4. Discussion

The EGFR-targeting drug gefitinib has been approved in many countries for the treatment of NSCLC patients who have previously received chemotherapy. Previous preclinical models have demonstrated the synergistic effects of gefitinib and platinums or taxanes [8,9]. However, no significant difference in survival was demonstrated in two randomized placebo-controlled phase II trials examining over 2000 previously untreated patients with NSCLC. In these trials, gefitinib was given in combination with paclitaxel and carboplatin or with gemcitabine and cisplatin [10,11]. Different administration schedules for gefitinib and cytotoxic agents may be necessary for select populations.

EGFR gene mutations have been demonstrated in NSCLC, and patients with lung cancers expressing mutant EGFR are strongly suspected to be hypersensitive to gefitinib alone. An in-frame short deletion in exon 19 of EGFR is strongly related to hyperresponsiveness to gefitinib and other tyrosine kinase inhibitors [12,13]. Cells expressing this deletional EGFR mutation are hypersenstivie to EGFR-targeted tyrosine kinase inhibitors [5]. On the other hand, the treat-

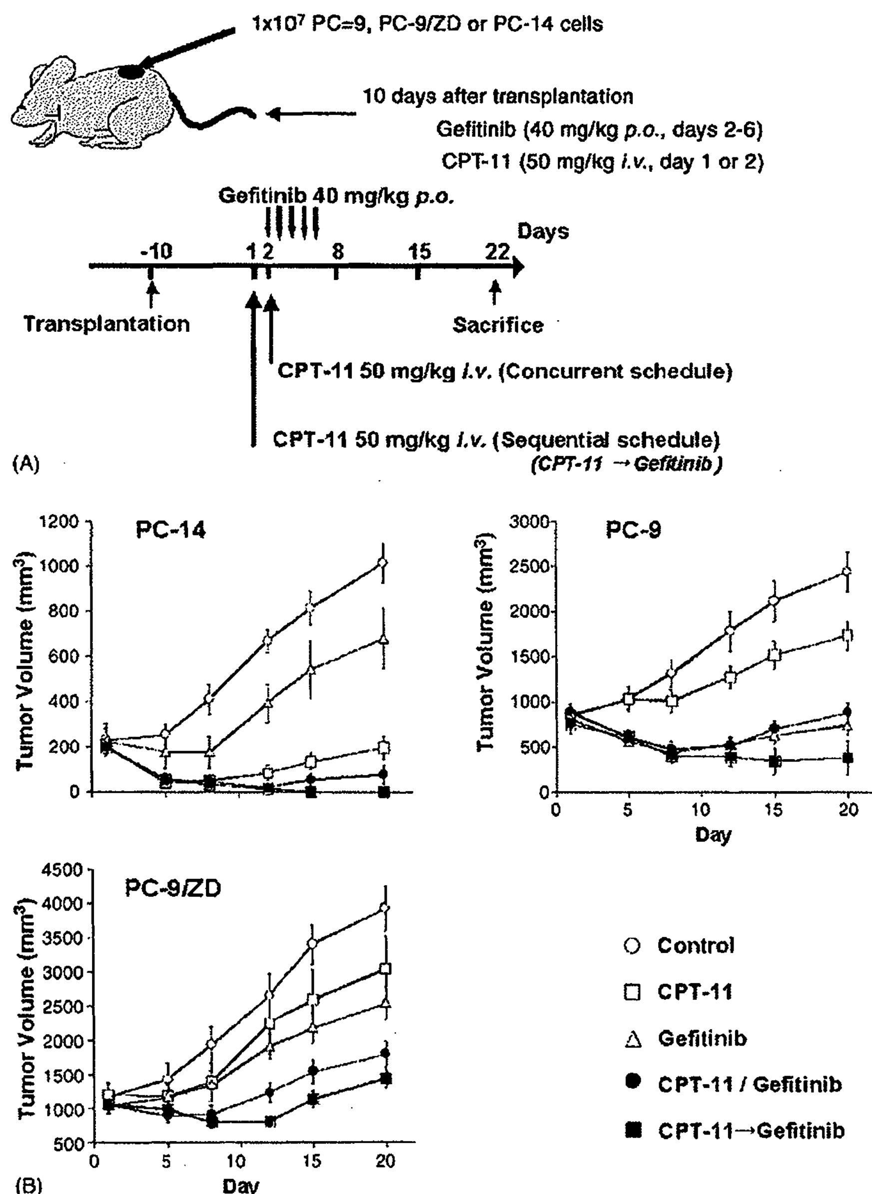

Fig. 2 Dose-dependent effects of combination therapy in PC9 and PC9/ZD cells in vivo. (A) Treatment schedule; (B) significant tumor growth-inhibition was observed in mice treated with the combination of gefitinib and CPT-11. Mice were allocated to five groups (6 mice/group) (○: 5% (w/v) glucose solution; □: CPT-11 50 mg/kg; △: gefitinib 40 mg/kg; ■: ZD1839 40 mg/kg + CPT-11 50 mg/kg concurrently; ●: CPT-11 50 mg/kg followed by ZD1839 40 mg/kg). (C) Treatment-related body weight loss in mice treated with gefitinib and/or SN-38. (○: 5% (w/v) glucose solution; □: CPT-11 50 mg/kg; △: ZD1839 40 mg/kg; ■: ZD1839 40 mg/kg + CPT-11 50 mg/kg concurrently; ●: CPT-11 50 mg/kg followed by ZD1839 40 mg/kg). Bars: ±S.D.

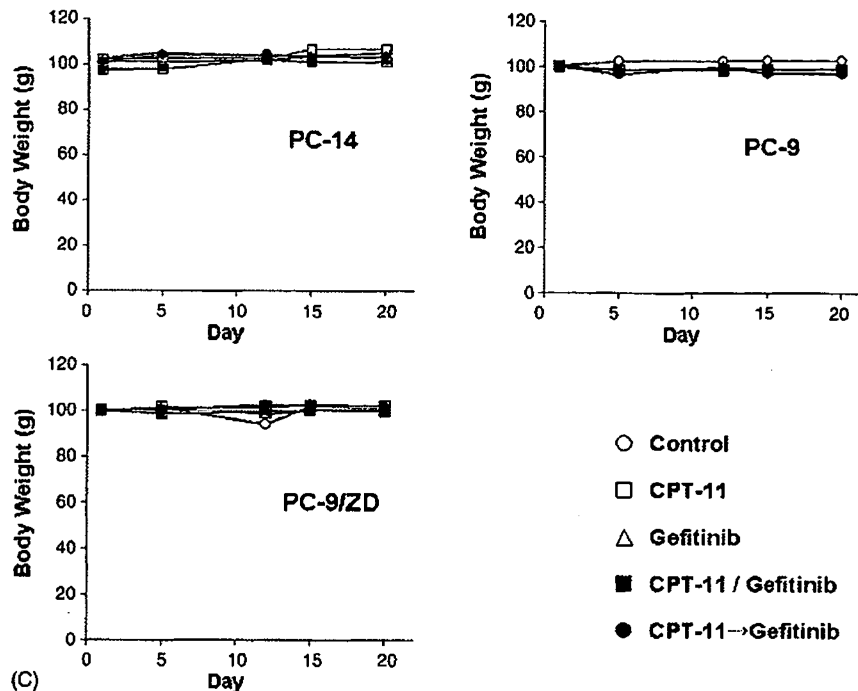

Fig. 2 *(Continued)*.

ment of lung cancers expressing wild-type EGFR is a major obstacle. Combined therapies are still considered to be a major strategy against lung cancer expressing wild-type EGFR. Our previous preclinical study demonstrated that gefitinib and CPT-11 have synergistic effects in colorectal cancer cell lines [14]. Here, we reevaluated the combined effects of gefitinib and cytotoxic agents based on the status of EGFR mutations in lung cancer.

