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Phase I/II study of amrubicin, a novel 9-aminoanthracycline, in patients with advanced non-small-cell lung cancer

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Summary

Purpose: Amrubicin is a novel, totally synthetic 9-aminoanthracycline. The present phase I/II study was performed to define its maximum-tolerated dose (MTD), efficacy and toxicity in the treatment of previously untreated patients with advanced non-small-cell lung cancer (NSCLC). *Patients and Methods*: Chemonaive patients were required to have cytologically or histologically proven measurable NSCLC, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 2, and adequate organ functions. Amrubicin was administered by daily intravenous injection for 3 consecutive days every 3 weeks. *Results*: In a phase I study, four patients were enrolled at dose level 1 (40 mg/m^2/day) and four at dose level 2 (45 mg/m^2/day). No dose limiting toxicity (DLT), which was defined as toxicity consisting of grade 4 neutropenia and leukopenia lasting four days or more, and grade 3 or 4 toxicity other than neutropenia, leukopenia, anorexia, nausea/vomiting, and alopecia, was observed at these dose levels. Subsequently, at dose level 3 (50 mg/m^2/day), 3 of 5 patients experienced DLTs (leukopenia, neutropenia, thrombocytopenia, or gastrointestinal complications). The MTD and recommended dose (RD) were determined to be 50 mg/m^2/day and 45 mg/m^2/day, respectively. Three partial responses (PRs) were achieved in 13 patients (response rate, 23.1%) in a phase I study. In a phase II study, 15 patients were assessable for efficacy and toxicity at the RD, and four PRs were obtained (response rate, 26.7%). The major toxicities were leukopenia and neutropenia, while non-hematologic toxicities were mild. The overall response rate in the combined patient population of the phase I/II study was 25.0% (7 PRs in 28 patients), with a 95% confidence interval of 10.7% to 44.9%. *Conclusion*: Amrubicin exerted promising antitumor activity on NSCLC with acceptable toxicity.

Introduction

Amrubicin is a novel, totally synthetic 9-aminoanthracycline, (+)-(7S, 9S)-9-acetyl-9-amino-7-[(2-deoxy-β-D-erythro-pentopyranosyl)oxy]-7,8,9,10- tetrahydro-6,11-dihydroxy-5,12-naphthacenedione hydrochloride, and is similar to doxorubicin in chemical structure, as shown in Figure 1 [1]. Amrubicin showed more potent antitumor activity than doxorubin on several human tumor xenografts implanted in nude mice [2]. Its toxic profile was qualitatively similar to that of doxorubicin in terms of acute toxicities [3], but amrubicin rarely caused delayed-type toxicity as observed with doxorubicin, especially cardiotoxicity [4, 5]. In an early phase II study of single-dose intravenous injection of 120 mg/m^2 every 3 weeks, amrubicin exhibited promising antitumor activity on non-small-cell lung cancer (NSCLC) with a response rate of 25% (95% confidence interval, 8.7% to 49.1%) [6].

A major characteristic of amrubicin that is closely associated with the efficacy and toxicity is that it is converted to an active metabolite, amrubicinol, via reduction of its C-13 ketone group to a hydroxy group. The *in vitro* cytotoxic activity of amrubicinol was almost equipotent to that of doxorubicin, and 20 to 220 times more potent than that of its parent compound, amrubicin [7]. The *in vivo* antitumor activity of amrubicin was closely related to the tumor concentration of amrubicinol [8]. In addition, the experimental data have shown that amrubicin yields greater efficacy in daily treatment for 5 consecutive days than in a single treatment, due to accumulation of greater amounts of amrubicinol in tumor tissues [9].

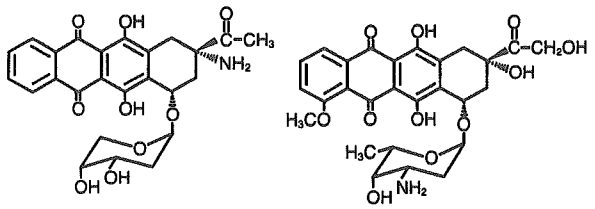

Figure 1. Chemical structures of amrubicin and doxorubicin

These data suggest that amrubicin may exert more potent effect against NSCLC in the divided treatment schedule than in the single-dose treatment schedule.

In addition, it has been reported that epirubicin, the same anthracycline derivative as amrubicin, could be administered at higher doses in 3-day consecutive treatment every 3 weeks than in single-dose treatment every 3 weeks, and consequently the high dosage of epirubicin in the former treatment schedule resulted in a higher response rate, compared with standard dosages of epirubicin in the latter treatment schedule, in previously untreated patients with advanced NSCLC [10].

In the present phase I/II study, therefore, daily treatment for 3 consecutive days every 3 weeks was chosen as the divided treatment schedule, and the efficacy and safety of amrubicin were evaluated in previously untreated patients with advanced NSCLC.

Patients and methods

Patient eligibility

This study involved patients with histologically or cytologically confirmed unresectable NSCLC in stages IIIA, IIIB, and IV. Eligibility criteria included no prior treatment, measurable lesions, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 2, an estimated life expectancy of at least 2 months, and age less than 75 years. Adequate organ function was required and defined as: white blood cell (WBC) count $\geq$4,000/μL, platelet count $\geq$100,000/μL, hemoglobin level $\geq$10 g/dL, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) $\leq$ 2 times the upper limit of normal, serum creatinine level $\leq$ normal limit, and electrocardiography (ECG) within normal limits.

The following patients were excluded: those with symptomatic brain metastasis or bone metastasis accompanying pain, those with plural fluid retention requiring treatment like drainage, those with continuous long term treatment with non-steroidal anti-inflammatory agents, glucocorticoids, or morphine derivatives, those with serious complications or other active cancer, and those judged by the investigators to be inappropriate for the study. Patients who were pregnant, breast-feeding, or taking inadequate contraceptive precautions were also ineligible. All eligible patients were required to provide signed informed consent prior to entering this study. The individual investigational review board at each institution approved the treatment protocol.

Drug administration

Amrubicin (Sumitomo Pharmaceuticals Co., Ltd, Osaka, Japan) was supplied as a freeze-dried powder in vials containing 20 mg each, reconstituted in 20 mL of physiological saline or 5% glucose solution, and administered intravenously over 5 minutes on 3 consecutive days every 3 weeks. At least 2 cycles were instituted, except in case of disease progression, unacceptable toxicity or patient refusal.

Dose levels

The phase I study was started at a dosage of 40 mg/m^2/day to determine the dose limiting toxicity (DLT), maximum-tolerated dose (MTD) and recommended dose (RD) of amrubicin given on 3 consecutive days (120 mg/m^2/course). The starting dosage was set at the same dosage per cycle as that used in the early phase II study for NSCLC in which amrubicin was given once every 3 weeks [6], because experimentally, amrubicin could be administered at a higher total dosage in the divided treatment schedule than in the single treatment schedule [10].

The dosage of amrubicin was escalated by 5 mg/m^2/day (15 mg/m^2/course). At least four patients were entered at each dose level until the MTD was reached. The dose escalations were determined based on the tolerability observed during the first 3 weeks of treatment as follows. The dose at which none or one patient experienced a DLT was escalated, and the MTD was the dose at which at least two patients developed a DLT, i.e., the dose at which at least 2/4, 2/5 or 2/6 patients experienced a DLT. Dosages were not escalated for individual patients.

The following phase II study was performed at the RD estimated in the phase I study.

Definition of DLT, MTD, and RD

DLT was defined as toxicity consisting of grade 4 neutropenia and leukopenia lasting four days or more, and grade 3 or 4 toxicity other than neutropenia, leukopenia, anorexia, nausea/vomiting, and alopecia. MTD was defined as the dose level at which at least one-third of patients experienced a DLT. The RD was chosen as the dose one-level lower than the MTD.

Adjustment of dosage and schedule modification

The treatment was repeated if the WBC count recovered to $\geq$3,000/μL and the platelet count recovered to $\geq$100,000/μL. In incomplete recovery, the treatment was delayed until the WBC count recovered to $\geq$3,000/μL and the platelet count recovered to $\geq$100,000/μL. If the WBC count and platelet count did not recover within 5 weeks after administration of amrubicin, the trial was discontinued. If the WBC nadir was $<$1,000/μL for $\leq$3 days, or $\geq$1,000/μL and the platelet nadir was $\geq$50,000/μL, the treatment was conducted at the same dosage as the previous course. If the WBC nadir was $<$1,000/μL for $\geq$ 4 days and/or the platelet nadir was $<$50,000/μL, the dosage was reduced by 5 mg/m^2/day from the dosage of the previous course.

Treatment evaluation

Before treatment, all patients underwent medical history review, physical examination, hematology and serum biochemistry tests, urinalysis, ECG, and baseline tumor measurements (e.g. chest radiography, computed tomography (CT) scan, bone scintigraphy, abdominal CT, brain CT). All measurable and assessable lesions were evaluated within 2 weeks before start of treatment.

