Table 2 Common ADRs occurring in two or more patients

AE (MedDRA term)[a]	Gefitinib 250 mg/day (*n*=18)	Placebo (*n*=20)
Abnormal hepatic function	4	0
Acne	2	0
Anorexia	5	1
Cough	2[b]	1
Diarrhea	9	2
Dry skin	3	0
Eczema	8	2
Elevated ALT/AST	2	0
Fatigue	2	0
Gastritis	3[b]	0
Loose stools	4	0
Nausea	3	0
Rash	5	3
Sputum	0	2
Stomatitis	2	0

[a]A patient could have more than one AE.
[b]All were associated with post-operative complications.

Table 3 Grade 3/4 ADRs

AE (MedDRA term)	Grade	Gefitinib 250 mg/day (*n*=18)	Placebo (*n*=20)
Abnormal hepatic function	3	1	0
Eczema	3	1	0
Elevated ALT	3	1	0
Neutropenia	3	0	1
Pneumonitis	4	1	0

Table 4 Exposure of patients to gefitinib

	Gefitinib 250 mg/day (*n*=18)	Placebo (*n*=20)
Median duration of treatment [days (range)]	86.5 (4–195)	144.0 (20–197)
Dosing period (*n*)		
<60 days	6	2
60–120 days	9	4
≥ 120 days	3	14
No. dose interruptions (*n*)		
1	5	6
2	2	2
≥ 3	2	2

Table 5 ADRs leading to patient withdrawals

Adverse event (MedDRA term)	Grade	Gefitinib 250 mg/day (*n*=18)	Placebo (*n*=20)
Eczema	2	1	0
Elevated ALT/AST	2	1	0
	3	1	0
Hepatic function abnormalities	2	1	0
	3	1	0
ILD	1	0	1
Impetigo	2	1	0
Neutropenia	3	0	1
Paronychia	2	1	0
Pneumonitis	4	1	0
Pulmonary fibrosis	1	0	1

and one patient experienced grade 4 ILD-type events (pneumonitis) 107 days after starting gefitinib and was withdrawn from the study. The patient with pneumonitis had taken concomitant shosaikoto, a Chinese herbal medicine, and loxoprofen, both of which have previously been shown to induce pneumonitis [15,16]. Twenty-one days later bacterial pneumonia related to methylprednisolone therapy was diagnosed, and the patient subsequently died 37 days later due to both pneumonitis and bacterial pneumonia. In the placebo arm, one patient who experienced cough and grade 1 pulmonary fibrosis had had interstitial changes on their chest X-ray at enrollment, and in a second patient, pre-existing non-specific interstitial pneumonia was exacerbated resulting in grade 1 ILD. In both patients, these conditions persisted following withdrawal of study drug.

Interruptions and withdrawals due to ADRs

ADRs requiring interruptions in therapy were similar between patients receiving gefitinib or placebo (Table 4) and were usually for less than 14 days, although four patients in the gefitinib arm required treatment to be interrupted for 14 days (including one patient whose treatment was interrupted for 20 days). The majority of ADRs leading to withdrawal were usually mild-to-moderate grade 1/2 in severity (Table 5). Grade 3 ADRs leading to withdrawal occurred in two patients receiving gefitinib (hepatic function abnormalities, elevated ALT) and in one patient receiving placebo (neutropenia), and grade 4 pneumonitis led to the withdrawal of one patient who was receiving gefitinib. Following withdrawal of gefitinib treatment, grade 3 abnormal hepatic function and elevated ALT resolved, and grade 3 neutropenia persisted.

AEs associated with post-operative complications

As there are no safety data regarding the use of gefitinib in the post-operative setting, AEs associated with the healing process were examined to provide preliminary safety data on the start of the dosing timing in the adjuvant setting for gefitinib. AEs related to post-operative complications were observed in six patients in the gefitinib arm and four patients in the placebo arm. In the gefitinib arm, the most frequent AEs were grade 1/2 cough (four patients) and gastritis (three patients), and in the placebo arm grade 1/2 pain (three patients). Grade 1 cough, grade 1 supraventricular arrhythmia and grade 2 dyspnea were also experienced by three out of four patients receiving placebo.

Discussion

This trial was designed to compare survival rates in patients with completely resected stage IB–IIIA NSCLC who had received adjuvant therapy with gefitinib 250 mg/day or placebo. However, incidences of ADRs of ILD-

type events in the advanced disease setting have been increasingly reported since gefitinib was launched in Japan, and new recruitment was put on hold on 23 October 2002 at the request of the Ministry of Health, Labor and Welfare. In order to evaluate the ILD and ensure the safety of the trial patients, two separate Co-ordination Committee and IDMC meetings (December 2002 and January 2003) were conducted to discuss the feasibility of continuing the study and management of the trial patients. Based on the updated information on ADRs of interstitial pneumonia, the committees concluded that the study could be continued because the possibility of risk did not exceed that of benefit to enrolled patients. The IDMC also suggested that top priority should be given to assure the safety of the patients receiving gefitinib, and that discontinuation should be considered if flu-like symptoms including difficulty in breathing, fever and coughing occurred.

A 'Supplemental Explanation Sheet and Informed Consent Form' was provided four times to enrolled patients, offered updated information and methods to assure and manage any safety issues, and confirmed the patients' willingness to continue participating in the study. In December 2002, AstraZeneca KK gave the principal investigators the option to suspend gefitinib treatment at once. With the extensive monitoring of the trial patients in terms of safety, there were still an increasing number of withdrawals. In addition, enrollment could not be resumed until the prospective investigation on gefitinib-related ILD was completed. Based on these facts, the sponsor finally decided to terminate the trial in March 2003.

The types of AEs reported in this trial were similar to that already reported in the large phase II IDEAL 1 and 2 trials for patients with locally advanced or metastatic NSCLC [10,11]. Three patients experienced ILD-type events – two in the placebo arm and one patient in the gefitinib arm (this patient was also taking two other medications known to induce ILD) [15,17]. It has generally been observed that a higher frequency of ILD-type events are reported in Japanese patients taking gefitinib compared with those in other south-east Asian countries and the rest of the world (1.6, 0.3, and 0.3%, respectively) [18]. The occurrence of ILD in Japanese patients and the reasons for such an ethnic stratification in ILD incidence following gefitinib treatment require further clarification.

The most common reason for withdrawal in both treatment arms was due to toxicity, with the majority of drug-related AEs being grade 1/2 in severity. In the advanced or metastatic disease setting, few patients who experience grade 1/2 drug-related AEs withdraw from treatment with gefitinib, and in IDEAL 1, which recruited Japanese patients, two out of 103 patients who received gefitinib 250 mg/day withdrew from therapy due to ADRs [18]. Several factors may explain the high number of withdrawals (including withdrawal of treatment for less severe ADRs) reported in this trial data compared with previously reported studies. These reasons include the fact that patients with early-stage NSCLC may be less tolerant of AEs compared with patients with advanced NSCLC who have received prior chemotherapy. In contrast to the other studies, the impact of heavy media coverage surrounding gefitinib-related ILD cannot be ignored.

It has been suggested that the dosage and schedule of gefitinib used in this study may not best suit patients with completely resected NSCLC in terms of tolerability and a number of adjustments may need to be taken into consideration when planning an adjuvant study of gefitinib in the future. It is unlikely that the time frame of 4–6 weeks is too short before starting adjuvant treatment, as other adjuvant trials conducted in Japanese patients have used similar time frames [3,4]. It may be possible to lengthen the duration by which gefitinib could be interrupted for toxicity, since 14 days may be too short for patients recovering from AEs such as hepatic enzyme elevation, or to reduce the dose following toxicity to perhaps 250 mg every other day, although this would require further study into the efficacy of such an approach.

With no experience of using gefitinib in post-operative patients there was a concern that EGFR-TKIs might impact on surgery-related complications (especially on the healing process) due to their mode of action. In order to assess this, the trial was designed to allow a safety review of the first 60 patients. Due to the early termination of the study, we have only 38 patients' (18 on gefitinib) data for review; however, there does not seem to be any impact on surgery-related complications when gefitinib was administered within 4–6 weeks after surgery, as evidenced by a similar number of these AEs that occurred in both groups. This indicates that it may be feasible to administer gefitinib in the adjuvant setting within this time frame.

In conclusion, this is the first study to investigate the use of EGFR-TKIs as adjuvant therapy. Despite the absence of survival data, there were no unexpected AEs seen in the adjuvant setting compared with those already reported for patients with locally advanced or metastatic NSCLC. However, it was observed that there were more AEs leading to withdrawal in the gefitinib arm, even though the majority of AEs were grade 1/2 in severity, suggesting that a daily dose of gefitinib 250 mg may not best suit patients with completely resected NSCLC in terms of tolerability.