We demonstrated that gefitinib and SN-38, the active form of CPT-11, have synergistic or additive effects in lung cancer cells expressing wild-type EGFR. The combination of gefitinib and CPT-11 may be useful against lung cancers expressing wild-type EGFR. On the other hand, this combination had antagonistic effects in PC-9 cells expressing mutant EGFR, even though PC-9 cells are basically hypersensitive to gefitinib alone.

The concurrent administration of gefitinib and SN-38 also had an antagonistic effect in the PC-9/ZD cells. The PC-9/ZD cells developed an acquired resistance to gefitinib after exposure to gefitinib in vitro. New treatment strategies for patients who are refractory to gefitinib treatment are clinically needed. We demonstrated that the sequential administration of SN-38 (CPT-11) and gefitinib improved the combined effects in PC-9/ZD cells both in vitro and in vivo.

The above results led us to propose a combined gefitinib and CPT-11 treatment strategy based on the EGFR mutation status of lung cancers: (1) combined treatment according to any schedule for lung cancers expressing wild-type EGFR, (2) gefitinib treatment alone for lung cancers expressing mutant EGFR, and (3) the sequential administration of gefitinib and CPT-11 for patients who are refractory to gefitinib treatment. Based on the above preclinical evidence, we are preparing to begin a clinical phase II trial for combined gefitinib and CPT-11 treatment in Japan.

We previously demonstrated that CPT-11 and gefitinib have a synergistic effect against colorectal cancer [14]. EGFR mutations are rarely observed in colorectal cancer cells [15]. Therefore, the combined effects of these agents against colorectal cancers were consistent with those against the lung cancers expressing wild-type EGFR in this study.

Different combined effects were observed for the concurrent and sequential schedules in vitro and in vivo. While the mechanisms responsible for the combined effects remain unclear, cell cycle distributions might explain some of the differences. In cells treated according to the sequential gefitinib followed by SN-38 (CPT-11) treatment schedule, treatment with gefitinib resulted in an increase in the G0—G1 phase and a decrease in the S phase populations (data not shown). The decreased S phase population was not sensitive to CPT-11 [16]. Thus, the antagonistic effects of the sequential administration of gefitinib followed by CPT-11 (SN-38) could be explained by this mechanism. On the other hand, in cells treated according to the sequential SN-38 followed by gefitinib treatment schedule, SN-38 treatment induced an increase in the S phase population. If the S phase population is sensitive to gefitinib, this might explain the synergistic effects of this sequential schedule [17]. An increase in EGFR phosphorylation induced by CPT-11 is another previously reported possible mechanism responsible for this synergistic action [14].

In conclusion, we demonstrated the different effect on lung cancer cell expressing mutant EGFR according to the

