grossly near the tumor-rich lesion on an unstained slide to enrich the tumor cell population as much as possible.

We performed mutational analyses of exon 19 deletion and the L858R point mutation of the EGFR gene, as previously described.[29] Briefly, exon 19 deletion was determined by common fragment analysis using polymerase chain reaction (PCR) with an FAM-labeled primer set, and the PCR products were electrophoresed on an ABI PRISM 310 (Applied Biosystems, Foster City, CA). The shorter segment of DNA amplified by PCR showed a deletion mutation in a new peak in an electropherogram. The L858R mutation was detected by the Cycleave real-time quantitative PCR technique using the Cycleave PCR core kit (Takara Co. Ltd., Ohtsu, Japan) with an L858R-specific cycling probe and a wild-type probe. Fluorescence intensity was measured with a Smart Cycler system (SC-100, Cepheid, Sunnyvale, CA).

Statistical Analysis

Data were analyzed using the chi-square test; $p < 0.05$ was regarded as significant. Confidence intervals (CI) were calculated using binomial CIs. PFS and OS were calculated using the Kaplan–Meier method and compared between two EGFR mutation groups using log-rank test. All the analyses were performed with Stata 8.2 for Macintosh (Stata Corp, College Station, TX).

RESULTS

Sampling Procedure for Detecting EGFR Mutations

Sixty-six consecutive patients with NSCLC were examined to detect the EGFR mutations from November 2004 through August 2005 at Aichi Cancer Center Hospital. Of these patients' samples, 23 specimens were obtained from bronchoscopic biopsy, 22 from computed tomography/ultrasound-guided needle biopsy, 13 from percutaneous aspiration (seven from pleural effusion, four from lymph nodes, and two from skin metastases), two from biopsy (one from tonsil metastasis and one from skin metastasis), and six from surgery with general anesthesia (three from thoracotomy, two from thoracoscopy, and one from mediastinoscopy (Table 1). Sixty samples (91%) were obtained from the biopsy or aspiration method. Tumor tissues or aspirates were procured at the time of initial diagnosis in 52 patients and at the time of tumor progression in 14 patients.

Patient Characteristics and EGFR Mutations

Mutations of the EGFR gene were detected in 27 (41%) of 66 patients. Ten of these had the deletion in exon 19, and

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TABLE 1. Patient Characteristics and Sample Procurement According to EGFR Mutation Status

	EGFR Mutation Status			
	All	Mutation	Wild type	*p*
All cases	66	27 (21)	39	
Sex				0.175
Male	36	10 (8)	26	
Female	30	17 (13)	13	
Age (yr)				0.5084
≤64	31	14 (11)	17	
>64	35	13 (10)	22	
Histology				0.0199
Adenocarcinoma[a]	59	27 (21)	32	*p* ([a] vs. [b])
Squamous cell[b]	2	0	2	
Large cell[b]	2	0	2	
Pleomorphic[b]	1	0	1	
NSCLC NOS[b]	2	0	2	
Smoking status				0.0002
Never smoker[c]	24	17 (13)	7	*p* ([c] vs. [d])
Former smoker[d]	17	9 (7)	8	
Current smoker[d]	25	1 (1)	24	
Stage at initial diagnosis				0.6348
IA[e]	2	1	1	*p* ([e] vs. [f])
IIB[e]	4	2 (2)	2	
IIIA[f]	3	0	3	
IIIB[f]	16	3 (2)	13	
IV[f]	41	21 (17)	20	
Performance status				0.6059
0/1	51	20 (14)	31	*p* (0/1 vs. ≥2)
2	7	3 (3)	4	
3	3	1 (1)	2	
4	5	3 (3)	2	
Prior first treatment				ND
No	8	5 (5)	3	
Surgery	3	3 (1)	0	
Thoracic irradiation	4	2 (2)	2	
Chemoradiotherapy	10	2 (1)	8	
Bone irradiation	6	3 (3)	3	
Brain irradiation	6	3 (2)	3	
Sclerotherapy for effusion	1	1 (1)	0	
Chemotherapy	28	8 (6)	20	
Prior chemotherapy				0.4337
0	28	13 (12)	15	*p* (0 vs. ≥1)
One regimen	28	10 (6)	18	
Two regimens	8	4 (3)	4	
Three regimens	2	0	2	
Method for sample procurement				ND
Bronchoscopic biopsy	23	11	12	
CT/US-guided needle biopsy	22	6	16	
Pleural effusion aspiration	7	4	3	
LN/skin aspiration	6	2	4	
Tonsil/skin biopsy	2	0	2	
Thoracotomy	3	2	1	
VATS	2	1	1	
Mediastinoscopy	1	1	0	

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; NOS, not otherwise specified; ND, not done; CT/US, computed tomography/ultrasound; LN, lymph node; VATS, video-assisted thoracoscopy. Superscript letters indicate groups compared in the statistical analysis. Numbers in parentheses represent the numbers of patients receiving gefitinib treatment.