Complete and differential blood cell counts, platelet counts, and hematocrit values were obtained two times a week as a rule, and biochemical data [AST, ALT, alkaline phosphatase, LDH, total bilirubin, BUN, creatinine, serum bilirubin, albumin, total protein, and electrolytes (Na, K, Cl, and Ca)], and urinalysis findings (protein, glucose, urobilinogen, and occult blood), were recorded weekly. ECG was performed every treatment cycle.

Subjective symptoms and objective signs were checked daily for 5 consecutive days from the start of treatment in each cycle, and thereafter ad libitum.

Response and toxicity evaluation

Response was assessed according to the "Criteria for the evaluation of the clinical effects of solid cancer chemotherapy" of the Japan Society for Cancer Therapy [11], which is almost equal to the World Health Organization criteria [12]. A complete response (CR) was defined as the disappearance of all lesions. A partial response (PR) was defined as a reduction by 50% or more in the size of lesions measurable in two dimensions, objective improvement in any evaluable lesions, and no new lesions. CR and PR required response durations of at least four weeks. No change (NC) was defined as lesions unchanged (a reduction of $<$25% or an increase of $<$25% in the size of lesions) for at least four weeks. Progressive disease (PD) was defined as failure, with an increase of $\geq$25% in the size of lesions and appearance of new lesions. The Kaplan-Meier product-limit method was used to estimate the survival time.

Toxicity grading was recorded based on the side effect record form in the "Criteria for the evaluation of the clinical effects of solid cancer chemotherapy" of the Japan Society for Cancer Therapy [11], which is almost equal to the World Health Organization criteria [12]. For toxicity items that were not included on the record form, only their presence or absence was recorded, without grading.

Results

Patient characteristics

Thirteen patients were entered in the phase I study, and subsequently 17 patients in the phase II study, between November 1992 and September 1994. Of the 13 patients entered in the phase I study, 4 were treated at dose level 1 (40 mg/m^2/day $\times$ 3), 4 at level 2 (45 mg/m^2/day $\times$ 3), and 5 at level 3 (50 mg/m^2/day $\times$ 3); all were assessable for efficacy and safety.

In the phase II study, 15 of 17 patients were assessable for efficacy and safety; 2 of them were ineligible because one had suffered from serious complications of pneumonitis and arrhythmia, a deviation against the inclusion criteria in the protocol, and another had been treated without registration prior to the study.

The characteristics of the eligible patients are listed in Table 1.

Phase I study

Toxicity. Hematologic toxicity is shown in Table 2. Dose-related leukopenia and neutropenia were noted. At dose level 1 (40 mg/m^2), one patient experienced grade 4

Table 1. Characteristics of eligible patients

Characteristic	No. of patients	
	Phase I study	Phase II study
No. of patients entered	13	17
No. of eligible patients	13	15
Gender(Male/Female)	8/5	10/5
Median age, years (range)	69 (45–74)	65 (29–72)
ECOG performance status		
0/1/2	5/3/5	1/12/2
Histology		
Squamous cell carcinoma	5	6
Adenocarcinoma	7	8
Large cell carcinoma	1	1
Stage (IIIA/IIIB/IV)	2/1/10	1/3/11

Table 2. Hematologic toxicity of amrubicin in phase I study

	Grade of toxicity (No. of patients)											
	40 mg/m² (n = 4)				45 mg/m² (n = 4)				50 mg/m² (n = 5)			
Toxicity	1	2	3	4	1	2	3	4	1	2	3	4
Hemoglobin, decrease	1	0	1	0	2	1	1	0	2	1	2	0
Leukopenia	1	1	1	1	1	0	3	0	0	0	3	2
Neutropenia	0	1	1	1	0	1	0	3	0	0	0	5
Thrombocytopenia	1	0	0	0	0	1	1	0	3	0	1	1

neutropenia and leukopenia, which did not last for 4 days or longer. At dose level 2 (45 mg/m²), three of four patients also experienced grade 4 neutropenia, lasted 4 days or longer in only one. At this dose level, no grade 4 leukopenia was observed. Dose-limiting leukopenia and neutropenia lasting for more than 4 days were seen in two and in all five patients at dose level 3 (50 mg/m²), respectively. Grade 3 or 4 hemoglobin decrease and thrombocytopenia each occurred in two patients at the highest dose level. Three patients required blood transfusion or platelet transfusion or both.

As shown in Table 3, non-hematologic toxicities observed frequently in this study were anorexia, nausea/vomiting, fever, diarrhea and alopecia, but no grade 3 or 4 toxicity was seen at dose level 1 or 2. On the contrary, at dose level 3, grade 3 or 4 toxicity was noted in three of five patients; grade 3 nausea/vomiting and melaena and grade 4 hematemesis in one patient each. Because the grade 3 melaena and grade 4 hematemesis were noted in

Table 3. Non-hematologic toxicity of amrubicin in phase I study

	Grade of toxicity (No. of patients)											
	40 mg/m² (n = 4)				45 mg/m² (n = 4)				50 mg/m² (n = 5)			
Toxicity	1	2	3	4	1	2	3	4	1	2	3	4
Stomatitis	0	0	0	0	0	0	0	0	1	1	0	0
Anorexia	2	1	0	–[a]	1	0	0	–[a]	0	2	0	–[a]
Nausea/vomiting	2	0	0	–[a]	3	0	0	–[a]	1	1	1	–[a]
Diarrhea	3	0	0	0	1	0	0	0	1	0	0	0
Fever	1	0	0	0	0	1	0	0	1	4	0	0
Alopecia	1	0	0	–[a]	1	3	0	–[a]	2	3	0	–[a]
Melaena	0	0	0	0	0	0	0	0	0	0	1	0
Hematemesis	0	0	0	0	0	0	0	0	0	0	0	1
AST, increase	1	0	0	0	1	0	0	0	2	0	0	0
ALT, increase	1	0	0	0	1	0	0	0	2	0	0	0
ALP, increase	0	0	0	0	1	0	0	0	0	0	0	0
BUN, increase	0	0	0	0	0	0	0	0	1	0	0	0

Abbreviation: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urine nitrogen.
[a]No grading.

Table 4. Efficacy of amrubicin in phase I study

	No. of patients						
Dose	Total	CR	PR	NC	PD	ORR (%)	95% CI (%)
40 mg/m²	4	0	1	1	2	25.0	
45 mg/m²	4	0	2	1	1	50.0	
50 mg/m²	5	0	0	5	0	0.0	
Total	13	0	3	7	3	23.1	5.0–53.8

Abbreviation: CR, complete response; PR, partial response; NC, no change; PD, progressive disease; ORR, overall response rate (CR + PR); 95% CI, 95% confidence interval

two patients who had received indomethacin or diclofenac sodium over 50 days, these episodes were considered to be associated with the long-term treatment of nonsteroidal anti-inflammatory agents. Therefore, the criteria for entry into the study were revised in the subsequent studies to exclude patients who had been treated with nonsteroidal anti-inflammatory agents for a long period. There was no toxicity to renal or cardiac function but a mild effect on hepatic function was observed. As uncommon toxicities, two episodes of grade 1 vitreous floaters occurred at 40 and 45 mg/m², and one episode of grade 1 eruption occurred at 50 mg/m².

Based on the above results, the MTD and RD of amrubicin in a 3-day consecutive administration were determined as 50 mg/m² (150 mg/m²/course) and 45 mg/m² (135 mg/m²/course), respectively. The DLTs were leukopenia, neutropenia, thrombocytopenia and digestive dysfunction including nausea/vomiting, melaena, and hematemesis.

Efficacy. Antitumor response is shown in Table 4. One of four patients (25.0%) at dose level 1 (40 mg/m²) and two of four patients (50.0%) at dose level 2 (45 mg/m²) showed PR. At dose level 3 (50 mg/m²), three patients discontinued treatment after the first cycle because of toxicity, and none of five patients responded. In total, three of the 13 patients had PR, an overall response rate of 23.1%. One of five patients with squamous cell carcinoma (20.0%) and two of seven with adenocarcinoma (28.6%) responded.

Phase II study

Efficacy. In the phase II study, amrubicin was administered daily for 3 consecutive days at 45 mg/m², which was the RD determined in the phase I study. The responses to amrubicin in patients with previously untreated NSCLC are shown in Table 5. Of 15 patients, four (26.7%) achieved PR. Of these responders, one patient (1/6, 16.7%) had a histology result indicating squamous cell carcinoma and three (3/8, 37.5%) had adenocarcinoma.

Table 5. Efficacy of amrubicin in phase II study

Histology	No. of patients					ORR (%)	95% CI (%)
	Total	CR	PR	NC	PD		
Adenocarcinoma	8	0	3	3	2	37.5	
Squamous cell	6	0	1	5	0	16.7	
Large cell	1	0	0	1	0	0.0	
Total	15	0	4	9	2	26.7	7.8–55.1

Abbreviation: CR, complete response; PR, partial response; NC, no change; PD, progressive disease; ORR, overall response rate (CR + PR); 95% CI, 95% confidence interval.