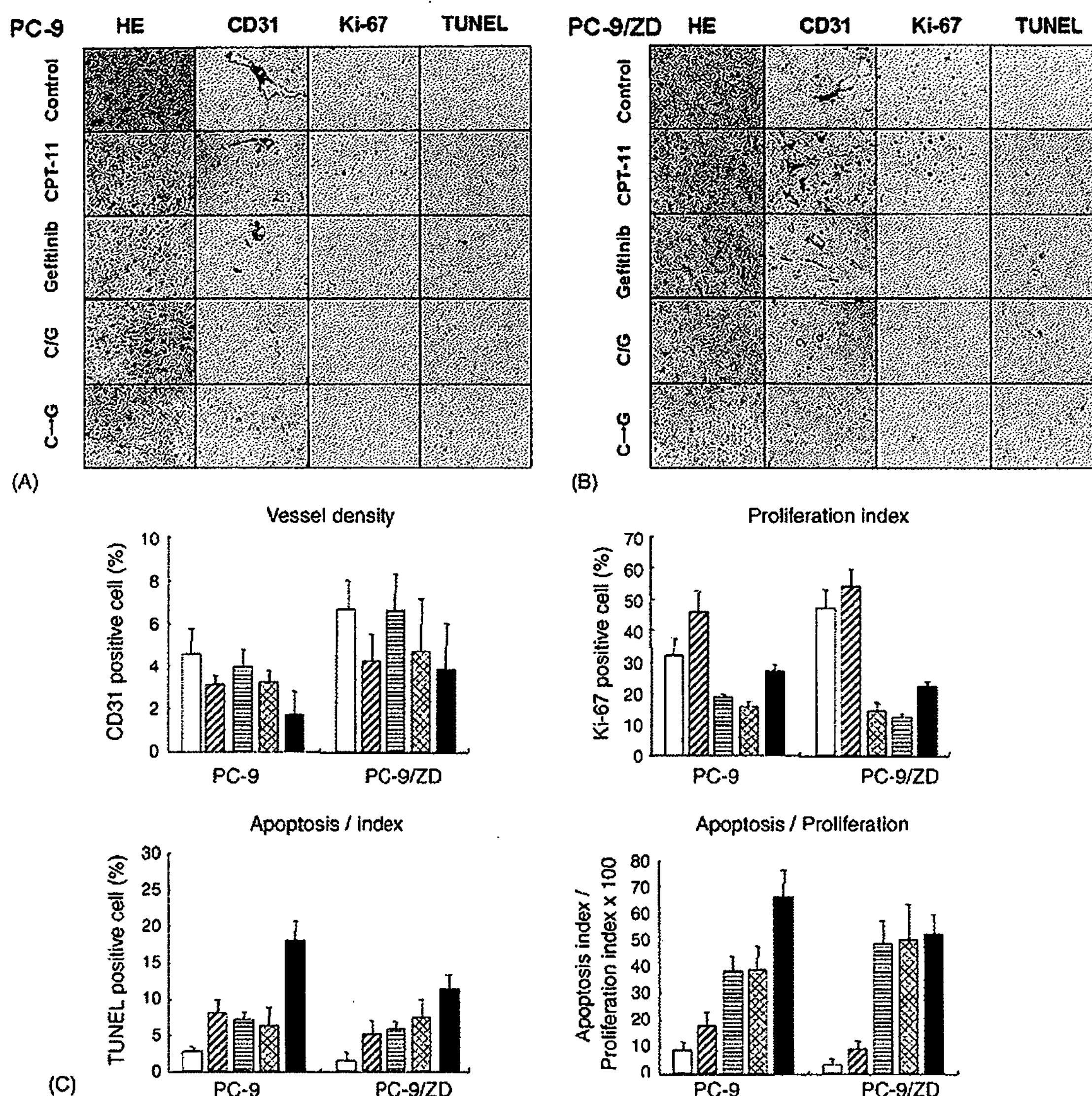

Fig. 3 (A) Historical examination of PC-9 tumor xenografts (day 22) stained with H&E, anti-CD31 vessel staining, TUNEL staining (magnification: 400×) and anti-Ki-67 nuclear antigen (magnification: 200×). The number of Ki-67-positive cells increased with the administration of CPT-11. The number of Ki-67-positive cells decreased with the gefitinib-alone and combination treatments. C/G: concurrent combination, C→G: sequential combination. (B) Historical examination of PC-9ZD tumor xenografts (day 22) stained with H&E, anti-CD31 vessel staining, TUNEL staining (magnification: 400×) and anti-Ki-67 nuclear antigen (magnification: 200×). The number of Ki-67-positive cells increased with the administration of CPT-11. The number of Ki-67-positive cells decreased with the gefitinib-alone and combination treatments. C→G: sequential combination; C/G: concurrent combination. (C) Quantitation of CD31 vessel staining, Ki-67 proliferation index, apoptosis index, and apoptosis: proliferation ratio. The columns represent the mean population of positive cells in five fields. Bars: ±S.D. Tumors from mice treated with vehicle (white), CPT-11 (diagonal hatched), Gefitinib (horizontal hatched), concurrent combination of CPT-11 plus Gefitinib (cross-hatched), or sequential combination of CPT-11 plus Gefitinib (cross-hatched).

combination schedule of gefitinib and CPT-11. The sequential combined treatment also active against lung cancer cell expressing wild-type EGFR.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Cancer Research and the 3rd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Tokyo, Japan.

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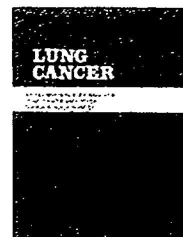

Eg5 expression is closely correlated with the response of advanced non-small cell lung cancer to antimitotic agents combined with platinum chemotherapy

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Received 28 February 2006; received in revised form 4 June 2006; accepted 22 June 2006

KEYWORDS
Eg5;
Antimitotic agents;
Platinum-based chemotherapy;
Advanced non-small cell lung cancer;
Cyclin B1

Summary
Background: Eg5 is a microtubule motor protein that functions in bipolar spindle assembly. We investigated the relationship between Eg5 expression and the response to chemotherapy of patients with advanced non-small cell lung cancer (NSCLC).
Patients and methods: Eg5 expression was investigated immunohistochemically in 122 formalin-fixed tumor samples from untreated stage IIIB or IV NSCLC patients. We also investigated cyclin B1 expression, which is involved in the G2/M transition. All patients received antimitotic agents combined with platinum chemotherapy. The response to chemotherapy was compared in relation to Eg5 and cyclin B1 expression and in relation to clinicopathological factors.
Results: The response rate to chemotherapy of patients with Eg5-positive tumors was 37%, as opposed to 10% for patients with Eg5-negative tumors, and Eg5 expression was significantly associated with the response to chemotherapy ($P = 0.002$). The response rate of patients with cyclin B1-positive tumors (53%) was higher than that of patients with cyclin B1-negative tumors (23%) ($P = 0.009$), and Eg5 expression was significantly correlated with cyclin B1 expression ($P = 0.005$). A multivariate analysis confirmed Eg5 status to be an independent variable related to response to chemotherapy ($P = 0.008$).
Conclusions: Eg5 expression can predict a response to antimitotic agents combined with platinum chemotherapy among patients with advanced NSCLC.
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doi:10.1016/j.lungcan.2006.06.018

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1. Introduction

Lung cancer is a major cause of death from cancer worldwide, and non-small cell lung cancer (NSCLC) accounts for ~85% of all cases of lung cancer. More than half of patients with NSCLC have advanced stage IIIB or IV disease at presentation, and patients with advanced NSCLC are candidates for systemic chemotherapy [1]. Meta-analyses have demonstrated that cisplatin-based chemotherapy for metastatic NSCLC statistically improves patient survival, compared with supportive care alone [2]. However, the response rate to chemotherapy has been poor, and very few patients survive for 5 years [3]. During the 1990s, five new drugs became available for the treatment of metastatic NSCLC: paclitaxel, docetaxel, vinorelbine, gemcitabine, and irinotecan. Each of these drugs has since been evaluated in combination regimens with cisplatin or carboplatin and has produced responses in 20–30% of patients [1]. Unfortunately, despite the increasing number of active chemotherapeutic agents, none of these chemotherapeutic regimens has offered a significant advantage over the others in the treatment of advanced NSCLC in randomized studies [4,5], and advanced NSCLC patients still have a median survival time of <1 year. Several reasons have been offered to explain the response to chemotherapy, such as the presence of drug-resistant tumor cells [6] and the redistribution of tumor cells within the cell cycle after chemotherapy. However, the molecular basis of the response to chemotherapy remains to be explored.

A network of microtrabecular filaments forms the cytoplasmic matrix, giving rise to the concept of the cytoskeleton, which comprises microtubules, actin, and intermediate filaments. Microtubules display a remarkable versatility of function and are involved in multiple biologic phenomena, including mitosis, cell shape determination, cell locomotion, and the movement of intracellular organelles [7]. Microtubule-polymerizing agents, including paclitaxel and docetaxel, and microtubule-depolymerizing agents, including vinorelbine, target preliminary tubulin and can induce disrupting kinetic stabilization of microtubules' polymerization–depolymerization, thus blocking the cell cycle in the mitotic phase [8].

Microtubule motors bind to and move unidirectionally on microtubules, and they have been proposed to generate the force required for spindle assembly and maintenance, attachment of the chromosomes to the spindle, and movement of chromosomes toward opposite poles. The microtubule motor proteins, which are members of the kinesin, dynein, or myosin families, can account for many of the movements of the spindle and chromosomes in dividing cells. Kinesin motors have been shown to be necessary to establish spindle bipolarity, position chromosomes on the metaphase plate, and maintain forces in the spindle [9]. Evidence that kinesin motors facilitate microtubule depolymerization also exists, raising the possibility that the motors modulate microtubule dynamics during mitosis. Eg5, which is a part of the kinesin-5 molecule (a member of the kinesin superfamily), is a microtubule motor protein. Eg5 accounts for many of the movements of the spindle and chromosomes in dividing cells and localizes to the spindle in mitotically dividing cells. It has been implicated in spindle function by both its cellular localization and the effects of mutations. Eg5 function in centrosome or spindle pole body separation is necessary for bipolar spindle assembly [10]. The latest antimitotic agent, named monastrol, is an inhibitor of mitotic kinesin Eg5 [11,12]. Monastrol arrests mitosis by reversibly inhibiting mitotic kinesin Eg5 and impairing bipolar mitotic spindle formation. Prolonged mitotic arrest leads to apoptosis in tumor cells and to senescence or apoptosis in primary cells, and the inhibition of mitotic kinesin Eg5 results in the formation of monoaster spindles leading to mitotic arrest [13].