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Phase I Study of Irinotecan and Amrubicin in Patients with Advanced Non-Small-Cell Lung Cancer

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Abstract. *Background: Combination chemotherapy of irinotecan and amrubicin for advanced non-small cell lung cancer (NSCLC) has not been fully evaluated. To determine the maximum-tolerated dose (MTD), a phase I study in patients with advanced NSCLC was conducted. Materials and Methods: Patients with stage IIIB/IV NSCLC were enrolled in this study. Both patients with and without prior chemotherapy were also eligible. The drugs were administered on days 1 and 8, every 3 weeks. The starting doses of irinotecan and amrubicin were 60 and 35 mg/m^2, respectively. Results: Nineteen patients received a total of 53 courses. Grade 4 neutropenia was observed in 23% of courses. Anaemia and thrombocytopenia were generally mild. Grade 3 febrile neutropenia occurred in 5 courses. Other grade 3 or greater non-haematological toxicities were observed in only 4 out of 52 courses (grade 3 infection and hyponatremia). The maximum-tolerated doses (MTDs) of irinotecan and amrubicin were 100 and 45 mg/m^2, respectively. Objective response was obtained in 2 patients (10.5%), who had received prior chemotherapy. Conclusion: This combination was well tolerated, but produced only a modest anti-tumour effect for advanced NSCLC. Further investigation into the role of this regimen as a salvage chemotherapy may be warranted in relapsed patients.*

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Key Words: Irinotecan, amrubicin, non-small cell lung cancer, phase I study, non-platinum regimen, topoisomerase inhibitor.

Lung cancer is the leading cause of cancer deaths in many countries (1). Although cisplatin-based chemotherapy has been conducted extensively in patients with advanced non-small cell lung cancer (NSCLC) over the past two decades, the survival benefit remains modest and further improvement of treatment outcome is needed (2). Recently, several new agents with novel mechanisms have been developed and have shown to be highly effective for NSCLC (3). Irinotecan, a unique semi-synthetic derivative of camptothecin, is a topoisomerase I inhibitor shown to have favourable anti-tumour activity for advanced NSCLC as a single agent, with a response rate of 21-32% (4, 5). Amrubicin, a totally synthetic anthracycline, is a topoisomerase II inhibitor and also effective for NSCLC as a single agent, with a response rate of 25% (6), although other anthracyclines are now considered to have little benefit in the treatment of advanced NSCLC. Combined use of topoisomerase I and II inhibitors has been demonstrated to be complementary in preclinical studies (7, 8). The toxicity profiles of these two drugs are different (4-6). Although several large randomized phase III studies have been conducted to compare survival, response and toxicity from a platinum-based doublet containing a single new agents to a non-platinum-based doublet consisting of two new agents, it has not yet been determined which is more effective (9). Thus, there is still room for investigation of non-platinum regimens.

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Table I. *Planned dose level.*

Dose level	Irinotecan (mg/m^2)	Amrubicin (mg/m^2)
1	60	35
2	80	35
3	80	40
4	100	40
5	100	45
6	100	50

Table II. *Patient characteristics.*

Number of patients		19
Age	median (range)	67 (48-74)
Gender	male	12
	female	7
Performance status	0 / 1	11 / 8
Histology	adenocarcinoma	16
	squamous cell carcinoma	3
Stage	IIIB	2
	IV	7
	postoperative recurrence	10
No. of prior chemotherapy regimens		
	0 / 1 / 2 / 3	12/ 6 / 0 / 1

Based on such a background, a phase I study of combination chemotherapy in patients with advanced NSCLC was designed. The primary objective was to determine the maximum-tolerated dose (MTD) for each drug, with a secondary objective of assessing anti-tumour activity.

Patients and Methods

Eligibility criteria. Patients were required to fulfil the following eligibility criteria: pathologically proven, advanced and inoperable NSCLC; Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0, 1, or 2; age ≤75 years; presence of evaluable lesions; adequate reserves of haematological function (white blood cell (WBC) count >4000 /μL, neutrophil count >2000 /μL, haemoglobin level >9.5 g/dL, platelet count >10x10^4 /μL), renal function (serum creatinine <1.5 mg/dL), hepatic function (total bilirubin <1.5 mg/dL, serum transaminases <2.5x upper limit of normal range) and pulmonary function (PaO_2 ≥60 Torr); and acquisition of written informed consent. Both patients with and without prior chemotherapy were included. Patients with symptomatic brain metastasis were excluded from the study. Baseline pretreatment evaluations included a complete history, physical examination, laboratory tests, chest radiograph, electrocardiogram, computed tomography (CT) scans of the chest and abdomen, magnetic resonance imaging (MRI) of the brain, and a radionuclide bone scan. Staging was assessed according to the tumour, node and metastasis system (10). The protocol was approved by the institutional review board of each participating institute.

Treatment schedules. Amrubicin, diluted in 20 mL physiological saline, was intravenously administered over 5 minutes on days 1 and 8. Soon after completion of amrubicin infusion, irinotecan, diluted in 250 mL physiological saline, was intravenously administered over 1 hour on the same days. Each patient was premedicated with intravenous administration of dexamethasone (8 mg) and granisetron (3 mg) 30 minutes before bolus infusion of amrubicin. Treatment was repeated every 3 weeks, with 5 dose levels planned. The starting doses of irinotecan and amrubicin were 60 and 35 mg/m^2, respectively, which were two-thirds of the recommended doses as single agents (Table I). Administration of irinotecan and amrubicin on day 8 was delayed until day 15 if haematological toxicity of grade 3 or greater, non-haematological toxicity of grade 2 or greater, or diarrhoea was observed on the day of administration. If these toxicities did not improve by day 15, this administration was cancelled in that course. Patients were treated with at least 2 courses of chemotherapy unless there was disease progression, unacceptable toxicity, or withdrawal of informed consent. Initiation of the next course of chemotherapy was delayed until recovery of WBC count to 3000 /μL or greater, neutrophil count to 1500 /μL or greater, platelet count to 10x10^4 /μL or greater, and resolution of non-haematological toxicities to grade 1 or less.

Assessment of toxicity and dose escalation. All toxicities were graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC, Version 2.0). Dose-limiting toxicity (DLT) was defined as development of at least one of the following adverse events: any non-haematological toxicity of grade 3 or greater, except alopecia, nausea, or vomiting; platelet count <2x10^4 /μL; grade 4 leukopenia; grade 4 neutropenia lasting for 4 days or longer; cancellation of irinotecan and amrubicin administration on day 8; or inability to begin the next course of treatment by day 56 due to failure to recover from toxicity.

For each dose level, 3 or 6 patients were scheduled to enter. If fewer than 2 out of 3 or 3 out of 6 patients experienced DLT, the next group of patients was treated at the next higher dose level. MTD was defined as the dose level that produced any DLT in 3 or more patients out of a maximum of 6 patients, and further dose escalation was not permitted. All treatment courses were analysed to determine DLT and MTD, although the decision to elevate dose level was based on toxicity in the first course. Dose escalation above starting doses in an individual patient was not allowed. The recommended dose was defined as the dose level below MTD. If grade 4 leukopenia, grade 4 neutropenia, or febrile neutropenia were observed, use of granulocyte colony-stimulating factor was permitted. The dose could be reduced in subsequent courses if patients experienced DLT in the previous course.

Table III. *Haematological toxicity of grade 2 or greater (all courses).*

		Dose level				
Toxicity	Grade	1	2	3	4	5
No. of treated patients		3	3	3	6	4
No. of courses evaluated		4	5	8	31	5
No. (%) of courses encountered						
Leukopenia	2	2 (50%)	2 (40%)	3 (38%)	9 (29%)	0
	3	2 (50%)	0	0	5 (16%)	4 (80%)
	4	0	0	0	0	1 (20%)
Neutropenia	2	0	3 (60%)	1 (13%)	9 (29%)	0
	3	4 (100%)	0	3 (38%)	8 (26%)	1 (20%)
	4	0	1 (20%)	0	7 (23%)	4 (80%)
Thrombocytopenia	2	0	0	0	1 (3%)	0
	3	0	0	0	1 (3%)	0
	4	0	0	0	0	0
Anaemia	2	1 (25%)	4 (80%)	0	3 (10%)	2 (40%)
	3	0	0	0	0	0
	4	0	0	0	0	0

Assessment of anti-tumour activity. Standard Response Evaluation Criteria in Solid Tumours (11) was used to evaluate responses. The best overall response was defined as the best response recorded from the start of treatment until disease progression or recurrence.

Results

Patient characteristics and treatment delivery. Nineteen patients with advanced NSCLC were enrolled between May, 2003 and January, 2004 in 4 institutes. The patient characteristics are listed in Table II. A total of 53 courses were evaluated, with a median number of 2 courses (range: 1 to 8). Eight patients (42%) received only 1 course of chemotherapy, because of disease progression in 6 patients, patient refusal and unacceptable toxicity (1 patient each). The median total delivered dose of amrubicin was 140 mg/m^2, ranging from 70 to 640 mg/m^2. Administration of irinotecan and amrubicin on day 8 was delayed for 1 week in 1 course at dose level 4 because of diarrhoea. All patients and courses were assessable for safety.

Haematological toxicity. Grade 4 neutropenia occurred in 12 (23%) out of 53 courses (Table III). In 2 courses, grade 4 neutropenia continued for 4 and 6 days, and grade 4 leukopenia continued for 4 days in 1 course despite G-CSF support, but they were not accompanied by any febrile episodes. Anaemia and thrombocytopenia were relatively mild, and no transfusions were required.

Non-haematological toxicity. Non-haematological toxicity was generally mild and no patient experienced grade 4 or greater toxicitiy (Table IV). Febrile neutropenia occurred in 5 (9%) out of 53 courses. Grade 3 infection occurred in 3 out of 53 courses; 2 in the first course, on day 4 at dose level 2 and on day 25 at dose level 4. These toxicities were reversible with appropriate supportive care. Grade 3 hyponatremia was observed in 1 out of 53 courses, but was reversible and mild. No other severe toxicities, such as diarrhoea or cardiotoxicity, occurred.