Table 6. Hematologic toxicity of amrubicin in phase II study

Toxicity	No. of pts.	Grade (No. of pts.)				≥ Grade 3	
		1	2	3	4	No. of pts.	%
Hemoglobin, decrease	15	4	3	3	1	4	26.7
Leukopenia	15	2	5	5	3	8	53.3
Neutropenia	15	0	4	3	8	11	73.3
Thrombocytopenia	15	0	1	3	1	4	26.7

Table 7. Non-hematologic toxicity in phase II study

Toxicity	No. of pts.	Grade (No. of pts.)				≥ Grade 3	
		1	2	3	4	No. of pts.	%
Stomatitis	15	3	0	0	0	0	0.0
Anorexia	15	8	3	0	—[a]	0	0.0
Nausea/vomiting	15	9	2	0	—[a]	0	0.0
Diarrhea	15	3	0	0	0	0	0.0
Fever	15	0	3	1	0	1	6.7
Phlebitis	15	2	0	0	0	0	0.0
Alopecia	15	4	5	0	—[a]	0	0.0
Peripheral neuropathy	15	0	1	0	0	0	0.0
ECG abnormalities	12	0	4	0	0	0	0.0
Arrhythmia	15	0	1	0	0	0	0.0
Palpitation	15	0	1	0	0	0	0.0
Pneumonia	15	0	1	0	0	0	0.0
AST, increase	15	3	0	0	0	0	0.0
ALT, increase	15	3	0	0	0	0	0.0
Total bilirubin	15	4	0	0	0	0	0.0
Proteinuria	15	1	0	0	0	0	0.0

Abbreviation: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urine nitrogen.
[a]No grading.

The two studies of phase I and II were combined, and the overall data were analyzed for response. Of 28 patients, seven achieved PR, accounting for an overall response rate of 25% (95% confidence interval, 10.7% to 44.9%). Median survival time was 9.1 months (95% confidence interval, 6.8 months to 12.1 months), and 1-year and 2-year survival rates were 35.7% (95% confidence interval, 18.0% to 53.5%) and 12.1% (0% to 24.6%), respectively.

Toxicity. Hematologic toxicity was common, as shown in Table 6. In particular, neutropenia and leukopenia developed in all patients, with grade 3 or 4 leukopenia at 53.3% and neutropenia at 73.3%. Hemoglobin decrease and thrombocytopenia were also frequently noted, but these were less severe, compared with leukopenia and neutropenia. Grade 3 or 4 hemoglobin decrease and thrombocytopenia were each observed in four patients (26.7%). Blood transfusion was required by two patients, and platelet transfusion by one.

Non-hematologic toxicity seen in the phase II study is summarized in Table 7. Stomatitis, anorexia, nausea/vomiting, diarrhea, fever and alopecia were commonly observed, but there were no grade 3 or 4 episodes except for one of grade 3 fever (6.7%). AST, ALT and total bilirubin levels, which were the referenced indices of hepatic function, were slightly increased, but no effect was seen on BUN or serum creatinine levels, the indices of renal function. There were four patients (33.3%) with abnormal ECG, showing nonspecific decreases in T-wave level without ST change. Other effects on cardiac function were palpitation and arrhythmia, occurring in one patient each. No patient had reactions such as abnormal visual system (i.e., myodesopsia), eruption, melaena, or hematemesis, all observed in the phase I study.

Discussion

The present study was performed as a 3-day consecutive administration every 3 weeks, on the basis of encouraging experimental findings that amrubicin exerted more potent antitumor activity on human tumor xenografts implanted in nude mice in the divided treatment schedule than in the single treatment schedule [9]. When given on 3 consecutive days every 3 weeks, amrubicin achieved an overall response rate of 25% (7PRs in 28 patients) in previously untreated patients with advanced NSCLC. It has been reported that amrubicin also demonstrated an overall response rate of 25% (5 PRs in 20 patients) in an early phase II study which was conducted in chemotherapy-naöve patients by single-dose intravenous injection of 120 mg/m^2 every 3 weeks [6]. The data, therefore, indicate that there was no difference in the response rate between two clinical studies conducted under different treatment schedules, but the scales were too small to evaluate which of the two treatment schedules is superior; single-dose treatment or 3-day consecutive treatment, because only 20 or 28 patients were enrolled into each study. Subsequent, larger scale clinical studies are needed for confirmation.

Currently, NSCLC is treated with newer agents such as taxanes, gemcitabine, vinorelbine, and irinotecan, in combination with cisplatin and carboplatin, and these agents have single-agent reproducible response rates of more than 20% for NSCLC [13, 14]. Amrubicin showed response rates of more than 20% in two clinical studies conducted independently and under differing treatment schedules, as described above. These reproducible results strongly suggest that amrubicin is an anticancer agent with promising single-agent activity on NSCLC, comparable to the newer agents for NSCLC in efficacy, and further clinical trials are warranted to evaluate it. In addition, amrubicin is different from other newer agents in mode of action [15], in that it is an potent inhibitor of topoisomerase II, so that amrubicin is expected to play an important role in combination therapy, differently from other agents.

The major toxicity of amrubicin was hematologic, and especially neutropenia and leukopenia were remarkable. In the phase II study, 53.3% and 73.3% of patients experienced grade 3 and 4 leukopenia and neutropenia, respectively. On the other hand, non-hematologic toxicity such as anorexia, nausea and vomiting, diarrhea, fever, and alopecia was frequently observed, but relatively mild; grade 3 or 4 episodes were not seen other than in one patient (6.7%) who experienced grade 3 fever.

As noteworthy toxicity, grade 3 melaena and grade 4 hematemesis were noted in one patient each in the phase I study, although these episodes were not observed in the clinical trials using single-bolus treatment [6, 16]. These toxicities were considered to be associated with the long-term treatment of nonsteroidal anti-inflammatory agents, because these two patients had received indomethacin or diclofenac sodium for more than 50 days. The criteria for entry into the study was therefore revised to exclude patients who had been treated with nonsteroidal anti-inflammatory agents for a long period, and thereafter such episodes have not been experienced. As uncommon toxicity, two episodes of grade 1 myodesopsia and one episode of grade 1 eruption occurred in a phase I study, but these episodes were not observed in the subsequent phase II study.

In a phase II study, 4 patients (33.3%) experienced ECG abnormality, showing nonspecific decreases in T-wave level without ST change. Other effects on cardiac function were palpitation and arrhythmia, which occurred in one patient each. All these effects seemed to be different from cardiomyopathy caused by cumulative doses of doxorubicin, but these data show that amrubicin might affect cardiac function in a different manner from doxorubicin. Therefore, careful observation might be needed concerning the effects of amrubicin on cardiac function in subsequent clinical studies.

Appendix

Amrubicin has showed reproducible response rates of 18.3% (11/60) and 27.9% (17/61) in two subsequent phase II studies when used as single agents in previously untreated patients with advanced NSCLC. Amrubicin, therefore, is considered to be comparable to newer agents such as paclitaxel, docetaxel, gemcitabine, vinorelbine, and irinotecan in efficacy for NSCLC. The clinical study of amrubicin in combination with other agents, in particular cisplatin, is currently planned.

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ORIGINAL ARTICLE

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Non-small cell lung cancer: radiation therapy for locoregional recurrence after complete resection

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Abstract
Background. We investigated patterns of failure after radical radiation therapy in relation to the radiation field in patients with postsurgical locoregional recurrence of non-small cell lung cancer.
Methods. Between 1992 and 2002, 31 patients with locoregional recurrence were treated with radiation therapy. At the time of radiation therapy, the sites of recurrence were the bronchial stump, the regional lymph nodes, the chest wall, and both the regional lymph nodes and the chest wall in 7, 20, 3, and 1 patient, respectively. The prescribed dose was 60 Gy in 30 fractions over 6 weeks in all patients.
Results. The response rate was 87%. The overall 1-year, 2-year, and 4-year Kaplan-Meier survival rates were 61%, 30%, and 15%, respectively, and the median survival time was 14 months. Locoregional relapse with or without distant metastasis occurred in 15 patients (in-field, 7; marginal, 7; out-field, 1), and distant metastasis alone occurred in 7 patients. The sites of marginal relapse were the upper margin in two patients, the ipsilateral margin in one patient, the contralateral margin in one patient, and the lower margin in three patients, respectively (in one patient, the data for marginal relapse overlapped). In all patients with relapse on the lower margin, the mediastinal lymph nodes were dissected at the initial surgery.
Conclusion. Postoperative recurrent non-small cell lung cancer showed distinctive features: the response rate was high, and the incidence of marginal relapse was also high, as in small cell lung cancer. The incidence of lower marginal relapse was high, in contrast to that in surgery-naive patients.

Key words Non-small-cell lung cancer · Radiation therapy · Surgery · Recurrence

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Introductions

Stereotactic radiotherapy is rapidly spreading as a definitive treatment for stage I non-small cell lung cancer.[1] However, until recently, surgery has been a standard treatment for patients with early stage non-small cell lung cancer. After surgery, 5%–20% of patients develop locoregional recurrence as the first site of the failure.[2–5] For locoregional recurrence, radiation therapy is the treatment of choice, and several reports have shown that 2- and 5-year survival is comparable to those for radiation therapy alone in patients with primary stage III non-small cell lung cancer.[6–8] Therefore, we have treated these patients with radical radiation therapy when possible.