Cyclin and cyclin-dependent kinase complexes play an important role in the control of the cell cycle [14], and the cyclin B1/cdc2 complex has a role as a maturation/mitosis-promoting factor in the G2–M phase transition during the cell cycle [15]. Thus, lack of regulation of cyclin B1 expression may be involved in uncontrolled cell growth and malignant transformation. Overexpression of cyclin B1 has been reported in various malignant tumors and has been shown to predict a poor outcome in NSCLC, esophageal carcinoma, and head and neck cancer [16–18].

In this retrospective study, we investigated the level of expression of Eg5, in addition to cyclin B1—a molecule involved in the G2/M transition, in clinical samples from patients with advanced NSCLC who were subsequently treated with antimitotic agents and investigated whether its expression predicts response to chemotherapy and outcome.

2. Materials and methods

2.1. Subjects

A total of 122 stage IIIB or IV NSCLC patients received platinum-based combination chemotherapy combined with docetaxel, paclitaxel or vinorelbine at the National Cancer Center Hospital East between August 1997 and July 2004 because of PS 0 or 1 on the Eastern Cooperative Oncology Group scale. Adequate tumor biopsy specimens were obtained from all 122 of these patients before chemotherapy and were analyzed in this study. All of the tumor specimens were obtained before chemotherapy, by bronchoscopy in 83 patients, by percutaneous needle biopsy in 31 patients, by thoracotomy in five patients, and by mediastinoscopy in three patients. The histological classification was based on the third edition of the WHO classification. Clinical staging was based on an initial evaluation consisting of a clinical assessment, chest radiography, computed tomography of the chest and abdomen, computed tomography or magnetic resonance imaging of the brain, and bone scintigraphy. The current international staging system was used for clinical disease staging [19]. The clinicopathological characteristics of all the patients are listed in Table 1. Their median age at diagnosis was 62 years (range, 42–78 years). Seven of the 43 stage IIIB patients were women, and 32 of the 79 stage IV patients were women. All of the patients were treated with antimitotic agents combined with platinum chemotherapeutic regimens in what were considered standard regimens for patients with metastatic NSCLC [20]. Nine of the 43 stage IIIB patients received thoracic radiotherapy after the completion of chemotherapy; three of these patients were women. The median follow-up time of the 122 patients was 26 months (range, 18–54 months).

Table 1 Characteristics of 122 patients with advanced NSCLC

Characteristics	No. of patients
Total no. of patients	122
Gender	
Male	83
Female	39
Age (years)	
Median	62
Range	42–78
Histology	
Adenocarcinoma	80
Squamous cell carcinoma	28
Large cell carcinoma	13
Others	1
Stage	
IIIB	43
IV	79
Performance status	
0	32
1	90
Chemotherapeutic regimen	
Cisplatin + vinorelbine	76
Cisplatin + docetaxel	20
Carboplatin + paclitaxel	26
Smoking history	
Positive	91
Negative	31

NSCLC: non-small cell lung cancer.

After obtaining informed consent in accordance with our institution's guidelines, all of the patients underwent a tumor biopsy and chemotherapy.

2.2. Chemotherapy

The platinum-based regimens were vinorelbine (25 mg/m^2) on days 1 and 8 plus cisplatin (80 mg/m^2) on day 1 of a 21-day cycle (76 patients), docetaxel (60 mg/m^2) on day 1 plus cisplatin (80 mg/m^2) on day 1 of a 21-day cycle (20 patients), and paclitaxel (200 mg/m^2 administered over 3 h) on day 1 plus carboplatin (dosed with an area under the curve of 6) on day 1 of a 21-day cycle (26 patients). All of the patients received two or more courses of chemotherapy before the appearance of progressive disease. We used the RECIST guidelines [21] to evaluate the response to chemotherapy. A complete response was defined as the disappearance of all clinically detectable lesions for at least 4 weeks. A partial response required a minimum of a 30% reduction in the greatest diameter of all of the measurable lesions for a minimum of 4 weeks. Progressive disease was defined as the appearance of new lesions or an increase in disease of >20% measured in the same manner as for partial response. All other results were classified as "no change". The response rate was defined as the total of the complete response cases and partial response cases expressed as a percentage of all cases. PFS (progression-free survival) was measured from the start of chemotherapy until the documentation of progressive disease or death.

2.3. Immunohistochemistry

Immunostaining was performed on 4-μm formalin-fixed, paraffin-embedded tissue sections. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series. For antigen retrieval, the slides for cyclin B1 were immersed in 10 mM citric buffer solution (pH 6.0) and the slides for Eg5 were immersed in 1 mM EDTA retrieval fluid (pH 8.0). All of the slides were heated to 95 °C by exposure to microwave irradiation for 20 min. The slides were then cooled for 1 h at room temperature and washed in water and PBS. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 15 min. Non-specific binding was blocked by preincubation with 2% BSA plus 0.1% NaN_3 for 30 min; after draining off the blocking serum, the slides were incubated overnight at 4 °C with anti-Eg5 monoclonal antibody (Clone, 20; Dilution, 1:50; BD Biosciences, NJ, USA) or with anti-cyclin B1 monoclonal antibody (Clone, 7A9; Dilution, 1:20; Novocastra Laboratories, Newcastle upon Tyne, UK). The slides were then washed three times in PBS and incubated with a labeled polymer Envision+ (DAKO, Glostup, Denmark) for 60 min. The chromogen used was 2% 3,3′-diaminobenzidine in 50 mM Tris buffer (pH 7.6) containing 0.3% hydrogen. Slides were counterstained with hematoxylin [22,23]. Normal human lung tissue was used as a positive control.

Eg5 staining was considered positive if the cytoplasm of >10% of the tumor cells stained positive. Cyclin B1 staining was considered positive if the nuclei of >10% of the tumor cells stained positive, because the cyclin B1/cdc2 complex translocates from the cytoplasm into the nucleus during the G2/M transition [24–26]. Thus, the criteria for cyclin B1 positivity used in the present report differed from those used in other reports on non-small cell lung cancer, esophageal carcinoma and head and neck cancer. All of the slides were examined and scored independently by two observers (T.S. and G.I.) who had no knowledge of the patients' clinical data. When the antibody evaluations differed between the observers, the observers discussed the results, with or without re-evaluating the slides, until an agreement was reached.