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17 were the point mutation at codon 858. As previously reported,[12–14,17] the EGFR mutations were significantly associated with adenocarcinoma histology and never-smoking status (Table 1). However, the EGFR mutation status was not significantly correlated with sex, age, PS, stage at initial diagnosis, or prior chemotherapy. Twelve patients received gefitinib treatment as the first-line chemotherapy; five patients desired first-line gefitinib therapy, and the other seven were unfit for conventional chemotherapy because of age (one patient, age 84 yr), cardiac disease (one patient), widespread bone metastases (two patients), and poor PS (3–4 in three patients).

Clinical Response and Survival

Of 27 patients harboring EGFR mutation, 21 were treated with gefitinib and were assessable for objective responses (Table 2) and adverse events (Table 3). The median interval of gefitinib treatment was 5.9 months (range, 0.67 to 11.4 mo). Of the assessable 21 patients, 19 patients achieved objective responses (three complete response and 16 partial response), for an overall response rate of 90.5% (95% CI, 69.6–98.8%). One patient had stable disease, giving an overall disease control rate of 95.2% (95% CI, 76.2–99.9%). According to EGFR mutation classes and PS, the objective responses were seven of eight for the exon 19 deletion, 12 of 13 for the L858R point mutation, 13 of 14 in PS 0 to PS 1 patients, and 6 of seven in PS 2 to PS 4 patients. The response to gefitinib did not differ significantly according to the mutation class or PS.

The median PFS was 7.7 months (95% CI, 6.0 mo to not reached) (Figure 1A). The median OS has not been reached at present (Figure 1B). Subset analyses showed that PFS was greater in patients with the exon 19 deletion than in those with the L858R point mutation (log rank test, $p = 0.04$; Fig 2*A*). The median PFS for the exon 19 deletion group was 7.8 months (95% CI, 7.6 mo to not reached); for the L858R mutation group, median PFS was 6.0 months (95% CI, 2.6 to 7.7 mo). OS did not differ significantly between the two types of mutations (Figure 2B). No difference was observed in PFS and OS between never-smokers and current/former smokers (data not shown).

TABLE 2. Response of EGFR Mutation-Positive Patients to Gefitinib Treatment

	EGFR Mutation Status		
	Exon 19 Deletion ($n = 8$)	L858R Mutation ($n = 13$)	Total ($n = 21$)
CR	1 (12.5%)	2 (15.4%)	3 (14.3%)
PR	6 (75%)	10 (76.9%)	16 (76.2%)
Overall response rate (CR + PR)	7 (87.5%)	12 (92.3%)	19 (90.5%)
SD	1 (12.5%)	0	1 (4.8%)
Disease control (CR + PR + SD)	8 (100%)	12 (92.3%)	20 (95.2%)
Progressive disease	0	1 (7.7%)	1 (4.8%)

EGFR, epidermal growth factor receptor; CR, complete response; PR, partial response; SD, stable disease.

TABLE 3. Number (%) of Patients with Treatment-Related Adverse Events ($n = 21$)

	Grade				
	0	1	2	3	4
Skin toxicity	15 (71)	4 (19)	2 (10)	0	0
Diarrhea	13 (62)	3 (14)	3 (14)	2 (10)	0
Elevated aspartate aminotransferase/ alanine aminotransferase	15 (71)	1 (5)	2 (10)	3 (14)	0
Nail changes	17 (81)	3 (14)	1 (5)	0	0
Mucositis	20 (95)	1 (5)	0	0	0
Joint pain	20 (95)	1 (5)	0	0	0

Adverse Events

All 21 patients were evaluated for drug-related adverse events. The most common adverse events were skin toxicity, diarrhea, and elevated asparatate aminotransferase/alanine aminotransferase (AST/ALT) (Table 3). The grade 3 adverse events of diarrhea and elevated AST/ALT occurred in two (10%) and three (14%) patients, respectively. These events occurred slightly more frequently than in previous studies.[8,9] No grade 4 adverse events or pulmonary toxicity were observed. Seven patients required an interruption of treatment, lasting 2 to 4 weeks, because of grade 2/3 diarrhea or grade 3 elevated transaminases. Two patients withdrew: one after 3 weeks of gefitinib treatment because of grade 3 diarrhea, and the other after 9 weeks of gefitinib treatment because of grade 2 nail changes.