Maximum-tolerated dose. In the first course, DLT was observed in 1 of 3 patients at dose level 2 (grade 3

Table IV. *Non-haematological toxicity of grade 2 or greater (all courses).*

		Dose level				
Toxicity	Grade	1	2	3	4	5
No. of treated patients		3	3	3	6	4
No. of courses evaluated		4	5	8	31	5
No. (%) of courses encountered						
Febrile neutropenia	3	1 (25%)	0	0	2 (6%)	2 (40%)
Nausea/vomiting	2	0	1 (20%)	4 (50%)	0	0
	3	0	0	0	0	0
Hepatotoxicity	2	1 (25%)	0	0	0	0
	3	0	0	0	0	0
Infection	2	0	0	0	0	0
	3	0	2 (40%)	0	1 (3%)	0
Diarrhoea	2	0	2 (40%)	0	0	1 (20%)
	3	0	0	0	0	0
Hyponatremia	2	0	0	0	0	0
	3	0	1 (20%)	0	0	0

No patient developed grade 4 or greater toxicity.

infection), in 2 of 6 at dose level 4 (grade 3 febrile neutropenia and grade 3 infection) and in 3 of 4 at dose level 5 (persistence of grade 4 neutropenia and grade 4 leukopenia, persistence of grade 4 neutropenia, and grade 3 febrile neutropenia). There were no treatment-related deaths. Accordingly, the MTDs of irinotecan and amrubicin were determined to be 100 and 45 mg/m^2, respectively (dose level 5). The recommended doses of irinotecan and amrubicin were therefore concluded to be 100 and 40 mg/m^2, respectively (dose level 4).

Anti-tumour activity. All patients were assessable for response. Objective tumour response was obtained in 2 (11%) of 19 patients. Both patients were treated at dose level 4 and had received prior chemotherapy (single agent vinorelbine therapy and combination chemotherapy of carboplatin and gemcitabine, respectively).

Discussion

The present study demonstrated that the two-drug combination of irinotecan and amrubicin using a fractionated administration schedule was well tolerated in patients with advanced NSCLC. MTDs of irinotecan and amrubicin were 100 and 45 mg/m^2, respectively. Dose-intensities of irinotecan and amrubicin at the MTD were 67 and 30 mg/m^2/week, respectively. The MTD for irinotecan was higher than in the two-drug combination with cisplatin (12) and the MTD for amrubicin was slightly lower than when used as a single agent (6).

As expected, myelosuppression was the major toxicity in this study, however, it was reversible and not life-threatening. One of the major toxicities associated with anthracyclines is cardiotoxicity (13). However, in a preclinical study using dogs, Noda *et al.* reported that amrubicin had neither cardiotoxicity nor deteriorating effects on pre-existing cardiomyopathy (14). Also, previous clinical trials involving 74 patients with small cell lung cancer demonstrated that amrubicin had no cardiotoxicity, consistent with our results (15, 16). Similarly, irinotecan is not reported to have any major potential cardiotoxicity (17). Thus, this regimen may be used safely even in patients with pre-existing cardiac dysfunction. Additionally, it is of note that diarrhoea was mild in our study, since one of the DLTs of single agent irinotecan was diarrhoea (17). The difference in incidence of diarrhoea might be attributable to the difference in treatment schedule or in supportive care. Further confirmation is warranted.

Previous studies have shown that development of cellular resistance to topoisomerase II inhibitors confers an increased sensitivity to topoisomerase I inhibitors (7). The reverse effect, in which resistance to a topoisomerase I inhibitor enhances the sensitivity to topoisomerase II inhibitors, has also been reported (8). This enhanced sensitivity may be related to a compensatory role by each topoisomerase

enzyme to a deficiency in the other. Despite positive experimental findings, combined use of topoisomerase inhibitors has produced controversial *in vitro* results. When administered simultaneously, some reports have indicated synergistic or additive cytotoxic effects in various tumour cell lines (18), while others have demonstrated antagonistic effects (19). However, Bertrand *et al.* have shown that sequential administration of camptothecin and etoposide resulted in additive cytotoxicity in colon cancer cell lines (20). Therefore, further characterisation of the maximum attainable effect from a topoisomerase I and II inhibitors combination is required.

In Japan, two phase II studies investigating a combination of irinotecan and topoisomerase II inhibitor, etoposide, have been conducted (21, 22). Masuda *et al.* evaluated this combination chemotherapy in patients with relapsed small cell lung cancer (21). Irinotecan was administered at a dose of 70 mg/m^2 on days 1, 8 and 15, and etoposide was given at a dose of 80 mg/m^2 on days 1 to 3. This combination produced a response rate of 71%, far exceeding the response rates of 40-50% previously reported for the combination of cisplatin and etoposide (23). However, Oshita *et al.* investigated the same combination using a different schedule in chemo-naïve patients with metastatic NSCLC, in which both irinotecan and etoposide were administered on days 1 to 3 (22). This concurrent administration yielded a disappointing response rate of 21%, less than the 32% response rate of irinotecan alone (4). Considering the data, it may be preferable to avoid simultaneous administration of topoisomerase I and II inhibitors. This scheduled-dependency, as well as insufficient doses of the two drugs (15 out of 19 were treated with doses lower than MTD) probably contributed to the lower efficacy in this study. More preclinical and clinical investigations are needed to clarify the optimum sequence and administration schedule for both drugs.

In this study, objective response was obtained in 2 patients who had prior chemotherapy, whereas no chemo-naïve patients achieved objective response. This may be attributed to differences in the expression levels of target molecules of chemotherapeutic agents, in addition to the small sample size. Naruse *et al.* reported that K562/TPA, a human leukemic phorbol ester-resistant subline, was 400-fold more sensitive to the EGFR tyrosine kinase inhibitor gefitinib than the K562 parental cell, and that EGFR protein expression was detected in K562/TPA but not in K562 parent cells. They speculated that the high sensitivity of the multiple drug-resistant cell line K562/TPA is due to acquired EGFR expression from exposure to cytotoxic agents (24). Similarly, determining protein levels of topoisomerases before and after chemotherapy may be useful for characterising differences in response to this regimen between relapsed and chemo-naïve patients.

In conclusion, the combination of irinotecan and amrubicin was well tolerated, but produced only a modest anti-tumour effect for advanced NSCLC. However, further investigation into the role of this regimen as salvage chemotherapy may be warranted in relapsed patients, because relapsed patients responded to the regimen and there have been no reports evaluating topoisomerase I and II inhibitors combination trials in relapsed patient with NSCLC.

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Advanced Age Is Not Correlated With Either Short-term or Long-term Postoperative Results in Lung Cancer Patients In Good Clinical Condition*

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Objectives: **Several investigators have reported that operative mortality in the elderly is acceptable. However, their patients are potentially biased with regard to some factors such as performance status (PS) and comorbidity. In this study, we discuss surgical indications for the elderly and effects on perioperative mortality and prognosis.**

Study design: **A retrospective study was carried out by reviewing the records of 1,114 patients who were referred for treatment of non-small cell lung cancer between January 1993 and December 2002. The patients were classified into younger (≤ 75 years of age) and elderly (≥ 76 years of age) groups. The histologic subtype, TNM stage, Eastern Cooperative Oncology Group PS, and treatment were reviewed for members of each group, and the proportion of patients who underwent surgery was compared between the two groups. The surgical procedures, perioperative mortality, and prognosis of the two groups were also compared.**

Results: **There was a significant difference in the histologic distribution with no difference in TNM staging between the two groups. Regarding treatment, 51.0% of those in the younger group and 36.1% of those in the elderly group underwent surgery. The proportion of elderly patients who underwent surgery was significantly lower than that of the younger patients, mainly due to worse PS and comorbidity in the elderly patients. The perioperative mortality rates for the younger and elderly groups were 0.9% and 4.1%, respectively, with no significant difference, and the overall survival was similar between the two groups.**

Conclusions: **When compared to younger patients, fewer elderly patients underwent surgery because of worse PS and comorbidity. However, in elderly patients with good PS and no comorbidity, the rate of perioperative mortality and the prognosis were similar to those in the younger patients. Therefore, advanced age only is not a negative factor for surgery in elderly patients.** ***(CHEST 2005; 128:1557–1563)***

Key words: elderly; limited resection; lobectomy; mortality; non-small cell lung cancer; performance status; prognosis

Abbreviations: ECOG = Eastern Cooperative Oncology Group; NSCLC = non-small cell lung cancer; PS = performance status

Lung cancer is one of the most common forms of neoplasms and the leading cause of cancer-related death in Western countries as well as in Japan.[1] The peak incidence age was in the sixties in 1987 but shifted to the seventies in 2001 in Japan.[2] In addition, there is a general trend worldwide of an increasing incidence of lung cancer of the elderly.

The treatment of choice in patients with stages I, II, and some subsets of stage IIIA cancer is surgery, but this carries with it certain risks. Generally, the surgical risk becomes higher with age, since elderly patients usually have several types of comorbidities

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and poor performance status (PS), although the perioperative mortality rate is similar between younger and elderly patients.[3–5] The patients reported in previous studies[6–7] were potentially selected in terms of PS and comorbidity, and surgery was performed only in those in good clinical condition.