2.4. Statistical analysis

The correlations between immunohistochemical expression and the clinical variables and response to chemotherapy were evaluated by the χ^2-test or Fisher exact test, as appropriate. PFS was used as a clinical marker for duration of response to chemotherapy. Overall survival was measured from the start of chemotherapy to the date of death from any cause or the date the patient was last known to be alive. Survival curves were estimated using the Kaplan–Meier method, and any differences in PFS and survival between the subgroups were compared by using the log-rank test. The Cox proportional hazards model was used for a multivariate analysis. A multivariate analysis examining the correlation between variables and response to chemotherapy was performed by using logistic regression. P values <0.05 were

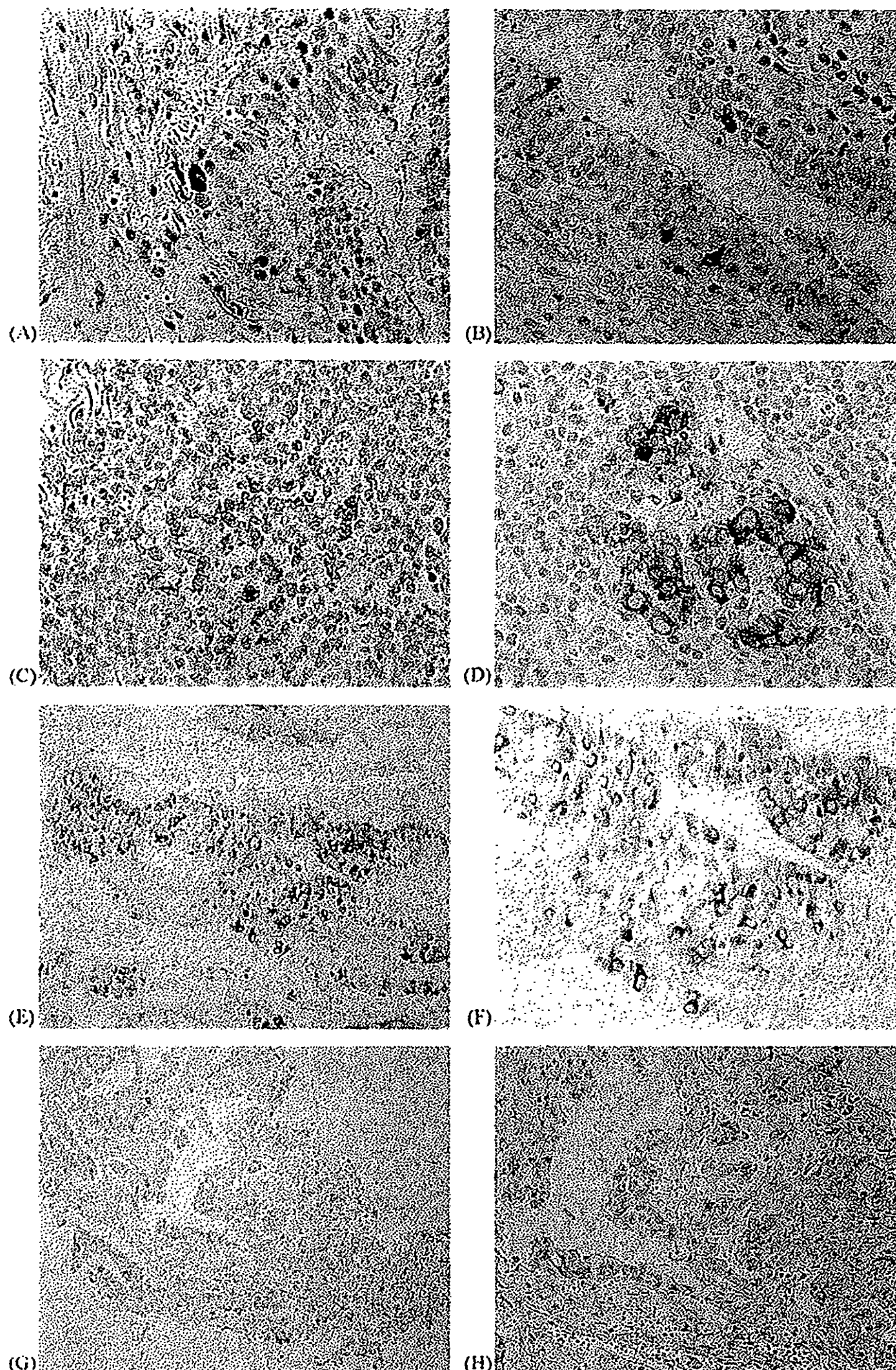

Fig. 1 (A–D) Immunohistochemical staining of Eg5 in normal lung tissue (A), Eg5 is present in part of the basal layer of the bronchial epithelium in this frozen section of normal lung tissue (400×). (B) Eg5 is also present in parts of the basal layer of the bronchial epithelium in this formalin-fixed, paraffin-embedded section of normal lung tissue (400×). (C) Eg5 expression is visible in germinal center lymphocytes giving rise to follicular hyperplasia in this frozen section of normal lung tissue (400×). (D) Eg5 expression is also visible in germinal center lymphocytes giving rise to follicular hyperplasia in this formalin-fixed, paraffin-embedded section of normal lung tissue (400×). (E–H) Immunohistochemical staining of Eg5 in NSCLC (E), low magnification (100×) of squamous cell carcinoma of the lung showing Eg5 immunoreactivity (F), high magnification (200×) of squamous cell carcinoma of the lung showing Eg5 immunoreactivity (G), Eg5 staining was considered to be negative in this adenocarcinoma of the lung: the cytoplasm of <10% of the tumor cells were stained (low magnification; 100×). (H) Eg5 staining was considered to be negative in this adenocarcinoma of the lung: the cytoplasm of <10% of the tumor cells were stained (high magnification; 200×).

considered significant. Two-sided statistical tests were used in all of the analyses. Statistical analysis software (StatView-J Ver. 5.0, Windows) was used for the analyses.

3. Results

3.1. Expression of Eg5 in normal lung tissue

To investigate the validation of immunostaining in the present experiment, we first evaluated Eg5 immunostaining in frozen sections and paraffin-embedded tissue sections of surgical specimens and confirmed that the staining intensity and specificity in the paraffin-embedded tissue sections were almost the same as in the frozen sections. Next, to choose the criteria for immunohistochemical positivity, normal lung tissue was used for Eg5 immunohistochemical staining. Representative immunohistochemical Eg5 staining in normal lung tissue is shown in Fig. 1A—D. In normal lung tissue, Eg5 expression was observed in some of the cells in the basal layer of the bronchial epithelium (Fig. 1A and B) and in germinal center lymphocytes exhibiting follicular hyperplasia (Fig. 1C and D). The frequency of positivity for bronchial epithelial cells and lymphoid germinal center lymphocytes were roughly more than 50% and 90%, respectively. We used these tissues as positive controls. Eg5 immunoreactivity was not detected in the pulmonary parenchyma.