To investigate the role of radical radiation therapy in this patient population, the data were reviewed for a single institution. In particular, patterns of failure in relation to the radiation field were investigated.

Patients and methods

Eligible for the current analysis were patients with locoregional recurrence of non-small cell lung cancer after curative surgery. Patients with distant metastasis or contralateral hilar lymph node metastasis were excluded from this analysis. Between 1992 and 2002, 31 eligible patients were treated with radical radiation therapy in our

Table 1. Characteristics of patients

Characteristics	Number of patients
Sex	
Male	26
Female	5
Age (median, 68 years; range, 44–83 years)	
<70 years	16
≥70 years	15
Histology	
Squamous cell carcinoma	20
Adenocarcinoma	9
Other	2
ECOG performance status	
0–1	26
2	4
3	1
Surgery	
Lobectomy	24
Pneumonectomy	6
Wedge resection	1
Recurrence site	
Stump	7
Regional lymph node	
N2	13
N3	8
Peripheral	4
Longest diameter of recurrent tumor	
8–19 mm	3
20–39 mm	14
40–59 mm	12
60–85 mm	2

ECOG, Eastern Cooperative Oncology Group
Recurrence sites overlap in one patient

institution. Oral informed consent was obtained from all patients.

Initial surgery was lobectomy in 24 patients (78%), pneumonectomy in 6 patients (19%), and wedge resection in 1 patient (3%). The mediastinal lymph nodes were dissected in 23 patients (74%). The median interval between initial surgery and radiation therapy was 15 months (range, 4–61 months).

Characteristics of the patients at the time of radiation therapy are summarized in Table 1. Recurrence was histologically diagnosed in 20 patients (65%). In other patients, obvious enlargement of the tumor was confirmed by postoperative follow-up computed tomography (CT). The sites of recurrence were the bronchial stump, the regional lymph nodes, the chest wall, or both the regional lymph nodes and the chest wall in 7 (23%), 20 (64%; N2, 12; N3, 8), 3 (10%), and 1 (3%; N2) patient, respectively. The longest diameter of the recurrent tumor, measured on CT, is also presented in Table 1.

Irradiation was performed with 10 MV photons from a linear accelerator. Lung density correction was not performed. The prescribed dose was 60 Gy in 30 fractions over 6 weeks in all patients. The radiation field contained the ipsilateral hilar lymph nodes and the mediastinal lymph nodes (from the subcarinal lymph nodes to the upper mediastinal lymph nodes) in 26 (84%) and 18 (58%) patients, respectively. Elective mediastinal irradiation was often omitted in patients with supraclavicular lymph node metastasis alone, or in patients who had undergone pneumonectomy. In these patients, the radiation field contained the recurrent tumor and margins of more than 20 mm. When the initial radiation field contained the spinal cord, off-cord (i.e., the spinal cord was outside the field) oblique boost fields were used after initial irradiation with a dose of 30 Gy or 40 Gy. Chemotherapy was performed sequentially or concurrently in five patients. The agents are listed in Table 2.

Table 2. Agents in chemotherapy

Characteristics	Number of patients
Cisplatin + vindesine	1
Gemcitabine + paclitaxel	1
Carboplatin + paclitaxel	1
Cisplatin + vinorelbine	1
Docetaxel	1

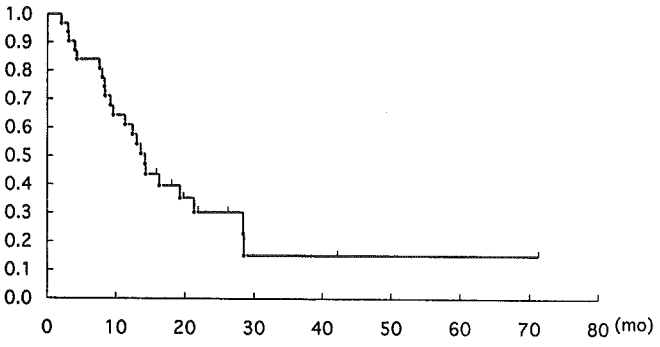

Fig. 1. Kaplan-Meier survival curve

Survival was calculated using the Kaplan-Meier method, and the differences between the curves were analyzed using the generalized Wilcoxon method. Tumor response to irradiation was evaluated with CT. A complete response (CR) was defined as 100% regression of the tumor, and a partial response (PR) was defined as more than 50% regression of the tumor, when evaluated 0–6 months after irradiation.

Results

One patient could not receive the full dose of radiation therapy owing to the presence of a bronco-esophageal fistula. The overall 1-year, 2-year, and 4-year Kaplan-Meier survival rates were 61%, 30%, and 15%, respectively, and the median survival time was 14 months (Fig. 1). The response rate was 87% (CR, 23%; PR, 64%).

The median survival times according to various prognostic factors are summarized in Table 3. The median survival time among patients with recurrence in the bronchial stump and the regional lymph nodes was 16 months and 13 months, respectively (generalized Wilcoxon, $P = 0.28$). No correlations were found between survival and extent of initial surgery, tumor histology, or radiation field.

Locoregional relapse with or without distant metastasis occurred in 15 patients, and distant metastasis alone occurred in 7 patients. Local relapse was subgrouped accord-

Table 3. Median survival time (MST) according to various prognostic factors

Factor	MST (months)	*P*
Age		
<70	16	0.06
≥70	9	
Histology		
Squamous cell carcinoma	14	0.66
Adenocarcinoma	14	
Performance status		
0–1	16	0.01
2–3	4	
Surgery		
Lobectomy	14	0.85
Pneumonectomy	14	
Recurrence site		
Stump	16	0.11
N2	9	
N3	19	
Radiation field (mediastinum)		
Yes	12	0.07
No	21	

P value for the recurrence site was between the stump and N2 lymph node metastasis

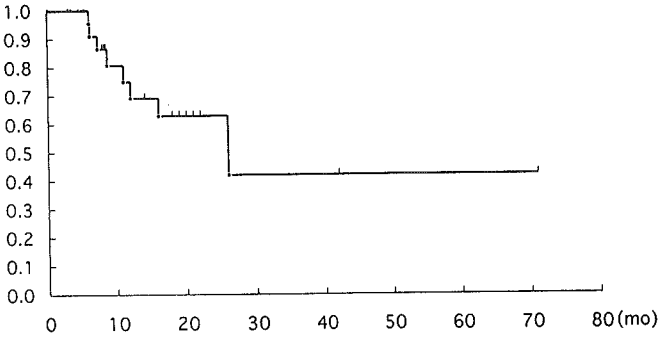

Fig. 2. Kaplan-Meier curve of the in-field control

ing to in-field relapse, marginal relapse, or out-field relapse, that is, relapse with respect to the radiation field (marginal relapse was defined as locoregional relapse at the edge of the radiation field). In-field relapse, marginal relapse, and out-field relapse occurred in seven, seven, and one patient, respectively (the out-field relapse was ipsilateral hilar lymph node metastasis; the lymph nodes had not been contained in the radiation field). The sites of marginal relapse were the upper margin in two patients, the ipsilateral margin in one patient, the contralateral margin in one patient, and the lower margin in three patients, respectively (in one patient, the data for marginal relapse overlapped). In four of the seven patients with marginal relapse, the radiation field contained the mediastinal lymph nodes. In all patients with relapse on the lower margin, the mediastinal lymph nodes were dissected at the initial surgery. The 2-year and 4-year in-field control rates were 62% and 41%, respectively (Fig. 2).

Discussion

There are several reports on the role of radiation therapy in the treatment of patients with postoperative locoregional recurrent non-small cell lung cancer. Although the number of patients was not large in the current study, the prescribed dose was uniform and patterns of recurrence in relation to the radiation field were investigated.

In surgery-naive patients, marginal relapse after radiation therapy occurred in 4% and 16% of patients with non-small cell lung cancer and with small cell lung cancer, respectively, in our institution.[9,10] However, the presented results showed that marginal relapse occurred in 23% of the patients with postoperative locoregional recurrent non-small cell lung cancer. A narrow radiation field did not cause the frequent marginal relapse since among 18 patients with the conventional radiation field, which contained the mediastinal lymph nodes, marginal relapse occurred in 22%. Furthermore, the response rate was 87%, which is higher than the usual response rate in surgery-naive non-small cell lung cancer. These features were similar rather to those of small cell lung cancer. However, causes for the distinctive features are unclear; invasively spread tumors might be specific to this population, or the nature of the tumor may have been changed by surgery.

In patients with surgery-naive small cell lung cancer, marginal relapse frequently occurs on the upper margin of the radiation field.[10] However, in the current study, the incidence of lower marginal relapse was high. In all patients with lower marginal relapse, the mediastinal lymph nodes were dissected. Therefore, a change in lymphatic circulation by surgery is considered to have caused the lower marginal relapse.