DISCUSSION

In the present study, we have observed that the objective response rate in our patients was similar to that in previous reports. We also found that PFS and OS seem promising in identifying gefitinib-sensitive patients regardless of whether the study includes patients unsuited for conventional cytotoxic chemotherapy because of age, cardiac disease, widespread bone metastases, or poor PS (3 to 4). Our favorable data might have resulted because we selected patients harboring one of two hotspot mutations (exon 19 deletion and exon 21 L858R mutation). Greulich et al.[31] examined NIH-3T3 cells transformed with various EGFR mutants and showed that a distinct EGFR mutation confers differential sensitivity to TKIs. They demonstrated greater sensitivity to TKIs in cell lines with the two hotspot mutations than with the G719S mutation, and insensitivity to TKIs in cell lines with exon 20 insertion (D770-N771 ins) mutation. These in vitro data may explain, at least partially, our promising results for detecting these two sensitive mutations.

We previously reported that patients with the EGFR exon 19 deletion respond significantly better to gefitinib than those with the L858R mutation ($p = 0.0108$).[17] Our current data show no difference in gefitinib sensitivity and OS after

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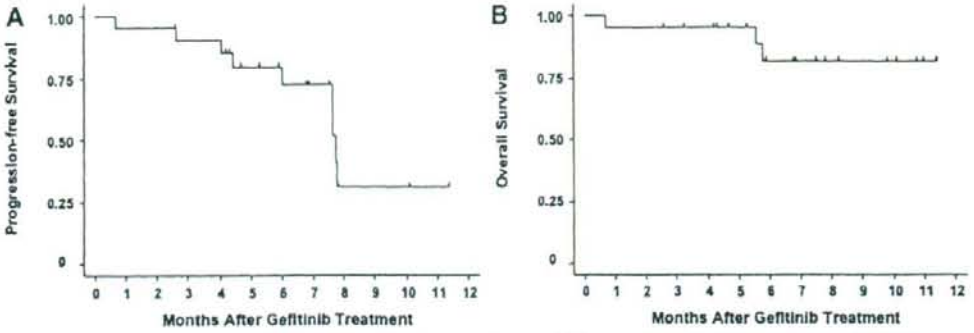

FIGURE 1. Kaplan–Meier estimates of (*A*) progression-free survival and (*B*) overall survival for patients with EGFR mutations ($n = 21$). The median progression-free survival was 7.7 months (95% CI, 6.0 mo to not reached). The median survival was not reached.

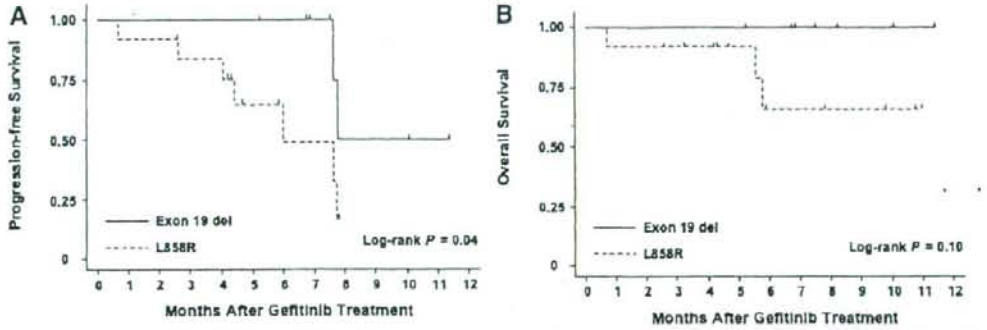

FIGURE 2. Kaplan–Meier estimates of (*A*) progression-free survival and (*B*) overall survival for patients with EGFR mutations according to the exon 19 deletion ($n = 8$) and L858R mutation ($n = 13$). The median PFS for the exon 19 deletion group was 7.8 months (95% CI, 7.6 mo to not reached); for the L858R mutation group, median PFS was 6.0 months (95% CI, 2.6 to 7.7 mo).

gefitinib treatment between these two groups of patients, although we observed a greater PFS in the EGFR exon 19 deletion group than in the L858R group. It is possible that the number of patients (eight with exon 19 deletion and 13 with L858R) was too small to detect a statistically significant difference in OS. Riely et al.[32] reported recently that patients with exon 19 deletion have a significantly longer survival after TKI treatment than those with the L858R mutation ($p = 0.01$). These findings suggest that the EGFR exon 19 deletion might be a better predictor of the efficacy of TKIs than the L858R mutation.