3.2. Expression of Eg5 in NSCLC

The tumors of 82 (67%) of the 122 patients were Eg5 positive. Cytoplasmic staining was observed in most of the Eg5-positive tumors, but some tumors also showed nuclear staining. The median of the percentage staining of the lung cancer cells for Eg5 was 35% (range, 0—100%). Representative immunohistochemical Eg5 staining in NSCLC is shown in Fig. 1E—H. Fig. 1E and F shows the staining results for an Eg5-positive squamous cell carcinoma of the lung. The cytoplasm of almost 80% of the cancer cells stained positive for Eg5. Fig. 1G and H shows an Eg5-negative adenocarcinoma of the lung; this adenocarcinoma of the lung was judged to be negative for Eg5 because the cytoplasm of <10% of the tumor cells showed evidence of staining.

The relationships between the expression of Eg5 and clinical variables are shown in Table 2. Eg5 expression was significantly higher in males than in females ($P = 0.03$), in squamous cell carcinoma than in non-squamous cell carcinoma ($P = 0.02$), and in current and former smokers than in non-smokers ($P = 0.03$).

The tumors of 18 (95%) of the 19 patients with cyclin B1-positive tumors were Eg5 positive, and the tumors of 39 (98%) of the 40 patients with Eg5-negative tumors were cyclin B1-negative (data not shown). Eg5 expression was significantly correlated with cyclin B1 expression ($P = 0.005$; data not shown).

3.3. Expression of Eg5 and clinical outcome

All 122 patients were assessed for response to chemotherapy and survival. The relationships between clinical variables, Eg5 expression, and cyclin B1 expression, and the response to chemotherapy and survival in this study are shown in Table 3.

The chemotherapy response rate of patients with Eg5-positive tumors was 37%, as opposed to 10% for patients with Eg5-negative tumors. Eg5 expression was significantly associated with response to chemotherapy ($P = 0.002$). The chemotherapy response rate of patients with cyclin B1-positive tumors was 53%, as opposed to 23% for patients

Table 2 Relationship between clinical variables and expression of primary antibodies

	n	Eg5-positive (%) patients	Cyclin B1-positive (%) patients
Total	122	82 (67)	19 (16)
Gender			
Male	83	61 (73)*	15 (18)
Female	39	21 (54)	4 (10)
Histology			
Sq	28	24 (86)**	6 (21)
Non-sq	94	58 (62)	13 (14)
Stage			
IIIB	43	30 (70)	8 19)
IV	79	52 (66)	11 (14)
PS			
0	32	20 (63)	1 (3)
1	90	62 (69)	18 (20)**
Smoking history			
Positive	91	66 (73)*	17 (19)
Negative	31	16 (52)	2 (6)

Sq: squamous; PS: performance status.
* $P = 0.03$.
** $P = 0.02$.

Table 3 Summary of the relationships between clinical variables and response to chemotherapy and survival

	n	Response rate (%)	P	PFS (months)	P	MST (months)	P
Total	122	28		5.0		12.0	
Gender							
Male	83	28	0.95	5.0	0.43	10.0	0.046
Female	39	28		7.0		15.0	
Histology							
Sq	28	32	0.57	5.0	0.72	9.0	0.64
Non-sq	94	27		5.0		13.0	
Stage							
IIIB	43	33	0.39	6.0	0.01	17.0	0.07
IV	79	25		5.0		11.0	
PS							
0	32	25	0.67	5.0	0.21	14.0	0.16
1	90	29		5.0		10.0	
Smoking history							
Positive	91	27	0.87	5.0	0.23	10.0	0.035
Negative	31	29		6.0		15.0	
Eg5							
Positive	82	37	0.002	5.0	0.08	10.0	0.006
Negative	40	10		6.0		13.0	
Cyclin B1							
Positive	19	53	0.009	5.0	0.77	8.0	0.31
Negative	103	23		5.0		13.0	

PFS: progression-free survival; MST: median survival time.

with cyclin B1-negative tumors, and cyclin B1 expression was also significantly associated with response to chemotherapy ($P = 0.009$).

The each of PFS and overall survival curves calculated using the Kaplan–Meier method according to Eg5 expression was shown in Fig. 2. The median PFS time for the Eg5-negative group was 6.0 months, as opposed to 5.0 months for the Eg5-positive group (Fig. 2A). The median survival time for the Eg5-negative group was 13.0 months, as opposed to 10.0 months for the Eg5-positive group (Fig. 2B). According to the overall survival data, the Eg5-positive group had a significantly poorer outcome than the Eg5-negative group ($P = 0.006$).

The median PFS time in both the cyclin B1-negative and the cyclin B1-positive group was 5.0 months (Fig. 2C). The median survival time in the cyclin B1-negative group was 13.0 months, as opposed to 8.0 months in the cyclin B1-positive group (Fig. 2D). Cyclin B1 expression was not associated with PFS or overall survival. Among the clinical variables, gender and smoking history were significantly associated with overall survival, and disease stage was significantly associated with PFS, also.

3.4. Multivariate analysis for response to chemotherapy, PFS, and overall survival

Following the univariate analyses for response to chemotherapy, PFS, and overall survival, we performed multivariate analyses. Table 4 shows the results of the multivariate analysis for response to chemotherapy, PFS, and overall survival. The multivariate analysis for response to chemotherapy was performed using logistic regression to determine the prognostic value of Eg5 when other prognostic factors were considered. A multivariate analysis that included gender, histology, stage, PS, smoking history, Eg5 expression and cyclin B1 expression, showed that Eg5 expression was the only significant independent variable correlated with response to chemotherapy ($P = 0.008$).

A multivariate analysis using the Cox proportional hazards model for PFS and overall survival was performed, using gender, histology, stage, PS, smoking history, Eg5 expression and cyclin B1 expression, as variables. No correlation between variables and PFS was found in the multivariate analysis. Stage was the only independent variable significantly correlated with overall survival ($P = 0.036$).

4. Discussion

This is the study to investigate the relationship between the level of expression of Eg5 and the clinical response to chemotherapy and outcome of previously untreated patients with advanced NSCLC. Eg5, a kinesin motor, accounts for many of the movements of the spindle and chromosomes in dividing cells. It localizes to the spindle in mitotically dividing cells and has been implicated in spindle function by both its cellular localization and the effects of mutations.