The median survival time of 14 months and the 2-year survival of 30% are comparable to results for radiation therapy alone in patients with surgery-naive locally advanced non-small cell lung cancer.[11,12] Therefore, radiation therapy is considered to play a role in the treatment of postoperative recurrent non-small cell lung cancer. However, the role of radiation therapy will be changed by progress in surgical techniques or in imaging techniques used for diagnosis, such as positron emission tomography.[13,14]

In conclusion, postoperative recurrent non-small cell lung cancer showed distinctive features: the response rate was high, and the incidence of marginal relapse was also high, similar to those of small cell lung cancer. The incidence of lower marginal relapse was high, in contrast to that in surgery-naive patients.

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ORIGINAL REPORT

Epidermal Growth Factor Receptor Gene Mutations and Increased Copy Numbers Predict Gefitinib Sensitivity in Patients With Recurrent Non–Small-Cell Lung Cancer

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Purpose
To evaluate epidermal growth factor receptor (*EGFR*) mutations and copy number as predictors of clinical outcome in patients with non–small-cell lung cancer (NSCLC) receiving gefitinib.

Patients and Methods
Sixty-six patients with NSCLC who experienced relapse after surgery and received gefitinib were included. Direct sequencing of exons 18 to 24 of *EGFR* and exons 18 to 24 of *ERBB2* was performed using DNA extracted from surgical specimens. Pyrosequencing and quantitative real-time polymerase chain reaction were performed to analyze the allelic pattern and copy number of *EGFR*.

Results
Thirty-nine patients (59%) had *EGFR* mutations; 20 patients had deletional mutations in exon 19, 17 patients had missense mutations (L858R) in exon 21, and two patients had missense mutations (G719S or G719C) in exon 18. No mutations were identified in *ERBB2*. Response rate (82% [32 of 39 patients] *v* 11% [three of 27 patients]; $P < .0001$), time to progression (TTP; median, 12.6 *v* 1.7 months; $P < .0001$), and overall survival (median, 20.4 *v* 6.9 months; $P = .0001$) were significantly better in patients with *EGFR* mutations than in patients with wild-type *EGFR*. Increased *EGFR* copy numbers (≥ 3/cell) were observed in 29 patients (44%) and were significantly associated with a higher response rate (72% [21 of 29 patients] *v* 38% [14 of 37 patients]; $P = .005$) and a longer TTP (median, 9.4 *v* 2.6 months; $P = .038$). High *EGFR* copy numbers (≥ 6/cell) were caused by selective amplification of mutant alleles.

Conclusion
EGFR mutations and increased copy numbers were significantly associated with better clinical outcome in gefitinib-treated NSCLC patients.

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INTRODUCTION

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase of the *ErbB* family that has been implicated in cell proliferation and survival and is frequently overexpressed in many solid tumors, including non–small-cell lung cancer (NSCLC). Gefitinib (Iressa; AstraZeneca, Osaka, Japan) is an orally active, selective EGFR tyrosine kinase inhibitor that binds to the adenosine triphosphate–binding pocket of the EGFR kinase domain and blocks downstream signaling pathways. Two phase II studies, IRESSA Dose Evaluation in Advanced Lung Cancer 1 and 2 (IDEAL 1 and 2), have demonstrated that gefitinib monotherapy exerts an antitumor activity in patients with advanced NSCLC who had previously received platinum-based chemotherapy.[1,2] Gefitinib was approved in Japan for the treatment of inoperable or recurrent NSCLC in July 2002.

The IDEAL trials and retrospective studies have revealed that women, never smokers, patients with adenocarcinoma, and Japanese patients have higher response rates to gefitinib.[1-4] Among patients with adenocarcinoma, histologic subtypes have been studied; one study showed that responses were more frequent in patients with bronchioloalveolar carcinoma (BAC) features (38% *v* 14%; $P < .001$),[3] whereas another study showed that the response rate was higher in patients with a papillary-dominant subtype (76% *v* 21%; $P = .002$).[5]

Although no predictive molecular markers had been identified at the time of approval, somatic mutations in the kinase domain of *EGFR* have been subsequently linked to gefitinib sensitivity. According to three initial reports, 20 of 24 gefitinib-responsive tumors contained *EGFR* mutations, whereas 19 nonresponsive tumors did not contain any mutations.[6-8] The mutations were detected in exons 18 to 21 of *EGFR*, close to the region coding the adenosine triphosphate–binding pocket of the kinase domain, and most of them were observed in two hotspots: in-frame deletions including amino acids at codons 747 to 749 in exon 19 and an amino acid substitution at codon 858 (L858R) in exon 21. Analyses of surgically resected NSCLC tumors revealed that such mutations were more frequent among women, never smokers, patients with adenocarcinoma, and Japanese or East Asian patients,[7-13] consistent with the known clinical predictors of gefitinib sensitivity.

To evaluate the exact predictive value, we studied consecutive patients with recurrent NSCLC who received gefitinib therapy. To insure high-quality genetic analyses of the archived tissues, we used methanol-fixed, paraffin-embedded surgical specimens, which are known to preserve DNA better than formalin-fixed tissues,[14] and performed laser capture microdissection (LCM).

Recently, some other biomarkers of NSCLC have been studied. The *EGFR* and chromosome 7 copy numbers in NSCLC were assessed using fluorescence in situ hybridization (FISH), and more than 3.0 *EGFR* copies per cell (balanced polysomy or gene amplification) were detected in 39 (22%) of 183 patients.[15] A correlation between an increased *EGFR* copy number and gefitinib sensitivity was also proposed in another study.[16] In yet other studies, mutations in the kinase domain of *ERBB2 (HER2)*, a gene coding another receptor tyrosine kinase of the ErbB family, were detected in 16 (3.6%) of 445 patients with lung adenocarcinoma.[17,18] In the current study, we also analyzed the *EGFR* copy number and the presence of *ERBB2* mutations to assess their impact on clinical outcome.

The expression of EGFR and related proteins has been more widely studied using immunohistochemistry. Some studies suggested that high expression of phosphorylated Akt[19,20] or low expression of phosphorylated mitogen-activated protein kinase[20,21] was associated with better outcome in gefitinib-treated patients, but in general, methods, criteria, and results were inconsistent among studies. We thought that protein expression should be analyzed in another exploratory study, and in the current study, we focused on the genetic analyses.

PATIENTS AND METHODS

Patients

After searching the pharmaceutical records of the National Cancer Center Hospital, 279 patients with NSCLC who had begun receiving gefitinib monotherapy (250 mg/d) between July 2002 and May 2004 were identified. Seventy-three of these patients had undergone surgical resection of primary NSCLC at the hospital and subsequently relapsed. Recurrences were not necessarily confirmed pathologically but were diagnosed clinically. Seven patients were ineligible for inclusion in this study because methanol-fixed tissues were not available (n = 5) or their informed consent to the genetic analysis was not obtained (n = 2); consequently, 66 patients were included.

Genetic Analyses of EGFR and ERBB2

On a protocol approved by the institutional review board of the National Cancer Center, we performed mutational analyses of exons 18 to 24 of *EGFR* and exons 18 to 24 of *ERBB2* and analyzed the *EGFR* copy number. Methanol-fixed, paraffin-embedded surgical specimens of primary NSCLC were collected retrospectively, and DNA was extracted from bulk tumor tissue, laser capture microdissected tumor tissue, and normal lung tissue from each patient. LCM was performed using a PixCell II LCM system (Arcturus Engineering Inc, Mountain View, CA) according to a previously described method.[22] If appropriate, tumor cells were captured separately from two areas with different histologic subtypes, such as an area with a BAC subtype and another area with stromal invasion. Nested polymerase chain reaction (PCR) was performed to amplify exons 18 through 24 of *EGFR* using previously described primers,[6] and standard PCR was used to amplify exons 18 through 24 of *ERBB2*. Direct sequencing of the PCR products was performed using ABI PRISM 3700 and 3100 DNA Sequencers (Applied Biosystems, Foster City, CA). All sequencing reactions were performed in both forward and reverse directions, and single nucleotide substitutions, insertions, and deletions were detected using an application program named NAMIHEI.[23] Pyrosequencing was performed to verify the sequencing data of the hotspots of *EGFR* and to assess the proportion of mutant alleles in the laser-captured tumor cells using a Pyrosequencing PSQ 96MA (Pyrosequencing, Uppsala, Sweden).[24] On the basis of the proportion of mutant alleles, *EGFR* mutations were divided into two patterns: balanced heterozygous (BH) pattern (< 60%) and mutant-allele-dominant (MD) pattern (≥ 60%). The cutoff level of 60% was decided because if more than 60%, the superiority of the mutant over the wild-type sequences was obvious on the direct sequencing chromatograms. Quantitative, real-time, TaqMan duplex PCR was performed to analyze the *EGFR* copy number using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The *EGFR* primers were 5′-GGAGGACCGTCGCTTGGT-3′ and 5′-AACACCGCAGCATGTCAAGA-3′; the probe (5′-CACCGCGACCTGGCAGCCA-3′) was labeled with the reporter dye 6-carboxyfluorescein (FAM). RNaseP was coamplified in the same reaction mixture as the endogenous reference gene using TaqMan RNaseP Control Reagents (6-carboxyrhodamine [VIC] dye; Applied

Biosystems). The average *EGFR* copy number per cell was calculated from the differences in the threshold amplification cycles between *EGFR* and RNaseP. Peripheral-blood samples obtained from healthy volunteers were analyzed as normal controls. Decreased, normal, moderately increased, and highly increased *EGFR* copy numbers were defined as less than 1.5, 1.5 to 3.0, 3.0 to 6.0, and ≥ 6.0 copies per cell, respectively.