Another issue to consider is the life span of the elderly, which is naturally shorter than that of younger patients. Lobectomy is now a standard surgical procedure for patients with lung cancer. However, the perioperative mortality rate associated with this procedure is approximately 1%. Lobectomy including bilobectomy is more invasive than limited resection such as segmentectomy and partial resection. When considering the perioperative risks and benefits of surgery, lobectomy might be less beneficial for the elderly compared to the young. To determine if this is true, we reviewed 1,114 non-small cell lung cancer (NSCLC) patients at a single institute and classified them into two groups by using the age of 75 years as an upper and lower cutoff. Differences were then examined for the selected surgical procedures between the young and elderly patients, with special interest paid to the conditions of the elderly patients when determining the surgical modality. We also evaluated the relationships between surgical procedures such as lobectomy or limited resection and the short-term and long-term results in the elderly group to discuss the optimal surgical procedure for these patients.

Materials and Methods

A retrospective study was carried out using the lung cancer database of the Shikoku Cancer Center on 1,114 patients with NSCLC who were treated between January 1993 and December 2002. Information regarding the Eastern Cooperative Oncology Group (ECOG) PS, histologic subtype, TNM stage, and treatment, including chemotherapy, radiotherapy, and surgical procedures, was carefully reviewed. The perioperative mortality rate and long-term results were also reviewed if surgery was carried out. TNM stage was determined according to the International Union Against Cancer classification.[8]

Our treatment diagram is shown in Figure 1. Chemotherapy and/or radiotherapy were performed in patients with nonresectable stage IIIA, IIIB, and IV cancer; patients with stage I, II, or resectable IIIA or IIIB were candidates for surgery. Preoperative examinations including spirometry, ECG, and arterial blood gas analysis were carried out, and more detail examinations including diffusion capacity of the lung for carbon monoxide, ultrasound cardiography, and a 6-min walking test were added if necessary. Lobectomy, bilobectomy, or pneumonectomy were indicated if a patient had a predicted FEV_1 of ≥ 0.8 L, a PS of 0 or 1, and no or slight comorbidity. For a predicted FEV_1 of < 0.8 L and/or moderate comorbidity, limited resection was considered. Surgical resection was not indicated for patients with PS 3 or severe comorbidity. Hilar and mediastinal lymphoadenectomy were performed in case more than segmentectomy was required.

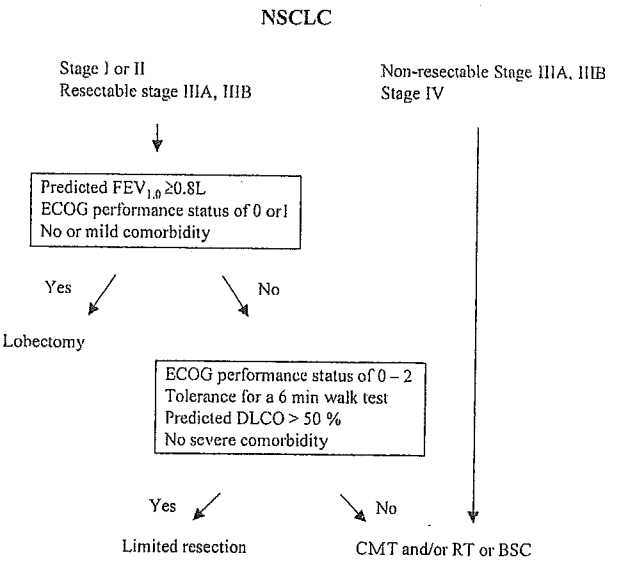

FIGURE 1. Flow chart of treatments. DLCO = diffusion capacity of the lung for carbon monoxide; CMT = chemotherapy; RT = radiotherapy; BSC = best supportive care.

Data Analysis

The differences in the proportions of histologic subtypes, TNM stage, PS, pulmonary function status, treatment, surgical procedure, and perioperative mortality rate were compared using the χ^2 method between the younger (≤ 75 years of age) and elderly (≥ 76 years of age) groups. The surgical procedures were classified into three groups: limited resection, lobectomy, and pneumonectomy. Segmentectomy and partial resection were classified as limited resection. Lobectomy and bilobectomy were classified as lobectomy. Death within 30 days postoperatively was considered to be perioperative death. In this study, prognostic data were analyzed only in patients who underwent limited resection and lobectomy, since the number of pneumonectomies was small. The overall survival and disease-specific survival curves were calculated using the Kaplan-Meier method and compared using the log-rank test. For disease-specific survival analysis, patients who died of any cause other than lung cancer were censored at the time of death. As subset analysis, in the elderly, the overall survival and disease-specific survival rates were compared in terms of the surgical procedures (lobectomy vs limited resection). In addition, the overall and disease-specific survival rates of the elderly treated by lobectomy were also compared to those of the younger patients treated in the same manner. A p value < 0.05 was considered significant. Statistical analysis was performed using statistical software (Version 13; SPSS; Chicago, IL).

Results

Demographics and Clinical Presentation

Patient characteristics are shown in Table 1. The younger and elderly groups consisted of 917 patients (82.3%) and 197 patients (17.7%), respectively, and there was no difference in the male/female ratio between the two groups. Histologic examination revealed that adenocarcinoma was predominant in

Table 1—*Patient Demographics and Clinical Data**

Variables	Younger Group (≤ 75 yr old)	Elderly Group (≥ 76 yr old)	p Value
Patients.	917 (82.3)	197 (17.7)	0.123
Gender			
Male	604 (65.9)	141 (71.6)	
Female	313 (34.1)	56 (28.4)	
Histology			< 0.001
Adenocarcinoma	620 (67.6)	94 (47.7)	
Squamous cell carcinoma	213 (23.2)	87 (44.2)	
Large cell carcinoma	37 (4.0)	3 (1.5)	
Others	47 (5.2)	13 (6.6)	
Final stage			0.225
I	358 (39.0)	62 (31.5)	
II	64 (7.0)	19 (9.6)	
IIIA	117 (12.8)	24 (12.2)	
IIIB	146 (15.9)	39 (19.8)	
IV	228 (24.9)	52 (26.4)	
Unknown	4 (0.4)	1 (0.5)	
ECOG performance status			< 0.001
0	485 (52.9)	73 (37.1)	
1	304 (33.1)	83 (42.1)	
2	50 (5.5)	17 (8.6)	
3	26 (2.8)	9 (4.6)	
4	17 (1.9)	9 (4.6)	
Unknown	35 (3.8)	6 (3.0)	
Smoking (Brinkman index)			0.007
None	231 (25.2)	30 (15.2)	
< 399	159 (17.3)	33 (16.8)	
> 400	522 (56.9)	133 (67.5)	
Unknown, No.	5	1	
Pulmonary function			
Vital capacity < 70%, % of patients	24.0	34.9	0.005
FEV_1 < 80 %, % of patients	18.1	37.5	0.225
Arterial blood gas, % of patients			
Pao_2 < 70 mm Hg	20.1	34.2	0.001
$Paco_2$ > 50 mm Hg	2.2	3.9	0.227
Treatment			< 0.001
Surgery	468 (51.0)	66 (36.1)	
Nonsurgical	449 (49.0)	131 (63.9)	
Chemotherapy	197 (43.9)	26 (19.8)	
Radiotherapy	60 (13.4)	56 (42.7)	
Chemotherapy and radiotherapy	91 (20.3)	0 (0)	
Best supportive care	101 (22.4)	48 (37.5)	

*Data are presented as No. (%) unless otherwise indicated.

both groups. However, the incidence of adenocarcinoma in the younger patients (67.6%) was significantly higher than that in the elderly patients (47.7%). In contrast, the incidence of squamous cell carcinoma in the elderly patients (44.2%) was higher than that in the younger patients (23.2%). There was no difference in the distribution of the TNM stages between the two groups ($p = 0.225$), and the PS of the elderly patients was significantly worse than that of the younger patients ($p < 0.001$). In addition, the proportion of elderly smokers was significantly higher than that of younger smokers ($p = 0.007$). Concerning respiratory function, the percentages of patients with vital capacity < 70% and Pao_2 < 70 mm Hg in the elderly patients were significantly greater than those in the younger patients ($p = 0.005$ and $p = 0.001$, respectively).

Five hundred thirty-four of the 1,114 patients underwent surgery, and the percentage of younger patients who underwent surgery (51.0%) was significantly higher than the percentage of the elderly patients (36.1%) [$p < 0.001$]. Surgical procedures are shown in Table 2. Limited resection, including partial resection and segmentectomy, was performed in 86 younger patients (18.4%) and in 18 elderly patients (24.3%), while lobectomy was performed in 369 younger patients (78.8%) and in 55 elderly patients (74.3%). There was no significant difference in the selection of surgical procedures.

Table 2— *Surgical Procedures and Perioperative Mortality*

Variables	Younger Group (n = 468)	Elderly Group (n = 74)	p Value
Surgery (n = 534)			0.412
Limited resection	86 (18.4)	18 (24.3)	
Partial	66	12	
Segmental	20	6	
Lobectomy	369 (78.8)	55 (74.3)	
Lobectomy	357	52	
Bilobectomy	12	3	
Pneumonectomy	13 (2.8)	1 (1.4)	
Perioperative death (n = 7)	4 (0.9)	3 (4.1)	0.087
Limited resection	0	0	
Lobectomy	4	3	0.071
Pneumonectomy	0	0	

*Data are presented as No. (%) or No.