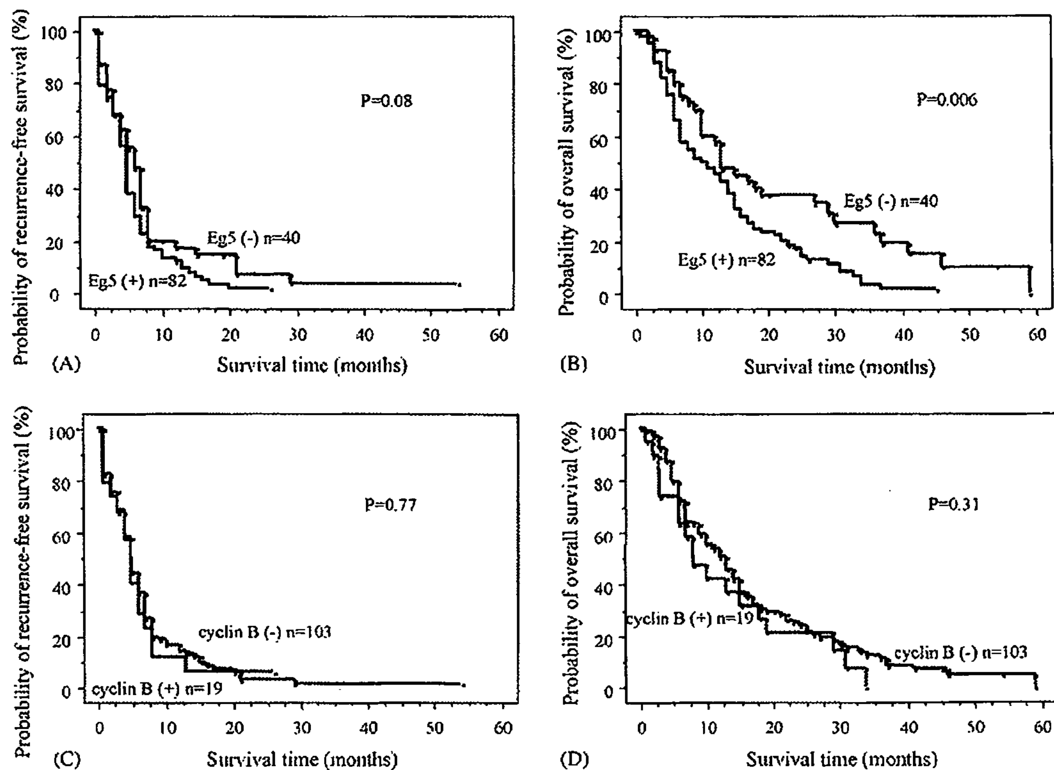

Fig. 2 (A) Progression-free survival curves of 122 patients with advanced non-small cell lung cancer, according to Eg5 expression. The median progression-free survival periods of Eg5-negative and -positive patients were 6.0 and 5.0 months, respectively. (B) Overall survival curves for 122 patients with advanced non-small cell lung cancer, according to Eg5 expression. The median survival periods for Eg5-negative and -positive patients were 13.0 and 10.0 months, respectively. (C) Progression-free survival curves of 122 patients with advanced non-small cell lung cancer, according to cyclin B expression. The median progression-free survival periods of Eg5-negative and -positive patients were 5.0 and 5.0 months, respectively. (D) Overall survival curves for 122 patients with advanced non-small cell lung cancer, according to cyclin B1 expression. The median survival periods for cyclin B1-negative and -positive patients were 13.0 and 8.0 months, respectively.

Eg5 function in centrosome or spindle pole body separation is necessary for bipolar spindle assembly [10].

In normal lung tissue, Eg5 expression was found to be present in some of the cells in the basal bronchial layer of the bronchial epithelium, but its expression in this region was not as strong as in lung cancer tissue. The overexpression of cyclin B1 has been reported in various malignant tumors and has been shown to predict a poor outcome in patients with NSCLC, esophageal carcinoma, and head and neck cancer [16—18]. It has been postulated that the overexpression of cyclin B1 is involved in uncontrolled cell growth and the malignant potential of carcinoma cells. Since the expression of Eg5 in lung cancer tissue has been found to be correlated with the expression of cyclin B1, lung cancer tissue that overexpresses Eg5 in comparison with normal lung tissue is assumed to have greater malignant potential than lung cancer tissue that does not.

Eg5 expression before chemotherapy was correlated with response to chemotherapy and Eg5 status was found to be an independent prognostic factor of response to chemotherapy in a multivariate analysis. Further investigation showed that Eg5 expression was correlated with the response to each type of regimen: the taxan regimens (CDDP + docetaxel: $n = 20$; CBDCA + paclitaxel: $n = 26$; $P = 0.046$), and the vinca alcaroid regimen (CDDP + vinorelbine: $n = 76$; $P = 0.02$) (data not shown). The mechanisms by which Eg5 overexpression affects chemotherapy have not been fully elucidated; nevertheless, Marcus et al. [27] recently reported that mitotic kinesin Eg5 inhibitors induce mitotic arrest and cell death in both paclitaxel-resistant and paclitaxel-sensitive cancer cells and that Eg5 was required for paclitaxel-induced microtubule aster formation (multi-polar spindle configuration) in an *in vitro* assay. They suggested that Eg5 functionality is necessary for paclitaxel-induced mitotic arrest and cell death. These findings may explain our result that Eg5 overexpression before chemotherapy was significantly correlated with response to chemotherapy. The results for docetaxel can be explained in the same manner as for paclitaxel because their modes of action are the same. On the other hand, vinorelbine inhibits the polymerization of tubulin. We suspect that some unknown interaction between tubulin and Eg5 may be modified by vinca alcaloids.

Although Eg5 expression was significantly correlated with response to chemotherapy, the Eg5-positive cases tended to have a poorer outcome in terms of overall survival than the Eg5-negative cases. The reason why the Eg5-positive cases had a poorer outcome remains unclear; despite their higher response to antimitotic agents, Eg5-positive cells may have

Table 4 Multivariate analysis

Variables	Category	Risk ratio	95% CI	*P*
Multivariate analysis for response of advanced NSCLC patients				
Gender	Male vs. female	0.77	0.245–2.42	0.66
Histology	Sq vs. non-sq	0.89	0.31–2.57	0.83
Stage	IIIB vs. IV	0.64	0.25–1.65	0.35
PS	0 vs. 1	0.98	0.34–2.82	0.97
Smoking history	(–) vs. (+)	0.59	0.18–1.95	0.39
Eg5	(–) vs. (+)	5.16	1.54–17.29	0.008
Cyclin B1	(–) vs. (+)	2.82	0.94–8.45	0.06
Multivariate analysis for PFS of advanced NSCLC patients				
Gender	Male vs. female	0.90	0.56–1.45	0.67
Histology	Sq vs. non-sq	0.89	0.55–1.43	0.63
Stage	IIIB vs. IV	0.60	0.39–0.93	0.02
PS	0 vs. 1	0.92	0.59–1.45	0.72
Smoking history	(–) vs. (+)	0.84	0.51–1.39	0.50
Eg5	(–) vs. (+)	0.77	0.50–1.19	0.24
Cyclin B1	(–) vs. (+)	1.09	0.62–1.89	0.77
Multivariate analysis for OS of advanced NSCLC patients				
Gender	Male vs. female	0.74	0.44–1.26	0.27
Histology	Sq vs. non-sq	1.03	0.63–1.67	0.92
Stage	IIIB vs. IV	0.63	0.41–0.98	0.04
PS	0 vs. 1	0.76	0.47–1.22	0.25
Smoking history	(–) vs. (+)	0.74	0.43–1.30	0.30
Eg5	(–) vs. (+)	0.62	0.39–0.97	0.04
Cyclin B1	(–) vs. (+)	1.03	0.59–1.78	0.93

PFS: progression-free survival; NSCLC: non-small cell lung cancer; PS: performance status; CI: confidence interval; OS: overall survival.

a higher malignant potential, contributing to a poor clinical outcome. This appears to be consistent with the expression of Eg5 being significantly correlated with the expression of cyclin B1, which may be involved in uncontrolled cell growth and the malignant potential of cancer cells.