Pathologic Evaluation

We reviewed the histologic features of the 66 patients using hematoxylin and eosin–stained slides of tumor samples. Two board-certified pathologists (K.T. and Y.M.) who were unaware of the patients' outcome and mutational status examined all the specimens independently; in case of discrepancy, final diagnoses were established by consensus. Adenocarcinoma was categorized in two ways. The first categorization was based on the WHO's classification of lung tumors,[25] which includes four major subtypes of adenocarcinoma: papillary, acinar, BAC, and solid; the dominant subtype in the total tumor mass of each case was documented. The second categorization was based on a report from the Memorial Sloan-Kettering Cancer Center,[26] in which adenocarcinomas were classified into adenocarcinoma without BAC features (Ad), adenocarcinoma with BAC features (AwBF), BAC with focal invasion (BwFI), and pure BAC (PBAC). If two or more tumors were present in one patient, the diagnosis of the most invasive tumor in each case was documented.

Radiologic Evaluation

In patients who had measurable lesions, imaging studies were performed at baseline, approximately 4 weeks after the initiation of gefitinib treatment, and periodically thereafter throughout the treatment. One board-certified radiologist (U.T.) who was unaware of the patients' mutational status reviewed the baseline, first follow-up, and confirmatory imaging studies and classified the tumor responses into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) using standard bidimensional measurements.[27] Responders were defined as patients with CR or PR. In this study, SD was subdivided into minor response (MR) and no response. MR was defined as a ≥ 25% decrease in the sum of the products of the perpendicular diameters of all measurable lesions at any point during gefitinib treatment. Time to progression (TTP) was defined as the time from the start of gefitinib administration to confirmed disease progression or death.

Statistical Analysis

The associations among mutational status, *EGFR* copy number, patient characteristics, and tumor response to gefitinib were assessed using a χ^2 test. The differences in TTP and overall survival (OS) according to the patient subgroups were compared using Kaplan-Meier curves and log-rank tests. Multivariate analyses using logistic regression models and Cox proportional hazard models were performed to assess the association between the biomarkers and clinical outcome while adjusting for the baseline patient characteristics. All analyses were performed using the SPSS statistical package (SPSS version 11.0 for Windows; SPSS Inc, Chicago, IL).

RESULTS

Patient Characteristics

The patient characteristics are listed in Table 1. All of the patients were Japanese. The proportions of women (39%), never smokers (47%), and patients with adenocarcinoma (94%) in this study were higher than those in a database of more than 1,000 patients with advanced or recurrent NSCLC treated at our hospital during the four most recent years (27%, 27%, and 73%, respectively). Twenty-two patients (33%) had been included in our phase II trial for first-line gefitinib therapy for patients with recurrent NSCLC, and the others had been treated with gefitinib in clinical practice settings. The operations for primary NSCLC were performed between February 1994 and August 2003, and the median time from the operations to the start of gefitinib was 2.3 years (range, 0.6 to 9.1 years).

Table 1. Patient Characteristics

	Patients (n = 66)	
	No.	%
Age, years		
Median	65	
Range	32-80	
Sex		
Female	26	39
Male	40	61
Smoking history*		
Never smokers	31	47
Former smokers	12	18
Current smokers	23	35
Histologic diagnosis		
Adenocarcinoma	62	94
Papillary/acinar/BAC/solid†	30/18/9/5	45/27/14/8
Ad/AwBF/BwFI/PBAC	15/45/2/0	23/68/3/0
Squamous cell carcinoma	3	5
Pleomorphic carcinoma	1	2
Performance status		
0/1	22/28	33/42
2/3	12/4	18/6
Prior chemotherapy regimens		
0	37	56
1	14	21
≥ 2	15	23

Abbreviations: BAC, bronchioloalveolar carcinoma; Ad, adenocarcinoma without BAC features; AwBF, adenocarcinoma with BAC features; BwFI, BAC with focal invasion; PBAC, pure BAC.
*Never smokers were defined as subjects who have never had a smoking habit, and former smokers were defined as subjects who had stopped smoking at least 1 year before diagnosis.
†Dominant subtype.

Clinical Outcome

Sixty-four patients had measurable lesions at the start of gefitinib administration. CR and PR were observed in two and 32 patients, respectively. MR was observed in three of nine patients with SD. Twenty-one patients had PD, including six patients who died before the first follow-up imaging studies. Two patients had only unmeasurable bone lesions at baseline; one patient showed rapid symptom improvement and continued to receive gefitinib therapy without progression for 13.8+ months, whereas the other

patient developed new lesions and died on day 71. These patients were included in the analysis as a responder and a nonresponder, respectively. The overall response rate was 53%. Forty-one patients died, and the median follow-up time for the 25 survivors was 14.6 months (range, 10.3 to 32.3 months). Eleven patients were still receiving gefitinib without progression at the time of the analysis. The median TTP and the median survival time (MST) for all patients were 5.2 and 16.3 months, respectively.

EGFR *and* ERBB2 *Mutations*

Forty-three mutations in the *EGFR* tyrosine kinase domain were detected in 39 (59%) of the 66 patients. All the mutations detected in this study are shown in Table 2. Twenty patients had deletional mutations in exon 19, and 17 patients had missense mutations (L858R) in exon 21. In exons 18 and 20, five types of missense mutations were detected. Two of them (G719S and G719C) occurred at a codon considered to be a third hotspot.[6,7,9-12] The others (L703V, E709K, and S768I) were detected in patients who also had mutations at the hotspots. Because these mutations were not detected in the normal lung tissues from the same patients, they were considered to be somatic mutations. No somatic mutations were detected in exons 22 to 24. Silent single nucleotide polymorphisms were identified at nucleotides 2361 (G/A; Q787Q), 2370 (G/A; T790T), and 2457 (G/A; V819V) in exon 20, and at nucleotide 2709 (C/T; T903T) in exon 23, but the association between these polymorphisms and the somatic mutations was not observed. In this study, no mutations and no polymorphisms were detected in exons 18 to 24 of *ERBB2*.

All 43 mutations were detected in LCM samples, but 11 (26%) of these mutations were not detected in the bulk tumor samples. In 13 patients, LCM was performed at separate areas with different histologic subtypes, but no heterogeneity was identified; the same mutations were detected in nine patients, and no mutations were detected in four patients. Mutational analyses of synchronous double lung cancers were performed in two patients; one patient had a tumor with wild-type *EGFR* and a more invasive tumor with L858R + S768I, and the other patient had a tumor with a 9-bp deletion (del L747-E749) and a more invasive tumor with a 15-bp deletion (del E746-T751insA) + L703V.

Among the 39 patients with *EGFR* mutations, the proportion of mutant alleles ranged from 29% to 94%. Nineteen patients showed a BH pattern and 20 patients showed an MD pattern.

EGFR *Copy Number*

The *EGFR* copy number in the laser-captured tumor cells ranged from 1.27 to 31.2 per cell, and increased *EGFR* copy numbers (≥ 3.0 per cell) were observed in 29 patients (44%). The relation between the copy number and the proportion of mutant alleles is shown in Figure 1. Increased copy numbers were observed more frequently in patients with *EGFR* mutations than in patients with wild-type *EGFR* (56% [22 of 39 patients] *v* 26% [seven of 27 patients]; $P = .014$). High copy numbers (≥ 6.0 per cell) were observed only in patients with an MD pattern of mutations. The copy number and the proportion of mutant alleles among patients with *EGFR* mutations was positively correlated (Spearman correlation coefficient = 0.643; $P < .001$), implying that the mutant alleles were selectively amplified in patients with an MD pattern. One patient with an MD pattern had a tumor with only approximately one copy per cell, indicating a hemizygous mutation with a loss of wild-type allele. No alterations in the gene copy number were observed in normal lung tissues.

Table 2. *EGFR* Mutations Detected in the Present Study

Exons	Amino Acids	Nucleotides	No. of Patients
19	del E746-A750	del 2235-2249	12
	del E746-A750	del 2236-2250	5
	del E746-T751insA	del 2237-2251	1
	del L747-E749	del 2239-2247	1
	del E746-S752insV	del 2237-2255 + ins T	1
21	L858R	T → G at 2573	17
18	G719S	G → A at 2155	1
	G719C	G → T at 2155	1
	L703V	C → G at 2107	1*
	E709K	G → A at 2125	1†
20	S768I	G → T at 2303	2‡

Abbreviations: *EGFR*, epidermal growth factor receptor; del, deletion; ins, insertion.
*A patient with del E746-T751insA.
†A patient with L858R.
‡A patient with L858R and a patient with G719C.