EGFR mutations are significantly associated with patients with adenocarcinomas, patients of Asian origin, females, and patients who had never smoked—clinical factors also associated with patients who respond to gefitinib.[13,14,24,33] A phase II trial using gefitinib monotherapy as the first-line therapy for patients with adenocarcinoma histology and never-smoking status was recently completed in South Korea and reported promising data (e.g., an objective response rate of 69% and estimated 1-year survival rate of 73%).[34] However, this trial did not select patients using biomarkers, and we believe the benefit of gefitinib therapy could be enhanced by selecting individual patients according to appropriate biomarkers. Very recently, two prospective phase II studies that had selected patients based on molecular biomarkers demonstrated that EGFR mutations[35] and gene copy number assessed by fluorescence in situ hybridization (FISH)[36] can predict clinical outcomes in TKI-treated NSCLC patients.

The grade 3 adverse events of diarrhea and elevated AST/ALT were observed in five patients (24%); this is a higher rate than that reported in two previous phase II studies that reported rates of adverse events of 1.5%[8] and 7%[9] at a gefitinib dose of 250 mg per day. The reasons for our higher rate of adverse events are unknown. Although adverse events related to gefitinib treatment are generally thought to be mild and tolerable, they should not be discounted.

Most studies have detected EGFR mutations using direct sequencing or single-strand conformation polymorphism analysis for exons 18 to 21.[37] These techniques are less sensitive when applied to a small amount of tumor cells from the biopsy or aspiration samples.[38] We were able to detect

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two hotspot mutations with our sensitive rapid screening assay in most biopsy or aspiration samples in the routine clinical setting. Although this assay needs precise assessment of tumor samples by a pathologist to enrich the tumor cells, it is very sensitive and accurate for detection, and it can be completed within 4 hours without need for microdissection or nested PCR process.[29]

The key genetic event for TKI sensitivity has not been perfectly identified and is the subject of a growing debate about the role of EGFR mutations versus EGFR gene amplification/copy number in NSCLC. EGFR mutant NSCLC cell lines are strongly associated with increased EGFR gene copy number.[39,40] Cappuzzo et al.[27] and Takano et al.[22] found that EGFR mutations in NSCLC patients correlate significantly with gene copy number assessed by FISH and quantitative real-time PCR, respectively. However, Cappuzzo et al.[27] demonstrated that in patients treated with gefitinib, a high EGFR gene copy number is a better predictor of survival than EGFR mutations.[27] In contrast, Takano et al.[22] reported that the status of the EGFR mutations, rather than gene copy number, is the major determinant of gefitinib efficacy. Recent reports of the molecular analyses from the largest phase III TKI monotherapy trials failed to show that the EGFR mutation is superior to gene copy number in predicting the efficacy of TKIs.[23,26] These conflicting results on EGFR mutations and gene amplification/copy number could be explained by (i) differences in the detection methodologies and assessment of mutation and gene amplification/copy number (e.g., direct sequence versus PCR-based DNA testing for detecting EGFR mutations, or FISH versus PCR-based amplification for detecting EGFR gene amplification/copy number), (ii) failure to reconfirm these results in other institutions, and (iii) other unknown factors underlying drug sensitivity, especially those related to ethnicity. Further prospective studies are needed to investigate the crucial molecular markers involved in the EGFR network, using adequate tissue samples and assays to more precisely detect molecular events.

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Genomic profiling of malignant pleural mesothelioma with array-based comparative genomic hybridization shows frequent non-random chromosomal alteration regions including *JUN* amplification on 1p32

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Genome-wide array-based comparative genomic hybridization analysis of malignant pleural mesotheliomas (MPM) was carried out to identify regions that display DNA copy number alterations. Seventeen primary tumors and nine cell lines derived from 22 individuals were studied, some of them originating from the same patients. Regions of genomic aberrations observed in >20% of individuals were 1q, 5p, 7p, 8q24 and 20p with gains, and 1p36.33, 1p36.1, 1p21.3, 3p21.3, 4q22, 4q34-qter, 6q25, 9p21.3, 10p, 13q33.2, 14q32.13, 18q and 22q with losses. Two regions at 1p32.1 and 11q22 showed a high copy gain. The 1p32.1 region contained a protooncogene, *JUN*, and we further demonstrated overexpression of *JUN* with real-time polymerase chain reaction analysis. As MPM cell lines did not overexpress *JUN*, our findings suggested that induction of *JUN* expression was involved in the development of MPM cells *in vivo*, which also might result in gene amplification in a subset of MPM. Meanwhile, the most frequent alteration was the 9p21.3 deletion, which includes the $p16^{INK4a}/p14^{ARF}$ locus. With polymerase chain reaction analysis, we determined the extent of the homozygous deletion regions of the $p16^{INK4a}/p14^{ARF}$ locus in MPM cell lines, which indicated that the deletion regions varied among cell lines. Our results with array comparative genomic hybridization analysis provide new insights into the genetic background of MPM, and also give some clues to develop a new molecular target therapy for MPM. (*Cancer Sci* 2007; 98: 438–446)