Four patients (0.9%) in the younger group and three patients (4.1%) in the elderly group died perioperatively, and there was a trend toward a higher perioperative mortality rate in the elderly group than in the younger group, although there was no significant difference (p = 0.087) [Table 2]. The background and cause of death of these seven patients are shown in Table 3. Causes of death were pulmonary embolism (n = 2), pneumonia (n = 1), and respiratory failure (n = 4), all associated with lobectomy.

The median follow-up interval was 30 months (range, 0 to 123 months). The overall and disease-specific survival curves are shown in Figure 2. There was a trend toward an inferior overall survival rate in the elderly group compared to the younger group, but this difference was not statistically significant (p = 0.193). In addition, there was no difference between the younger and elderly groups in disease-specific survival (p = 0.892). Lobectomy and limited resection in the elderly patients were compared with respect of the rates of overall survival and disease-specific death (Fig 3). In elderly patients, there were

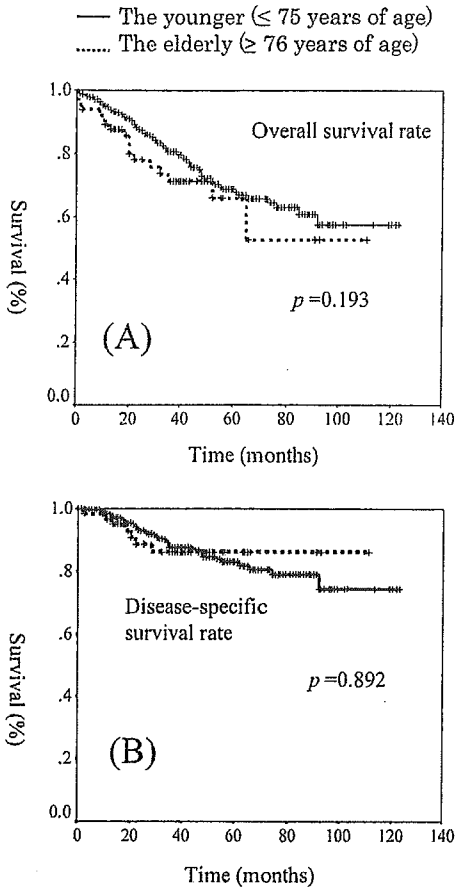

FIGURE 2. Overall survival (*top*, *A*) and disease-specific survival (*bottom*, *B*) for the younger and elderly groups.

no differences in both overall survival and disease-specific survival rates between the different surgical procedures. However, in the overall survival rate, during the early period after surgery, the elderly patients who underwent lobectomy had an associated inferior survival rate compared to those who underwent limited resection (Fig 3, *top*, *A*). Lobectomy between the younger and elderly groups was compared using the overall survival and disease-

Table 3—*Characteristics of the Seven Patients Who Died Perioperatively*

Age, yr	Gender	Comorbidity	Brickman Index	Surgical Procedure	Cause of Death
40	Female	None	None	Lobectomy	Pulmonary embolism
70	Female	Breast cancer	None	Lobectomy and partial lobectomy	Pulmonary embolism
72	Male	Hypertension, diabetes mellitus, tuberculosis, interstitial pneumonitis	1,000, active smoker	Lobectomy and partial lobectomy	ARDS
75	Male	Brain infarction, renal cell carcinoma	1,100, active smoker	Lobectomy	Interstitial pneumonitis
79	Male	Old myocardial infarction	1,060	Bilobectomy	Interstitial pneumonitis
79	Male	None	1,100	Lobectomy	Pneumonia
84	Male	Interstitial pneumonitis	800	Lobectomy	Interstitial pneumonitis

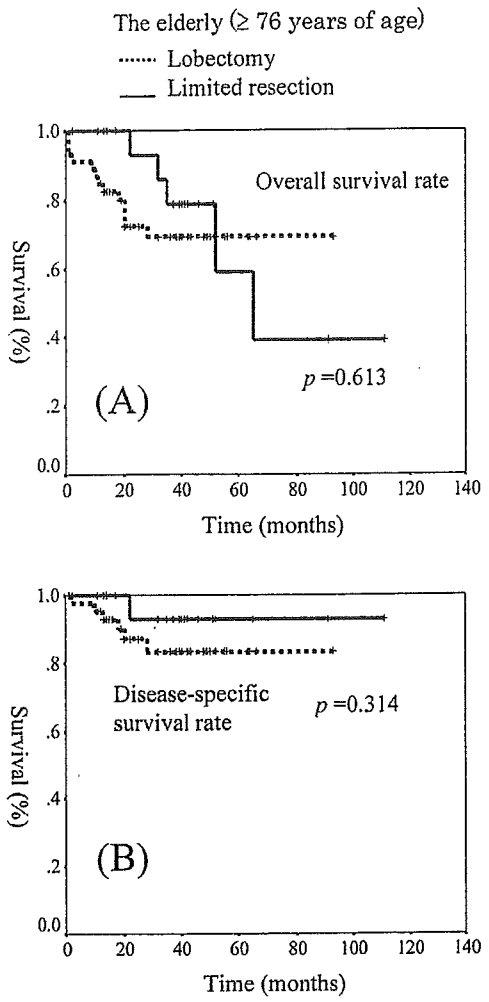

FIGURE 3. Overall survival (*top*, A) and disease-specific survival (*bottom*, *B*) for elderly patients according to the extent of surgery.

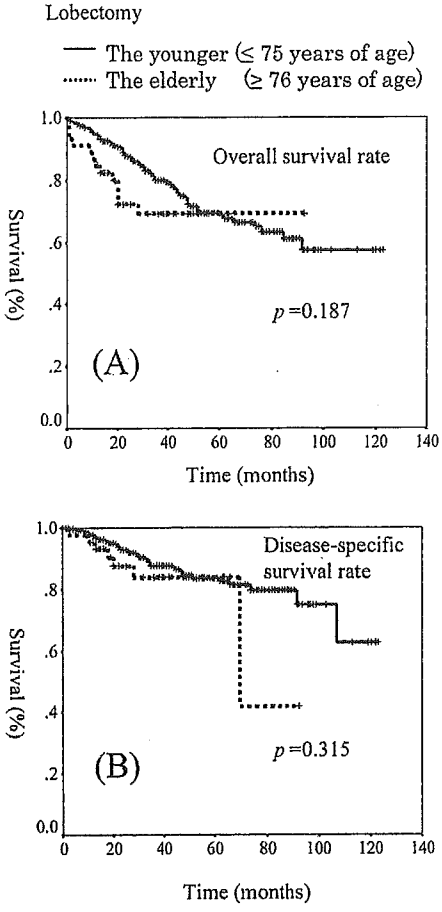

FIGURE 4. Overall survival (*top*, *A*) and disease-specific survival (*bottom*, *B*) after lobectomy for the younger and elderly groups.

specific survival rates (Fig 4), and there were no significant differences found. However, in the overall survival rate, during the early period after surgery, the elderly patients had an associated inferior survival rate compared to the younger patients.

DISCUSSION

The world population is aging, and the proportion of elderly patients with lung cancer is increasing. In this study, we found that elderly patients had worse PS and greater comorbidity than the younger patients, but the same surgical procedure can be performed and similar outcomes can be expected with respect to perioperative mortality and long-term results as long as patients who are > 75 years old have good PS and no or slight comorbidity.

In this study, we considered patients > 76 years old as elderly, but there is no clear definition for this distinction. In most of the literature, the age of 70 years is considered to be the cutoff.[9–12] In our data, 66% of patients would have been classified into the younger group and 34% would have been classified into the elderly group if the age of 70 years was used as the cutoff. We considered that it would have been difficult to clarify the differences between the younger and elderly groups with such a group balance. Therefore, in this study we classified the groups using the age of 75 years as the cutoff in order to emphasize the condition of highly aged patients.

Adenocarcinoma was the dominant histologic type of cancer found in both groups. The incidence of adenocarcinoma in the elderly patients was significantly lower than in the younger patients, but the incidence of squamous cell carcinoma was significantly higher, and this trend was also reported in other studies.[13–15] In our study, the percentage of smokers in the elderly group was significantly higher than in the younger group, and this might have had some influence on the higher percentage of cases of squamous cell carcinoma in the elderly group.

There was no difference in the TNM staging

between the groups, although several previous studies[16–21] showed that elderly patients more often have earlier stages, likely due to the slow progress of lung cancer in the elderly, and the tendency of younger patients to ignore or misinterpret nonspecific changes in their health. In contrast to these results, one study[11] conducted in Japan did not find a difference in the stage distribution, according to age. The method of lung cancer screening performed in Japan might affect the rate of early detection in the young and therefore led to no difference between the groups.

Several studies[22–25] noted that surgical mortality, morbidity, and long-term results in the elderly are acceptable. However, these were not randomized with respect to the patients, and there might have been bias. It is, therefore, not advisable to make generalizations regarding elderly patients. In our study, 51.0% of the younger patients underwent surgery compared to 33.5% of the elderly patients, although the two groups had the same TNM staging distribution. In addition, when looking at the nonsurgical treatments, the number of patients treated with chemotherapy and/or radiotherapy in the elderly patients was less than in patients < 75 years of age, suggesting that the elderly patients had greater comorbidity and worse PS than the younger patients, and that not only surgery but chemotherapy and radiotherapy were not indicated.