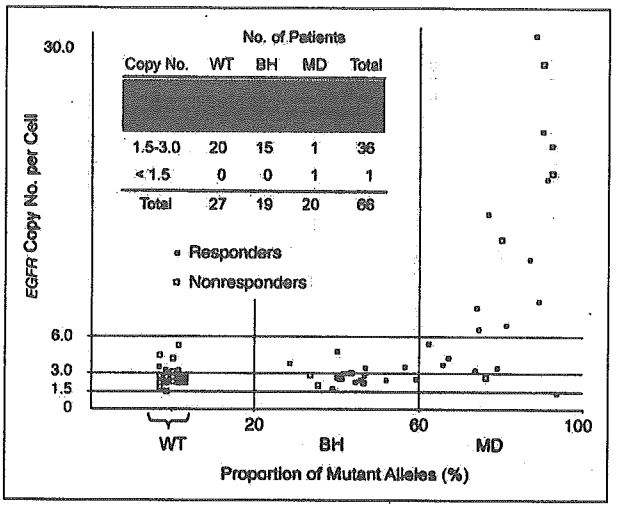

Fig 1. Relation between the epidermal growth factor receptor (*EGFR*) copy number and the proportion of mutant alleles. WT, patients with wild-type *EGFR*; BH, patients with a balanced heterozygous pattern of *EGFR* mutations; MD, patients with a mutant-allele-dominant pattern of *EGFR* mutations.

EGFR *Mutations,* EGFR *Copy Number, and Clinical Outcome*

The tumor responses to gefitinib according to the mutational status of *EGFR* are shown in Table 3. The response rates of patients with mutant and wild-type *EGFR* were 82% and 11%, respectively ($P < 10^{-7}$). Seven patients with *EGFR* mutations were nonresponders; three patients had PD at 0.3 (early death), 2.3, and 2.3 months, and four patients had SD. Three of the four patients with SD had MR (TTP, 2.5, 5.2, and 6.9 months), and the other patient continued to receive gefitinib therapy without progression for 24.2 months, whereas all SD tumors with wild-type *EGFR* progressed within 5 months without MR. Meanwhile, three patients with wild-type *EGFR* exhibited PR, and two of these patients were still receiving gefitinib therapy without progression at 10.9+ and 21.1+ months. The Kaplan-Meier plots of TTP and OS according to the presence of the *EGFR* mutations are shown in Figures 2 and 3, respectively. Patients with *EGFR* mutations had a significantly longer TTP and OS compared with those with wild-type *EGFR*.

Univariate analyses were performed to assess the correlations among patient characteristics, *EGFR* mutations, *EGFR* copy number, and clinical outcome (Tables 4 and 5). The response rates were significantly higher in women, never/former smokers, and patients with BAC features and were marginally higher in patients with a papillary-dominant subtype. The response rates among these subgroups were approximately consistent with the rates of *EGFR* mutations. An increased *EGFR* copy number was also significantly associated with a higher response rate and a longer TTP.

The results of multivariate analyses among 62 patients with adenocarcinoma are shown in Table 6. The presence of *EGFR* mutations was strongly associated with a higher response rate, a longer TTP, and a longer OS. An increased *EGFR* copy number was also a significant or marginally significant predictor of a higher response rate and a longer TTP. These results did not change substantially if any combinations of variables were included in the models.

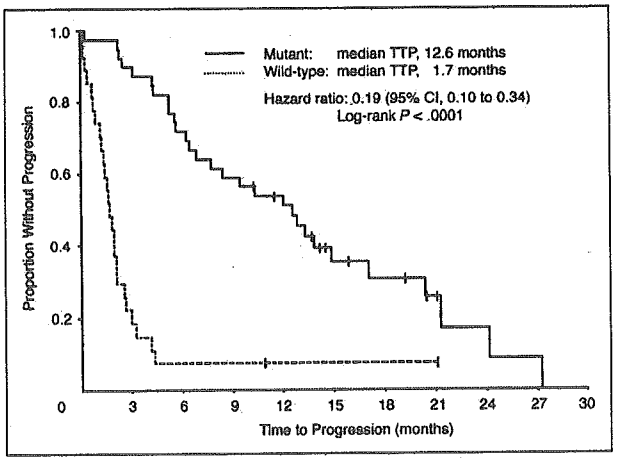

Fig 2. Kaplan-Meier plot of time to progression (TTP) according to epidermal growth factor receptor (*EGFR*) mutation status.

Among patients with wild-type *EGFR*, TTP was significantly longer in patients with increased *EGFR* copy numbers (median, 3.0 *v* 1.4 months; log-rank $P = .021$), and both of the two long-term responders had tumors with moderately increased *EGFR* copy numbers (3.20 and 3.45/cell). Among patients with *EGFR* mutations, TTP and OS were not significantly different according to the types of mutations, the presence of additional mutations, the proportion of mutant alleles, or the *EGFR* copy number (data not shown).

DISCUSSION

This study strongly implies that the mutational status of *EGFR* is a major determinant of gefitinib sensitivity in patients with NSCLC. The response rate was 82%, the median TTP was 12.6 months, and the MST was 20.4 months in gefitinib-treated patients with *EGFR*-mutant NSCLC. *EGFR* mutations might be a good prognostic factor independent of treatment, but these remarkable results suggest a

Table 3. *EGFR* Mutations and Tumor Response to Gefitinib

	Responders		Nonresponders			Responders/Total Patients	Response Rates (%)
	CR	PR	MR	SD	PD		
Mutant	2	30*	3	1	3†	32/39	82
DEL	0	18*	2	0	0	18/20	90
L858R	2	11	1	1	2†	13/17	76
G719	0	1	0	0	1	1/2	50
Wild-type	0	3	0	5	19	3/27	11
Total	2	33	3	6	22	35/66	53

Abbreviations: *EGFR*, epidermal growth factor receptor; CR, complete response; PR, partial response; MR, minor response; SD, stable disease without MR; PD, progressive disease; DEL, deletional mutations in exon 19; G719, G719S, or G719C.
*Including a clinical responder without measurable lesions.
†Including a patient who had no measurable lesions at baseline.

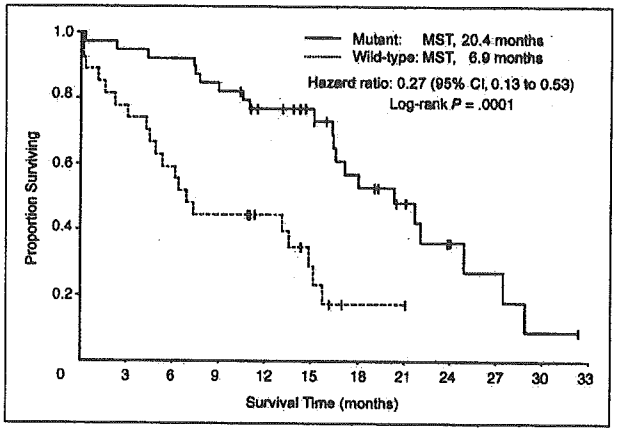

Fig 3. Kaplan-Meier plot of overall survival according to epidermal growth factor receptor (*EGFR*) mutation status. MST, median survival time.

survival benefit from gefitinib therapy in patients with *EGFR* mutations. Four of seven nonresponders with *EGFR* mutations also seemed to experience some clinical benefits because they had MR or a long SD (≥ 6 months). Among nine patients with SD, MR, or a long SD was observed only in patients with *EGFR* mutations. Although the sample size was too small to draw a firm conclusion, this finding suggests that *EGFR* mutations are also associated with clinical benefits in SD.

Table 4. *EGFR* Mutations Among Patient Subgroups

	EGFR Mutations		
	No. of Patients	%	*P*
Total	39/66	59	
Sex			.18
Female	18/26	69	
Male	21/40	53	
Smoking history			.003†
Never smokers	21/31	68	
Former smokers	10/12	83	
Current smokers	8/23	35	
Histologic diagnosis			—
Adenocarcinoma	38/62	61	
Squamous cell carcinoma	0/3	0	
Pleomorphic carcinoma	1/1	100	
Dominant subtype*			.059‡
Papillary	22/30	73	
Acinar	10/18	56	
BAC	5/9	56	
Solid	1/5	20	
BAC features*			.002
Yes	34/47	72	
No	4/15	27	

Abbreviations: *EGFR*, epidermal growth factor receptor; BAC, bronchioloalveolar carcinoma.
*Only patients with adenocarcinoma (n = 62).
†Comparison between never/former smokers and current smokers.
‡Comparison between patients with papillary-dominant adenocarcinoma and patients with other adenocarcinoma.

The *EGFR* mutations detected in this study were concentrated in three hotspots, deletions around codons 747 to 749, L858R, and G719S (or G719C), similar to the results of previous reports.[6-13] Some genetic variations existed among these mutations. Together with one of the hotspot mutations, additional missense mutations in exons 18 or 20 were detected in four patients. Among the 39 patients with *EGFR* mutations, an MD pattern was observed in 20 patients. Because the *EGFR* copy number in their tumor cells increased as the proportion of mutant alleles increased, this pattern was assumed to be caused not by homozygous mutations but by the selective amplification of the mutant alleles. Because one patient had a hemizygous mutation without amplification, the loss of wild-type alleles was also thought to be responsible for the pattern. The moderately increased copy number in patients with a BH pattern or wild-type *EGFR* can be explained by *EGFR* amplification and/or polysomy of chromosome 7.