MPM, a highly lethal neoplasm of the serosal lining of the pleural cavity, is thought to develop from superficial mesothelial cells.[1] In up to 80% of patients, MPM occurs within about 30 years of exposure to asbestos.[2–4] The incidence of MPM is expected to increase dramatically over the next few decades. It has been estimated that 250 000 people will die of MPM in Europe in the next three decades, and 2500–3000 new cases are diagnosed each year in the USA.[5,6] In Japan, a recent report has shown that there will be approximately 100 000 deaths due to MPM in the next 40 years using an age-cohort model.[7] Survival of patients with MPM is very poor, with a median survival of 7–11 months after diagnosis, especially in advanced-stage patients, regardless of a recent advancement in chemotherapeutic modalities that combines cisplatin and antifolate.[8–10]

The long latency period between asbestos exposure and tumor development implies that multiple, and likely diverse, genetic changes are required for the malignant transformation of mesothelial cells. Many studies have been conducted to determine underlying key genetic and epigenetic events responsible for the development of MPM, some of which may be directly caused by asbestos fibers. Traditional karyotype analysis using primary samples or cell lines uncovered multiple non-random chromosomal abnormalities that are frequently detected in most human MPM specimens, which include chromosomes 1p, 3p, 6q, 9p and 22q.[11–18] Subsequent studies of such common regions with allele loss, which indicate the sites of TSG, have identified the target genes of MPM, including $p16^{INK4a}/p14^{ARF}$ on chromosome 9p21 and *NF2* at 22q. The $p16^{INK4a}/p14^{ARF}$ gene, one of the most frequently mutated TSG of human malignancies, has been shown to be inactivated in ~90% of MPM, with most cases being targeted by homozygous deletion.[19,20] The *NF2* gene at the 22q12 locus, which is responsible for a familiar cancer syndrome of neurofibromatosis type II, has also been shown to be inactivated in 40–50% of MPM, mainly with nonsense mutation or homozygous deletion.[21,22] In contrast, the *p53* gene, another of the most frequently mutated TSG in human malignancies, is only occasionally mutated in MPM, with approximately 25% of MPM specimens being inactivated.[23,24] Meanwhile, MPM does not show frequent mutation of known protooncogenes including *KRAS*, *NRAS* and *EGFR*.[25–28] Thus, it has been suggested that there are other yet unidentified TSG or protooncogenes responsible for the development of MPM. Recently, a CGH technique introduced to search for additional genes that are potentially involved in MPM biology has identified other regions with alterations, including 1q, 4q, 5p, 6p, 7p, 8p, 8q, 10p13-pter, 13q, 14q, 15q, 17p12-pter, 17q and 20.[29–34]

In the present study, we carried out array CGH analysis with 17 resected MPM samples (from 16 patients) and nine MPM cell lines from a total of 22 individuals. We confirmed the same chromosomal alterations as described before in the literature and further identified new regions such as 8q24 and 13q33.2. We also identified high copy gain at 1p32, which includes the *JUN* protooncogene. The present study provides new insights

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Abbreviations: BAC, bacterial artificial chromosome; CGH, comparative genomic hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MPM, malignant pleural mesothelioma; PAC, P-1 derived artificial chromosome; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; STR, short tandem repeat; TSG, tumor suppressor gene.

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Table 1. Summary of malignant pleural mesotheliomas analyzed with array comparative genomic hybridization (CGH)