Table 4 lists the patients who were > 76 years old and candidates for surgery indicated by their TNM stage but who eventually did not undergo surgery. Factors considered were a poor PS in 5 patients and poor pulmonary function in 13 patients. The remaining 11 patients had moderate comorbidity and a PS of approximately 1 to 2, and were > 81 years old. Surgery could have been safely performed, but when considering their age, PS, comorbidity, and disease status together, we decided to treat them with radiotherapy instead.

A potential criticism of this study is the histologic distribution that was observed: the incidence of adenocarcinoma in younger patients was greater than that in the elderly patients. This could have had an impact on survival that favored the elderly because adenocarcinoma generally has worse prognosis than squamous cell carcinoma. Survival rates by histologic subtype are shown in Figure 5. Adenocarcinoma showed a better prognosis than squamous cell carcinoma. Therefore, we considered histologic distribution did not impact survival in favor of the elderly in this study at least.

The Kaplan-Meier curve for the overall survival rate in the elderly was inferior to that in the younger group, although this was not significant (Fig 2, *top*, *A*); and it was expected that the overall survival of the elderly was inferior to that of the younger, because of their advanced age. However, the Kaplan-Meier curves for disease-specific death were almost identical (Fig 2, *bottom*, *B*), suggesting that more elderly patients died of causes other than lung cancer and that other forms of disease had more influence on their prognosis compared with the younger patients.

The Lung Cancer Study Group[26] reported in 1995 that limited pulmonary resection does not result in improved perioperative morbidity, mortality, or late postoperative pulmonary function compared with lobectomy. In our study, there was no significant difference, but the early overall survival rate for lobectomy in the elderly was inferior to that for limited resection (Fig 3, *top*, *A*). In addition, 3 of 55 elderly patients (5.5%) but only 4 of 369 younger patients (1.1%) died perioperatively after lobectomy. There was a tendency for perioperative mortality after lobectomy in the elderly patients to be higher than that in the younger patients (p = 0.071) [Table

Table 4—*Factors Not Indicated for Surgery Among the Elderly With Stage I or II Cancer*

Factors	Patients, No.
PS ≥ 2	5
Poor pulmonary function	13
Others	11
Total	23

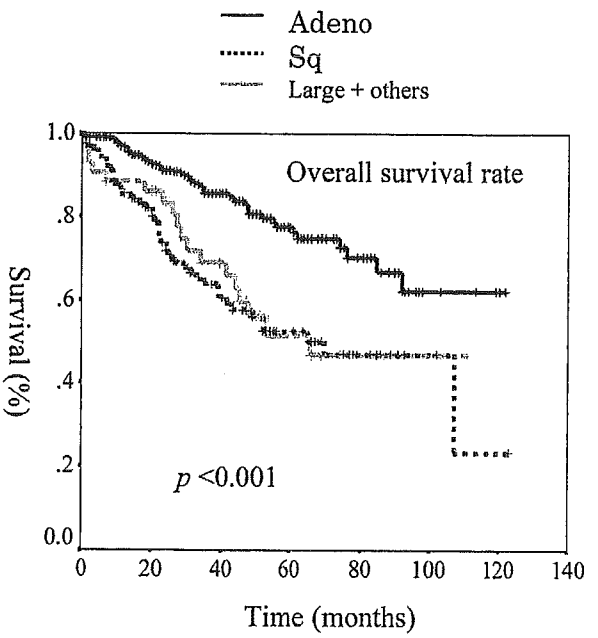

FIGURE 5. Overall survival according to histologic subtype. Adeno = adenocarcinoma; Sq = squamous cell carcinoma; Large = large cell carcinoma.

2]. Although the number of patients was not large and the study was not randomized, lobectomy including bilobectomy might have been too invasive; in some cases, limited resection may therefore have been preferable for those > 76 years old.

In our study, the elderly patients with a good PS and no or slight comorbidities could tolerate surgery and be expected to have a long-term survival rate similar to that of younger patients. However, when considering the drop in survival rate during the early survival period after lobectomy compared to limited resection, it is reasonable to consider limited resection as an alternative surgical management for the elderly.

Conclusions

There was a difference in the histologic distribution with regard to NSCLC between younger and elderly patients. The proportion of patients treated surgically was less than that in the younger patients due to severe comorbidity and poor PS, although the TNM staging distribution was similar between the groups. However, in elderly patients with a good PS and no or slight comorbidity, surgery was safely performed and long-term results were similar to those in the younger patients. Finally, we suggest that lobectomy including bilobectomy might be too invasive and that limited resection might be more beneficial for patients of advanced age.

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Activating Mutations in the Tyrosine Kinase Domain of the Epidermal Growth Factor Receptor Are Associated with Improved Survival in Gefitinib-Treated Chemorefractory Lung Adenocarcinomas

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Abstract

Purpose: Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) confer a strong sensitivity to gefitinib, a selective tyrosine kinase inhibitor of EGFR.

Experimental Design: We examined *EGFR* mutations at exons 18, 19, and 21 in tumor tissue from 68 gefitinib-treated, chemorefractory, advanced non–small cell lung cancer patients from the United States, Europe, and Asia and in a highly gefitinib-sensitive non–small cell lung cancer cell line and correlated their presence with response and survival. In addition, in a subgroup of 28 patients for whom the remaining tumor tissue was available, we examined the relationship among *EGFR* mutations, CA repeats in intron 1 of *EGFR*, *EGFR* and *caveolin-1* mRNA levels, and increased *EGFR* gene copy numbers.

Results: Seventeen patients had *EGFR* mutations, all of which were in lung adenocarcinomas. Radiographic response was observed in 16 of 17 (94.1%) patients harboring *EGFR* mutations, in contrast with 6 of 51 (12.6%) with wild-type *EGFR* ($P < 0.0001$). Probability of response increased significantly in never smokers, patients receiving a greater number of prior chemotherapy regimens, Asians, and younger patients. Median survival was not reached for patients with *EGFR* mutations and was 9.9 months for those with wild-type *EGFR* ($P = 0.001$). *EGFR* mutations tended to be associated with increased numbers of CA repeats and increased *EGFR* gene copy numbers but not with *EGFR* and *caveolin-1* mRNA overexpression (P = not significant).

Conclusions: The presence of *EGFR* mutations is a major determinant of gefitinib response, and targeting EGFR should be considered in preference to chemotherapy as first-line treatment in lung adenocarcinomas that have demonstrable *EGFR* mutations.

Platinum-based chemotherapy as first-line treatment in advanced non–small cell lung cancer (NSCLC) yields limited survival benefit. A retrospective analysis of advanced NSCLC patients showed that response rates decreased with each successive chemotherapy regimen: first line, 21%; second line, 16%; third line, 2%; fourth line, 0% (1). Aberrant epidermal growth factor receptor (EGFR) signaling limits sensitivity to anticancer agents, and ligand-independent tyrosine kinase activation of EGFR, often caused by EGFR mutations in the extracellular domain, has been observed in various tumor types, including glioblastoma multiforme (2). Pharmacologic inhibitors of EGFR, such as gefitinib (Iressa), disrupt EGFR activity by binding the ATP pocket within the catalytic domain containing a critical ATP-binding site, Lys^{745} (K745). Gefitinib and related tyrosine kinase inhibitors occasionally yield dramatic and durable "Lazarus responses" (3), yet response rates are variable, with higher rates in patients with adenocarcinoma, female gender, Asian origin, and never-smoker status (4, 5).

The value of EGFR inhibitors as an NSCLC treatment approach has been limited by the lack of reliable methods for predicting which patients are likely to respond. The logical supposition that tumors overexpressing EGFR would respond best to EGFR inhibitors has not been borne out either in preclinical models(6, 7) or in clinical trials (8, 9). However, recent discoveries of *EGFR* mutations in the tyrosine kinase

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domain have shed light on the relationship between EGFR and sensitivity to both gefitinib and the related kinase inhibitor erlotinib. Accumulated data from three studies (10–12) show that 25 of 31 (81%) tumors from NSCLC patients with partial response or marked clinical improvement contained mutations in the *EGFR* tyrosine kinase domain. In contrast, none of 29 specimens from patients refractory to EGFR inhibitors had such mutations ($P < 0.0001$). The mutations included small in-frame deletions (746-750) adjacent to K745 (ELREA amino acids) and missense mutations, mainly L858R adjacent to the DFG motif in the COOH-terminal lobe in the activation loop of the kinase (10–12). These *EGFR* mutations are bona fide somatic mutations in NSCLC and have not been identified in other primary tumor types (10, 13, 14), with the exception of colorectal tumors. One of 293 tumors contained a G719S point mutation (15) that had previously been reported in NSCLC (11), and recently, 4 of 33 tumors harbored point mutations in exons 19 and 20 (16). *In vitro* studies of lung cancer cell lines with endogenous *EGFR* mutations displayed elevated activation of downstream antiapoptotic targets like AKT and signal transducer and activation of transcription (STAT5 and STAT3), conferring enhanced gefitinib sensitivity and increased cisplatin resistance (17).