Among the patients with *EGFR* mutations, three patients had PD and eight of the other 36 patients had tumor regrowth within 6 months. This suggests the presence of other factors associated with intrinsic or acquired resistance to gefitinib. Although any genetic alterations of *EGFR*-mutant tumors at the time of primary surgery were not significantly associated with clinical outcome, that might be because further alterations occurred after the primary surgery or after gefitinib administration. Recently, a secondary mutation (C → T at nucleotide 2369; T790M) in exon 20 was detected in patients with *EGFR*-mutant NSCLC who had tumor regrowth during gefitinib therapy after exhibiting an initial response to the agent; this mutation was thought to be associated with acquired resistance.[28,29] To elucidate the determinants and the mechanism of resistance to gefitinib, genetic analyses of tumor samples obtained after gefitinib treatment are needed.

In this study, three (11%) of the 27 patients with wild-type *EGFR* responded to gefitinib. Various explanations for this result are possible: (1) the mutational analyses of the responders were false-negative, (2) the *EGFR* mutations occurred in their tumors after the primary surgery, (3) the recurrent tumors originated from a source other than the analyzed tumor cells, or (4) other determinants of gefitinib sensitivity were present.

The results of multivariate analyses suggest that the *EGFR* copy number is another independent predictor of gefitinib sensitivity. It is noteworthy that an increased *EGFR* copy number was observed in two of the three responders with wild-type *EGFR*, and was significantly associated with a longer TTP among patients with wild-type *EGFR*. Because patients with *EGFR* mutations had favorable clinical outcome regardless of *EGFR* copy numbers, the impact of increased copy numbers on *EGFR*-mutant NSCLC was unclear. In the overall population, an increased *EGFR* copy number was significantly associated with a higher response

Table 5. Clinical Outcome Among Patient Subgroups (univariate analyses)

	Response Rate			Time to Progression		Overall Survival	
	No.	%	*P*	Median (months)	Log-Rank *P*	Median (months)	Log-Rank *P*
Total	66	53		5.2		16.3	
Sex			.033		.35		.30
Female	26	69		6.2		16.5	
Male	40	43		3.3		15.1	
Smoking history			.007		.026		.37
Never/former smokers	43	65		6.9		16.4	
Current smokers	23	30		2.6		15.1	
Dominant subtype*			.070		.28		.65
Papillary	30	67		7.7		16.4	
Others	32	44		4.2		15.7	
BAC features*			.012		.12		.19
Yes	47	64		6.5		16.5	
No	15	27		2.1		15.7	
Performance status			.77		.012		< .0001
0-1	50	52		5.2		17.1	
2-3	16	56		3.1		6.1	
EGFR mutations			< .0001		< .0001		.0001
Yes	39	82		12.6		20.4	
No	27	11		1.7		6.9	
EGFR copy number			.005		.038		.33
≥ 3.0	29	72		9.4		16.4	
< 3.0	37	38		2.6		15.7	

Abbreviation: BAC, bronchioloalveolar carcinoma; *EGFR*, epidermal growth factor receptor.
*Only patients with adenocarcinoma (n = 62).

rate and a longer TTP, but not with a longer OS, which might be because an increased copy number had an unfavorable impact on prognosis, as suggested by another study.[15] In chronic myeloid leukemia, as well as *BCR-ABL* mutations that were structurally corresponding to T790M in *EGFR*, an increased *BCR-ABL* gene copy number was reported as a determinant of resistance to imatinib, a BCR-ABL tyrosine kinase inhibitor.[30] Therefore, we should consider the possibility that an increased *EGFR* copy number is associated with not only sensitivity but also resistance to gefitinib.

Among adenocarcinomas, the presence of BAC features was significantly associated with gefitinib sensitivity and *EGFR* mutations, but the BAC component was relatively small in most of the responders. The dominant subtype associated with a higher response rate was not BAC but papillary; both of the two patients with BwFI had PD, and all three patients with pure papillary adenocarcinoma without BAC features had PR. The association between pathologic features and gefitinib sensitivity or *EGFR* mutations is also the subject of further investigation.

Table 6. Univariate and Multivariate Analyses of the Association Between Biomarkers and Clinical Outcome in Patients With Lung Adenocarcinoma (n = 62)

	Odds Ratios for Response		Hazard Ratios for TTP		Hazard Ratios for OS	
	Univariate	Multivariate*	Univariate	Multivariate*	Univariate	Multivariate*
EGFR mutations, yes *v* no	31.0	27.9	0.21	0.13	0.30	0.16
95% CI	7.2 to 134	3.7 to 209	0.11 to 0.38	0.06 to 0.29	0.15 to 0.62	0.06 to 0.39
P	< .001	.001	< .001	< .001	.001	< .001
EGFR copy number, ≥ 3.0 *v* < 3.0	4.0	4.6	0.57	0.42	0.80	0.59
95% CI	1.4 to 12	0.84 to 25	0.32 to 1.0	0.21 to 0.84	0.42 to 1.5	0.26 to 1.4
P	.011	.079	.050	.014	.49	.22

Abbreviations: TTP, time to progression; OS, overall survival; *EGFR*, epidermal growth factor receptor.
*In the multivariate analyses, age (continuous variable), sex (women *v* men), smoking history (never/former smokers *v* current smokers), dominant subtype (papillary *v* others), bronchioloalveolar carcinoma features (yes *v* no), performance status (0 to 1 *v* 2 to 3), prior chemotherapy (yes *v* no), *EGFR* mutations (yes *v* no), and *EGFR* copy number (≥ 3.0 *v* < 3.0) were included as factors.

In never/former smokers, both the *EGFR* mutation rate and the response rate were significantly higher than in current smokers. We speculate that *EGFR* mutations occur equally throughout the entire population, regardless of smoking history, and account for smoking-unrelated carcinogenesis. Because many other genetic alterations, like *KRAS* mutations, occur and induce lung adenocarcinoma more frequently in smokers, the *EGFR* mutation rate seems to be relatively lower in smokers with lung adenocarcinoma.

The response rate of 53% and the *EGFR* mutation rate of 59% observed in this study were higher than previously reported rates. These results can partially be attributed to the fact that the physicians tended to select patients with characteristics known to be predictive for gefitinib sensitivity: women, never-smokers, and patients with adenocarcinoma. Consequently, this cohort was not necessarily representative of unselected NSCLC populations in Japan. However, other recent studies have also shown relatively high frequencies (32% to 55%) of *EGFR* mutations in Japanese or East Asian patients with lung adenocarcinoma who underwent surgical resection.[7,9-11,13] The reason why such somatic mutations occur selectively in East Asian people remains unknown. Environmental or genetic factors common among East Asian populations should be investigated to answer this question.

Recently, no significant survival benefit of gefitinib was reportedly observed in the initial analysis of the IRESSA Survival Evaluation in Lung Cancer (ISEL) trial, a phase III trial comparing gefitinib monotherapy to a placebo as a second- or third-line treatment for patients with advanced NSCLC.[31] Because subgroup analyses of the trial suggested survival benefits in never smokers or Asian patients, the selection of patients is thought to be crucial when considering gefitinib treatment. Because the present study showed that the *EGFR* mutation status is a major determinant of gefitinib sensitivity, mutational analyses in patients with advanced NSCLC should be considered before deciding on a course of treatment.

In this study, we performed LCM and direct sequencing using methanol-fixed surgical specimens to obtain high-quality data. If we had analyzed only bulk tumor samples without LCM, nine of the 39 patients with *EGFR* mutations would have been misjudged as having wild-type *EGFR*. Thus such procedures with LCM are presently recommended for the detection of *EGFR* mutations. However, obtaining appropriate tumor samples is often difficult in patients with advanced NSCLC, and performing LCM and direct sequencing in all patients is not practical. Thus more practical methods for detecting the major *EGFR* mutations using small tumor samples contaminated with normal tissue should be developed and validated.

Other than *EGFR* mutations, some candidate predictive biomarkers have been studied. The *EGFR* copy number is the leading candidate, and it can also be detected by FISH. Practicality and accuracy should be assessed comparing FISH and quantitative real-time PCR. The impact of *ERBB2* mutations on clinical outcome remains to be investigated because we could not detect any mutations in *ERBB2* in the present study. Protein expression analyses by IHC are easier to perform than the genetic analyses, but their significance is still controversial. Further studies are required to evaluate the predictive values of these biomarkers and to determine whether they are independent predictors of gefitinib sensitivity or surrogate markers of *EGFR* mutations.

In conclusion, this study indicates that *EGFR* mutations and increased copy numbers predict better clinical outcome in patients with NSCLC treated with gefitinib. Further research and clinical trials are needed to incorporate these markers into clinical practice appropriately.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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