KD number[1]	Sex	Subtype	Asbestos exposure	Cell line	*p16^{INK4a}/p14^{ARF}*[2]	*NF2*[3]	*JUN*
332	Male	Epithelioid	+		HL	(+)	No Amp
355	Male	Epithelioid	–			(+)	No Amp
471	Male	Epithelioid	Unknown		HL	(+)	No Amp
476	Male	Biphasic	–	Y-MESO-8 A, -8D	HD	+	No Amp
905	Male	Epithelioid	Unknown		HL	del(533–537)	No Amp
977	Male	Epithelioid	Unknown			(+)	No Amp
1032	Male	Biphasic	+			(+)	No Amp
1033	Male	Epithelioid	+			(+)	Amp
1038	Male	Epithelioid	+			(+)	No Amp
1039	Male	Duciduoid	+			(+)	Amp
1041	Male	Duciduoid	+		L	(+)	Amp
1043	Female	Epithelioid	+			del(468–479)	No Amp
1044	Male	Epithelioid	–		L	(+)	No Amp
1045	Male	Epithelioid	–		L	(+)	No Amp
1046	Male	Biphasic	+		L	(+)	No Amp
1048	Male	Epithelioid	+	Y-MESO-9	HD	del(527–528)	No Amp
1049	Male	Epithelioid	+			(+)	No Amp
	Female	Epithelioid	–	Y-MESO-12	HD	+	No Amp
	Female	Epithelioid	–	ACC-MESO-1	HD	Q389X	No Amp
	Male	Epithelioid	+	ACC-MESO-4	HD	+	No Amp
	Male	Unknown	Unknown	NCI-H28	HD	[+]	No Amp
	Male	Unknown	Unknown	NCI-H2052	HD	R341X	No Amp
	Unknown	Unknown	Unknown	MSTO-211H	HD	[+]	No Amp

[1]KD Number indicates primary tumors available for array CGH analysis. Two primary tumors were obtained from the same patient at surgical resection (KD1039) and autopsy (KD1041). [2]*p16^{INK4a}/p14^{ARF}* status was indicated as follows: HL, high-level loss; L, loss; HD, homozygous deletion (detected in cell lines). [3] +, No point mutation was detected with PCR sequencing analysis of exons 1–17 covering the entire open reading frame of *NF2*, and homozygous deletion was not detected in the corresponding cell line; (+), no point mutation was detected in exons 1–17, but homozygous deletion was not determined due to possible contamination of non-cancerous DNA; [+], undetectable point mutation for exons 2, 5, 7, 8, 9, 10, 11 and 12. Data of *p16^{INK4a}/p14^{ARF}* and/or *NF2* of Y-MESO-8A, Y-MESO-8D, ACC-MESO-1, ACC-MESO-4, NCI-H28, H2052 and MSTO-211H referred to Sekido *et al.* and Usami *et al.*[21,28] Amp, amplification.

into the genetic background of MPM, and also gives some clues to developing a new molecular target therapy for MPM.

Materials and Methods

Cell lines and tumor specimens. Twelve MPM cell lines and one non-malignant mesothelial cell line (MeT-5A) were used. ACC-MESO-1, ACC-MESO-4, Y-MESO-8A, Y-MESO-8D, Y-MESO-9 and Y-MESO-12 were established in our laboratory,[28] whereas NCI-H28 (CRL-5820), NCI-H2052 (CRL-5915), NCI-H2373 (CRL-5943), MSTO-211H (CRL-2081) and MeT-5 A (CRL-9444) were purchased from the American Type Culture Collection (Rockville, MD, USA). NCI-H290 and NCI-H513 were gifts from Dr Adi F. Gazdar. All MPM cell lines were maintained in RPMI-1640 medium (Sigma-Aldrich, Irvine, UK) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1× antibiotic–antimycotic (Invitrogen) at 37°C in a humidified incubator with 5% CO_2. MeT-5 A was cultured according to the instructions of the American Type Culture Collection. Nineteen MPM samples from 18 Japanese patients were obtained at Aichi Cancer Center Hospital, Nagoya University Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Second Red Cross Hospital and Kasugai City Hospital (KD332, KD355, KD471, KD476, KD905, KD977, KD1032, KD1033, KD1038, KD1039, KD1041, KD1042, KD1043, KD1044, KD1045, KD1046, KD1048, KD1049 and KD1050; of these, KD1039 and KD1041 originated from the same patient at surgery and autopsy, respectively). MPM samples and clinical data were collected after obtaining appropriate institutional review board approval and written informed consent from all patients. To confirm that there was no cross-contamination of clinical samples and cell lines, the uniqueness or identity of MPM tissues and established cell lines were evaluated by analysis of STR polymorphisms using the AmpFLSTR Identifier Kit (Applied Biosystems, Foster City, CA, USA), including the 16 STR loci D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, Amelogenin, D5S818, and FGA. Primary tumors and cell lines used in the present study are summarized in Table 1.

Preparation of DNA and RNA. Genomic DNA was extracted using a standard phenol–chloroform method.[35] Normal DNA was prepared from peripheral blood of healthy male donors and non-cancerous lung tissue of the patients. Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. DNase treatment was carried out on columns during RNA purification using an RNase-Free DNase Set (Qiagen, Germantown, MD, USA). Random-primed, first-strand cDNA was synthesized from 2 μg total RNA using Superscript II according to the manufacturer's instructions (Invitrogen).