The transcription activity of the *EGFR* gene is closely related to the enhancer region in intron 1 that is located near a polymorphic CA single sequence repeat containing 14 to 21 CA dinucleotides. Decreased numbers (<19) of CA dinucleotides in this CA sequence correlate with increased *EGFR* transcription (18, 19), and in breast cancer, this CA sequence is a frequent target for *EGFR* gene alterations (20). Moreover, interethnic studies have found that Japanese breast cancer patients carry increased numbers (>19) of CA dinucleotides than Caucasian patients (20). It has been shown that the number of repeats itself affects the mutation rate of nucleotide repeats (21).

A variety of cell surface receptors, including EGFR, as well as intracellular signaling molecules, are concentrated in specialized plasma membrane domains known as caveolae (22). *Caveolin-1* mRNA expression is elevated in multidrug-resistant cultured cancer cells (23), and up-regulation of caveolin-1 and caveolae organelles has been observed in drug-resistant human and ovarian cancer cell lines (24). In addition, high *caveolin-1* mRNA expression has been observed in potentially chemoresistant NSCLC cell lines established from metastatic NSCLCs (25). We therefore hypothesized that tumors harboring EGFR mutations might be associated with higher levels of *caveolin-1* mRNA.

In the present study, we examined *EGFR* mutations in tumor tissue from gefitinib-treated, chemorefractory, advanced NSCLC patients from the United States, Europe, and Asia and in a highly gefitinib-sensitive NSCLC cell line (26) and correlated their presence with response and survival. In addition, in a subgroup of patients for whom remaining tumor tissue was available, we examined the relationship among *EGFR* mutations, number of CA repeats, *EGFR* and *caveolin-1* mRNA levels, and increased *EGFR* gene copy numbers.

Materials and Methods

Patients. Patients with pretreated NSCLC received gefitinib, based on the attending oncologist's decision at the time of chemotherapy failure, at a daily dose of 250 mg given until disease progression. Patients were selected for the present study based on the availability of tumor tissue, without scoring tumor response at the time of selection. Acquisition of tissue specimens and examination of clinical records was approved by the ethics committees of participating institutions. A total of 68 patients were included: 32 Asians (19 Japanese and 13 Chinese) and 36 Caucasians (23 Spanish, nine German, three North American, and one English patient resident in Hong Kong). Assessment of *EGFR* mutations was done for all 68 patients. After this initial analysis, sufficient genomic DNA remained to perform additional related analyses in a subgroup of 28 patients.

Patients were divided into smokers and nonsmokers (having smoked <100 cigarettes in their lifetimes; ref. 27). Tumor response was defined according to the Response Evaluation Criteria in Solid Tumors (28). Survival was calculated from the start of gefitinib treatment. Follow-up was calculated from the start of gefitinib treatment; median follow-up was 11.4 months (range, 1.7-40.3 months).

Epidermal growth factor receptor sequencing. Pure tumor genomic DNA was derived from paraffin-embedded tissue obtained by laser capture microdissection (Palm, Oberlensheim, Germany). For isolation of DNA from deparaffinated, microdissected tissue, the material was incubated with proteinase K and DNA was extracted with phenol-chloroform and ethanol precipitation. Primers for PCR amplification in nested reactions for exons 18, 19, and 21 of EGFR (Genbank accession no. X00558) were as follows: exon 18 (first PCR, forward 5′-CAAATGAGCTGGCAAGTGCCGTGTC-3′ and reverse 5′-GAGTTTCCCA-AACACTCAGTGAAAC-3′; nested PCR, forward 5′-CAAGTGCCGTGTCC-TGGCACCCAAGC-3′ and reverse 5′-CCAAACACTCAGTGAAACAAAG-AG-3′); exon 19 (first PCR, forward 5′-GCAATATCAGCCTTAGGT GCGGCTC-3′ and reverse 5′-CATAGAAAGTGAACATTTAGGATGTG-3′; nested PCR, forward 5′-GTGCATCGCTGGTAACATCC-3′ and reverse 5′-TGTGGAGATGAGCAGGGTCT-3′); exon 21 (first PCR, forward 5′-CTAA-CGTTCGCCAGCCATAAGTCC-3′ and reverse 5′-GCTGCGAGCT-CACCCAGAATGTCTGG-3′; nested PCR, forward 5′-GCTCAGAGCCTGG-CATGAA-3′ and reverse 5′-CATCCTCCCCTGCATGTGT-3′). Sequencing was done using forward and reverse nested primers with the ABI Prism 3100 DNA Analyzer (Applied Biosystems, Foster City, CA). Electropherograms were analyzed for the presence of mutations using Seqscape v2.1.1 software in combination with Factura to mark heterozygous positions. The human NSCLC cell line (PC9) derived from an adenocarcinoma (Kyushu Cancer Center, Fukuoka, Japan) was also examined using the same methods.

CA repeats in intron 1. In the subgroup of 28 patients, genomic DNA from peripheral blood or adjacent normal lung tissue was used to determine the number of CA repeats in intron 1. PCR amplification was done with 50 ng of genomic DNA; the primer sequences specific for this microsatellite marker were as follows: forward 5′-FAMGGGCTCACAG-CAAACTTCTC-3′ and reverse 5′-AAGCCAGACTCGCTCATGTT-3′. One microliter of each PCR product was mixed with 0.5 μL of size standard (GenScan-350 Rox Standard, Applied Biosystems) and denatured in 18 μL of formamide at 95°C for 5 minutes. Separation was done with a four-color laser-induced fluorescence capillary electrophoresis system (ABI Prism 3100 DNA Analyzer, Applied Biosystems). The collected data was evaluated with the GeneScan Analysis Software (Applera, Norwalk, CT). DNA from the tumor cell line Hep-2 was used as a control for PCR amplified microsatellite fragment length.

Quantitative PCR. In the subgroup of 28 patients, total RNA was derived from paraffin-embedded tissue obtained by laser capture microdissection. After standard tissue sample deparaffinization using xylene and alcohols, samples were lysed in a Tris-chloride, EDTA, SDS, and proteinase K containing buffer. RNA was then extracted with phenol-chloroform-isoamyl alcohol followed by precipitation with isopropanol in the presence of glycogen and sodium acetate. RNA was resuspended in RNA storage solution (Ambion, Inc., Austin, TX) and treated with DNase I to avoid DNA contamination. cDNA was synthesized using Moloney murine leukemia virus retrotranscriptase enzyme. Template cDNA was added to Taqman Universal Master Mix (Applied Biosystems) in a 12.5-μL reaction with specific primers and

probe for each gene. The primer and probe sets were designed using Primer Express 2.0 Software (Applied Biosystems). Quantification of gene expression was done using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Primers and probe for *EGFR* and *caveolin-1* mRNA expression analysis were designed according to the Ref Seq NM_005228 and NM_001753, respectively (http://www.ncbi.nlm.nih.gov/LocusLink). The primers and labeled fluorescent reporter dye probe were as follows: β-actin, forward 5′-TGAGCGCGGCTACAGCTT-3′, reverse 5′-TCCTTAATGTCACGCACGATTT-3′, probe 5′-FAMACCACCACGGCCGAGCGG-3′TAMRA; EGFR, forward 5′-GGAAATTACCTATGTGCAGAGGAATT-3′, reverse 5′-TAACCAGCCACCCCCTGGAT-3′, MGB probe 5′-FAMTGATCTTTCCTTCTTAAAGAC-3′; Caveolin-1, forward 5′-CGACCCTAAACACCTCAACGA-3′, reverse 5′-GGTTCTGCAATCACATCTTCAAAG-3′, MGB probe 5′-FAMCGTGGTCAAGATTG-3′. Relative gene expression quantification was calculated according to the comparative C_t method using β-actin as an endogenous control and commercial RNA controls (Stratagene, La Jolla, CA) as calibrators. Final results were determined as follows: $2^{-(\Delta Ct\ sample\ -\ \Delta Ct\ calibrator)}$, where ΔC_t values of the calibrator and sample are determined by subtracting the C_t value of the target gene from the value of the *β-actin* gene. In all experiments, only triplicates with a SD of the $C_t < 0.20$ were accepted. In addition, for each sample analyzed, a retrotranscriptase minus control was run in the same plate to assure lack of genomic DNA contamination.

To distinguish between high and low gene expression levels, median levels obtained were used as cutoffs: 3.28 for *EGFR* and 0.52 for *caveolin-1* mRNA expression.

Fluorescence in situ hybridization assay. For each patient in the subgroup of 28 patients, two sections of 3- to 5-μm paraffin-embedded tumor tissue were placed over silenized treated slides. Another section was stained with H&E and confirmed to contain tumor tissue components. The silenized slides were left overnight at 60°C; deparaffinized in two changes of xylene for 10 minutes; rehydrated in 100% ethanol, 90% ethanol, and 70% ethanol for 1 minute each; and left in deionized water for 5 minutes. After tissue hydration, sections were placed in citrate buffer and heated in a microwave twice for 5 minutes at 800 W each. Slices were then digested by proteinase K treatment for 15 minutes at 37°C, fixed with formalin solution (pH 7.5), and washed in 2× SSC buffer. The hybridization was done using Vysis probes (LSI EGFR/CEP 7 Dual Color, Downers Grove, IL) following the manufacturer's instructions. Briefly, 5 μL of the probe solution were added to each slide and covered by a coverslip. Slides and probes were denatured for 3 minutes at 85°C in a slide warmer plaque (Hybrite, Vysis) and left at 37°C overnight. The coverslips were removed and the slides washed in 2× SSC/0.3%NP40 solution for 2 minutes at 72°C followed by an additional wash in 2× SSC/0.3%NP40 solution for 10 seconds at room temperature. Finally, slides were counterstained using a 4′-6′-diamidine-2-phenylindole-containing medium that specifically binds to DNA. For each patient, 100 nuclei from the selected tumor region were analyzed in a fluorescence microscope. The ratio of the average number of EGFR spots/nucleus by the average number of CEP 7 (centromeric chromosome 7) spots/nucleus was used for the scoring criteria. EGFR status in tumors was scored as follows: (a) single copy, up to four specific signals of both EGFR and CEP 7 probes with a ratio equal to 1; (b) polysomy, more than four specific signals of both probes per nucleus and a ratio <2; (c) amplification, more than four specific signals of EGFR probe per nucleus compared with CEP 7 with a ratio >2. Tumors scored as polysomy and/or amplification were labeled as having increased EGFR copy numbers.