Genome-wide array-based CGH. A genome-wide scanning array with 2304 BAC and PAC clones covering the whole human genome at a resolution of roughly 1.3 Mb was used as described previously.[36] In brief, clones were isolated from bacterial cultures containing the requisite antibiotics and extracted using a Plasmid Mini-kit (Qiagen). The location of all clones used for the array CGH was confirmed by standard fluorescence *in situ* hybridization analysis. BAC and PAC clones were amplified using degenerate oligonucleotide-primed PCR and spotted on glass slides. DNA preparation from cells, labeling, hybridization and scanning analysis were carried out as described previously[37] with minor modifications.[36,38,39] The data obtained were processed to detect chromosomal imbalances as described.[40]

Southern blot analysis. Genomic DNA from patient samples (7 μg) was digested with *Eco*RI restriction enzyme, electrophoresed,

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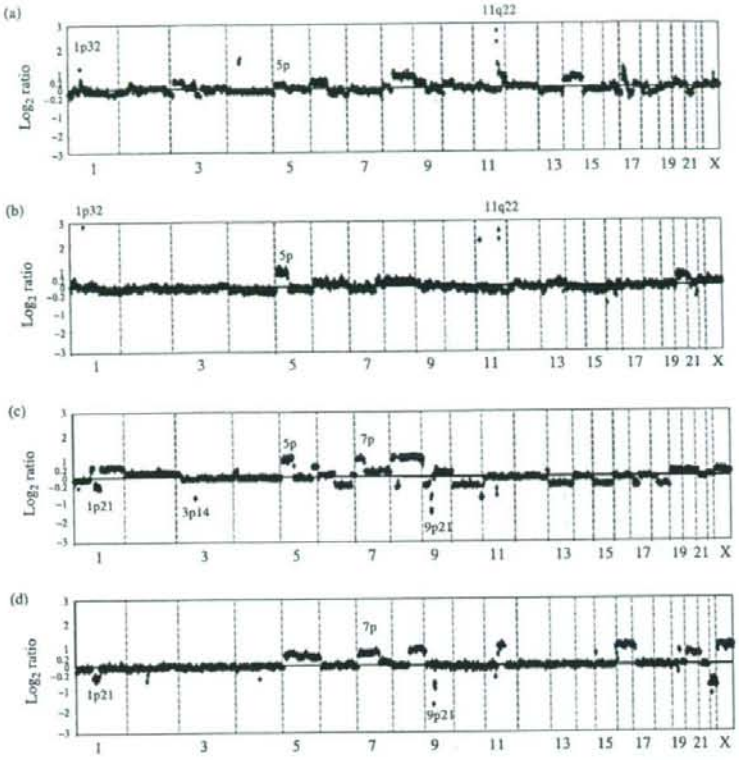

Fig. 1. Array comparative genomic hybridization profile of malignant pleural mesothelioma from three primary tumors and one cell line. Log_2 ratios are plotted for all clones based on chromosome position, with vertical dotted lines showing separation of the chromosome. Clones are ordered from chromosomes 1–22 and X within each chromosome on the basis of the Sanger Center Mapping Position, July 2004 version. (a) KD1033 sample shows chromosomal gain of 1p32.1-p32.3, 2p16, 3p22.2-pter, 3p12, 4q12, 5p, 6pter-q14.1, 8q, 9p, 10p, 11q22.1-q22.3, 11q23.3-qter, 14, 17p12-pter and 20p11.21-p12, and loss of 1p36.13-pter, 1q32-q42, 2q37.1-qter, 3q11-q13.31, 4q34.3-qter, 6q14.3-q21, 6q25-qter, 7q35-qter, 9q34.12-qter, 13q12.11-q13.3, 13q34, 16q23-qter, 17q11.2-qter, 18p, 18q12.2-qter and 21qcen-q22.2. (b) KD1041 primary sample shows chromosomal gain of 1p36.13-p36.32, 1p32.1, 5p, 6p22-pter, 6p12-p21.1, 8, 11p15.2-p15.3, 11q22.1, 20, 22q12-q13.2 and X, and loss of 3p21.31, 4q, 5q35.1-qter, 9p21.3, 11q23-qter, 13q12, 13q33.2, 15q22.3-qter, 16p13.2, 16q11-q12.2 and 21q22. (c) KD471 primary sample shows chromosomal gain of 1p22.2-p31.1, 1q, 2, 4p15-pter, 5p, 5q33.1-qter, 7, 8p21.1-pter, 8q, 9q, 12q24, 19 and 20, and loss of 1p36.31-p36.33, 1p36.13, 1p12-p22.1, 3p14.3-p21.31, 6q14-q25.1, 8p12-p21.1, 9p21.2-pter, 10, 11q12.1, 13, 15, 17p and 18q. (d) Y-MESO-12 cell line shows chromosomal gain of 5, 7pter-q21.3, 8q21-qter, 11qcen-q14.3, 15q11, 16, 19q13.2 and 20, and loss of 1p21-p31.1, 2p11, 4q22.1, 9p21.3, 11p12, 19p13.11 and 22.