Statistical methods. The primary objective of this study was to compare clinical characteristics, response rates, and survival in gefitinib-treated patients with and without mutations in the EGFR tyrosine kinase domain. In the subgroup of 28 patients, further analyses were done to examine the correlation among EGFR mutations, the number of CA repeats in intron 1 of EGFR in normal tissue, *EGFR* and *caveolin-1* mRNA expression levels in tumor tissue, and *EGFR* gene copy numbers.

The nonparametric Mann-Whitney *U* test and one-way ANOVA test were used to analyze differences in EGFR mutation status, number of CA repeats in intron 1 of EGFR, EGFR and caveolin-1 mRNA expression, and *EGFR* gene copy numbers. Normality of the distribution of continuous variables was assessed with the Kolmogorov-Smirnov test. The χ^2 and Fisher's exact tests were used to compare differences in response according to EGFR mutation status, number of CA repeats in intron 1, EGFR and caveolin-1 mRNA expression, and gene copy numbers. Univariate Cox regression models were used to measure hazard ratios. To identify relevant variables of influence, a multivariable logistic regression model was used, and the fit of the models was evaluated with the Hosmer-Lemeshow likelihood ratio test. The Wald test was used to test the statistical significance of each variable in the model. Survival curves were drawn with the Kaplan-Meier product limit method and *P* values assessed with the Tarone-Ware test. All reported *P* values are two sided; $P < 0.05$ was considered statistically significant. SPSS software version 11.5 (SPSS, Inc., Chicago, IL) was used for all analyses.

Results

Table 1 shows characteristics for all patients according to *EGFR* mutation status. Seventeen of the 68 patients harbored *EGFR* mutations in the tyrosine kinase domain. Mutations were not observed in DNA from peripheral blood or adjacent normal lung tissue, indicating that all mutations were somatic. All mutations were identified in adenocarcinomas (Table 1); 10 were heterozygous and six were homozygous (Table 2). Eleven tumors had in-frame nucleotide deletions in exon 19, adjacent to K745; five were delE746-A750, which was also observed in

Table 1. Characteristics of all patients according to *EGFR* mutation status

	EGFR mutation status		*P*
	Mutation	Wild-type	
No. patients	17	51	0.8
Age (range)	60 (34-84)	59 (39-86)	
Sex (%)			
Male	6 (35.3)	39 (76.5)	0.003
Female	11 (64.7)	12 (23.5)	
Histology (%)			
Adenocarcinoma	17 (100)	30 (58.8)	0.007
Large cell carcinoma	—	5 (9.8)	
Squamous cell carcinoma	—	11 (21.6)	
Other	—	5 (9.8)	
Smoking history (%)			
Smokers	3 (17.6)	43 (84.3)	0.0001
Nonsmokers	14 (82.4)	8 (15.7)	
No. prior regimens (range)	1 (0-3)	2 (0-6)	0.04
Response to gefitinib (%)			
Complete and partial response	16 (94.1)	6 (11.8)	<0.0001
Stable disease	1 (5.9)	8 (15.7)	
Progressive disease	—	34 (66.7)	
Not evaluable	—	3 (5.8)	
Duration of gefitinib treatment			
Months (range)	9.4 (1.1-23.1)	4.2 (0.2-41.9)	0.07

Table 2. Clinical characteristics and *EGFR* mutation status in 22 responders to gefitinib

Country of origin	Age	sex	Smoking status	Pathol	Prior regimens	Response	Overall survival (mo)	Survival status	*EGFR* mutation 1 AA sequence	*EGFR* mutation 2 AA sequence	Mutational status
Spain	60	F	Yes	ADC	1	PR	6.7	D	wt		wt
Spain	52	F	Yes	ADC	1	PR	8.3	D	wt		wt
Germany	65	M	Yes	ADC	1	PR	22.1	D	wt		wt
Japan	53	F	No	LCC	3	PR	18.4	D	wt		wt
Japan	76	M	Yes	ADC	3	PR	10.8	A	wt		wt
Japan	68	F	No	ADC	2	PR	24.3	D	wt		wt
Spain	71	F	No	ADC	1	PR	8.9	A	delE746.A750		Hetero
Spain	63	M	Yes	ADC	3	PR	17.8	A	delE746.T751InsA		Hetero
Germany	66	F	No	ADC	2	PR	13.7	D	delE746.S752InsV		Hetero
China	67	M	No	ADC	0	PR	22.0	A	delL747.P753InsS	L861Q	Hetero
China	34	F	No	ADC	2	PR	6.1	A	L858R		Homo
China	61	F	No	ADC	2	PR	14.3	A	L858R		Hetero
China	49	M	Yes	ADC	1	PR	25.4	A	L858R		Homo
China	37	M	No	ADC	0	PR	15.9	A	delE746.S752InsV		Hetero
Japan	71	F	No	ADC	1	PR	14.2	A	del719G(G)C to GC		Hetero
Japan	66	F	No	ADC	0	PR	9.5	A	delE746.T751		Homo
Japan	54	F	No	ADC	2	PR	18.7	D	delE746.A750		Homo
Japan	60	F	No	ADC	3	PR	15.3	A	L718P		Hetero
Japan	50	M	No	ADC	0	PR	18.4	A	delL747.T751InsF		Hetero
Japan	52	M	Yes	ADC	1	PR	8.9	A	delE746.A750		Homo
Japan	42	F	No	ADC	2	CR	18.9	A	delE746.A750		Homo
USA	84	F	No	ADC	1	PR	11.7	D	delE746.A750		Hetero

Abbreviations: Pathol, pathologic diagnosis; ADC, adenocarcinoma; LCC, large cell carcinoma; PR, partial response; CR, complete response; A, alive; D, dead; AA, amino acid; Homo, homozygogous; Hetero, heterozygous; wt, wild type.

the PC9 cell line; one was delE746-T751; four contained an amino acid insertion (one delE746-T751insF, one delE746-T751insA, and two delE746-752insV); and one tumor contained both an amino acid insertion (delL747-P753insS) and a missense mutation L861Q in exon 21. Four tumors contained an L858R mutation in exon 21. One tumor had an L718P mutation and another had a nucleotide deletion (guanine) in codon 719, both in exon 18. This second mutation was heterozygous. The G deletion affects the reading frame 5′ downstream of this position. The protein is nonfunctional, and the new sequence has a stop codon in codon 747 (TAA instead of TTA).

Twenty-two patients (32%) achieved a partial response to gefitinib. Table 2 shows the clinical characteristics of all responders. Sixteen of the 17 patients (94.1%) carrying EGFR mutations attained a partial radiographic response in contrast with 6 (12.6%) of the 51 patients with wild-type EGFR ($P < 0.0001$) Patients with *EGFR* mutations had 17.1 times greater probability of response ($P = 0.02$). Probability of response was also increased in nonsmokers, patients receiving a greater number of prior chemotherapy regimens, Asians, and younger patients (Table 3).

In general, patients harboring *EGFR* mutations obtained dramatic responses. For example, a Japanese female with adenocarcinoma underwent three pulmonary resections between 1999 and 2002; two of the three resected tumors contained an EGFR mutation (delE746-T751). The patient developed multiple bilateral lung metastases and did not respond to several chemotherapy regimens. After 2 months of gefitinib treatment, almost complete response was attained and the patient remains in remission at the time of submitting this article (Fig. 1). A second patient, a Spanish

Table 3. Adjusted odds ratio for the joint effect on response of different covariates

	Odds ratio (95% confidence interval)	*P*
Odds ratio adjusted by covariates		
EGFR mutations	17.1 (5.1-58.8)	0.002
EGFR mutations by sex (female)	8.7 (2.2-34.6)	0.0001
EGFR mutations by smoking status (nonsmoker)	37.4 (3.1-426)	0.005
EGFR mutations by no. prior chemotherapy regimens	73.1 (7.6-462)	0.005
EGFR mutations by ethnicity	61.7 (5.9-639)	0.001
EGFR mutations by age	105.0 (11.4-981)	0.00001
Crude odds ratio		
Sex (female)	1.4 (0.6-3.4)	0.4
Smoking status (smoker)	0.6 (0.4-0.8)	0.001
No. prior chemotherapy regimens	0.7 (0.5-0.9)	0.003
Ethnicity (Asian)	4.0 (1.7-9.2)	0.001
Age	0.9 (0.98-0.99)	0.008