and transferred to Hybond N+ (Amersham Biosciences, Piscataway, NJ, USA). Hybridization and washing were carried out using standard techniques.[35] The DNA probes were made by RT-PCR using normal lung cDNA. RT-PCR of *JUN* and *β-actin* were carried out using the primer sets: C-jun-S1, 5′-GACCTTATGGCTACAGTAACCC-3′ (sense) and C-jun-AS1, 5′-CTGCTCATCTGTCACGTTCT-3′ (antisense); and B-Actin-S, 5′-CTGTGGCATCCACGAAACTA-3′ (sense) and B-Actin-AS, 5′-AGGAAAGACACCCACCTTGA-3′ (antisense).

Quantitative real-time PCR. The reaction mixture for real-time PCR using first-strand cDNA contained TaqMan universal PCR Master Mix (Applied Biosystems) and 200 nM of each primer, *JUN* (Hs 00277190_s1; Applied Biosystems) and *FOS* (Hs 00170630_m1). All real-time PCR assays were done in MicroAmp optical 96-well reaction plates on an ABI PRISM 7900 Sequence Detector System (Applied Biosystems) according to the manufacturer's instructions. For normalization between samples, PCR amplification of *GAPDH* (Hs 00266705_g1; Applied Biosystems) was included for each sample at each run. Fluorescence measurements and melting curve analyses were carried out using SDS 2.1 software (Applied Biosystems). The relative quantification of gene expression was computed using the comparative threshold cycle method with a mathematical formula described previously, and results are shown as a fold induction of mRNA.[41] We classified them into high-level expresser of *JUN* or *FOS* (defined as >0.15 of *JUN* or *FOS* mRNA expression relative to *GAPDH* mRNA expression), middle-level expresser (defined as >0.025 but <0.15), and low-level expresser (defined as <0.025).

Deletion mapping of 9p21. Information on 16 microsatellite markers and one sequence-tagged site marker at 9p21 was searched, and their sequences were obtained from the Human Genome Database (GDB) and the Ensembl Genome Browser. Three primer sets for exons 1, 2 and 3 of $p16^{INK4a}$ were as described previously,[28] and the primer set of exon 1β of $p14^{ARF}$ was p14ARF-F, 5′-CACCTCTGGTGCAAAGGGC-3′ (sense) and p14ARF-R, 5′-CCTAGCCTGGGCTAGAGACG-3′ (antisense).

Mutation analysis of *NF2*. Mutation analysis of *NF2* was carried out by direct sequencing after PCR amplification of genomic DNA. Seventeen primer sets covering the entire coding region of *NF2* were described previously.[28]

Results

Genomic profiles and data analysis of MPM. Array CGH analysis was carried out using genomic DNA samples extracted from 19 MPM primary tumors and nine MPM cell lines (ACC-MESO-1, ACC-MESO-4, Y-MESO-8A, Y-MESO-8D, Y-MESO-9, Y-MESO-12, NCI-H28, NCI-H290 and MSTO-211H). Among 19 primary tumors, we did not detect any significant genomic alterations in two tumors, which was probably due to much contamination of genomic DNA from non-malignant cells, and we excluded these tumors for further analysis. Of the 26 MPM analyzed successfully, there were paired samples from the same individuals: the Y-MESO-8 A and Y-MESO-8D cell lines were established from the KD476 primary tumor, Y-MESO-9 was established from KD1048, and the other two primary tumors (KD1039 and KD1041) were obtained from the same patient at surgical resection and autopsy, respectively. Thus a total of 22 individual MPM were studied (Table 1). All of the clones on chromosome X were analyzed separately because of sex mismatching. Copy number changes were detected at high-resolution for genomes as a whole for primary tumor samples as well as cell lines. We defined regions of gain or amplification as $\log_2$ ratio > +0.2, and regions suggestive of heterozygous loss or deletion as $\log_2$ ratio < −0.2. Figure 1 shows representative data of the entire genomic profiles of three MPM primary tumors and one cell

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