The inhibition of Eg5 has recently been exploited as an aid to cancer treatment [12–14,27–32], and small cell-permeable molecules that inhibit mitotic kinesin Eg5 and do not target tubulin arrest cells in mitosis with monoastral spindles. Chromosomes in Eg5 inhibitor-treated cells frequently have both sister kinetochores attached to microtubules extending to the center of the monoaster. The mitotic kinesin Eg5 inhibitor also induces apoptosis and is effective in inhibiting the proliferation of cancer cells through mitotic arrest. The first small molecule inhibitor of Eg5 was monastrol [11,12], and second-generation Eg5 inhibitors like CK0106023 [29] and HR22C16 [27], which are specific allosteric inhibitors of Eg5 and exhibit anti-tumor activity *in vivo* or *in vitro*, have been discovered by drug screens. Therapeutic intervention with Eg5-specific inhibitors has also been reported, and SB-715992 has been shown to be a potent inhibitor of mitotic kinesin Eg5. Eg5 inhibitors may be used as new antimitotic agents to treat advanced NSCLC in the future.

In conclusion, our findings indicated that the expression of the mitotic kinesin Eg5 can predict a response to antimitotic agents combined with platinum chemotherapy among patients with advanced NSCLC. Our results have important implications for the treatment of NSCLC because Eg5 inhibitors, which cause tumor cell apoptosis, may be effective in patients with advanced NSCLC.

Acknowledgements

The authors thank Fumiko Koh for performing data management and Yukinori Murata for providing technical assistance.

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Int. J. Cancer: **118**, 180–184 (2006)
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EGFR and ErbB2 mutation status in Japanese lung cancer patients

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Much evidence has accumulated that the epidermal growth factor receptor *(EGFR)* and its family members are strongly implicated in the development and progression of lung cancers. Somatic mutations of the *EGFR* gene were found in about 25–40% of Japanese lung cancer patients. More recently, *erbB2* mutations are found in about 4% of European-derived lung cancer patients. We have investigated *EGFR* and *erbB2* mutation status in 95 surgically treated nonsmall cell lung cancer (NSCLC) cases from Nagoya City University Hospital. Seventy-five adenocarcinoma cases were included. The presence or absence of *EGFR* and *ernB2* mutations of kinase domains were analyzed by reverse transcription polymerase chain reaction (RT-PCR) amplifications and direct sequences. We have also investigated *erbB2* mutation status in 27 surgically treated NSCLC cases followed by treatment with gefitinib from Kinki-chuo Chest Medical Center. *EGFR* mutations (CTG→CGG; L858R) were found from 14 of 95 lung cancer patients. We also detected the deletion 1a-type mutations from 9 patients and deletion 4-type mutations from 6 patients in exon 19. In exon 20, 4 mutations including 2 novel mutations were found. Total *EGFR* mutations were present in 35 patients (36.8%). These mutation statuses were significantly correlated with gender (women 73.3% *vs.* men 20%, $p < 0.0001$), smoking status (never smoker 69.4% *vs.* smoker 16.9%, $p < 0.0001$), pathologic subtypes (adenocarcinoma 45.1% *vs.* nonadenocarcinoma 12.5%, $p = 0.0089$) and differentiation status of the lung cancers (well 51% *vs.* moderately or poorly 18.4%, $p = 0.0021$). On the other hand, *erbB2* mutation was only found from 1 of 95 patients, at exon 20. This patient was female and a never smoker with adenocarcinoma. This 12 nucleotide insertion mutation (2324–2325 ins ATACGTGATGGC) was located in the exon 20 at kinase domain (775–776 ins YVMA). There was no *erbB2* mutation in 27 gefitinib-treated NSCLC patients. In total, we have found only 1 *erbB2* mutation from 122 (0.8%) Japanese NSCLC patients. There was a significantly higher erbB2 positive (2+/3+) ratio in *EGFR* mutant patients (13/25, 52.0%) compared to *EGFR* wild-type patients (10/62, 16.1%; $p = 0.0247$). The NSCLC specimen with *erbB2* mutation showed 1+ immunoreactivity. The *EGFR* mutation status might correlate with the clinicopathologic features related to good response to gefitinib, such as gender, smoking history and pathologic subtypes of lung cancers. However, *erbB2* mutation is rare from Japanese lung cancer and is of limited value for molecular target therapy.
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Key words: EGFR; lung cancer; mutations; erbB2; Japanese

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy.[1] Lung cancer was the leading indication for respiratory surgery (42.2%) in 1998 in Japan.[2] More than 15,000 patients underwent surgical operations at Japanese institutions in 1998.[2] The clinical behavior of the lung cancer is largely associated with its stage. The cure of the disease by surgery is only achieved in cases representing an early stage of lung cancer.[3]

There is much accumulated evidence that epidermal growth factor receptor *(EGFR)* and its family members are strongly implicated in the development and progression of numerous human tumors, including lung cancer.[4,5] The erbB family comprises 4 structurally related receptors: ErbB1 (EGFR), ErbB2 (HER2-neu), ErbB3 and ErbB4. On ligand stimulation, the receptor forms either homodimers or heterodimers, which activate their cytoplasmic domain. This tyrosine-auto-phosphorylated region functions as a docking site for messenger proteins, which initiate cascades of cytoplasmic and nuclear mitogenic pathways.[6] Inhibition of this pathway is facilitated by several newly developed compounds that have shown promising results in preclinical and clinical trials.[7] The *EGFR* tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of nonsmall cell lung cancer (NSCLC) since 2002. Trastuzumab is a recombinant DNA-derived monoclonal antibody that selectively binds to p185 HER2, the protein product of *erbB2*. Trastuzumab was approved for breast cancer[8] and clinical trials for NSCLC is underway.[9,10]

Recently, we have found that novel *EGFR* mutations' status at ATP binding pockets in Japanese NSCLC patients were correlated with the clinicopathologic features related to good response to gefitinib.[11] These *EGFR* mutations are predominantly found in Japanese lung cancer patients (about 25%) when compared to USA patients (about 8%[12–14] to 10%[15]). Kasaoka *et al.* have reported that the *EGFR* mutation ratio is 40% of Japanese lung cancer patients.[16] Actually, *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy *in vivo* and *in vitro*.[11–13] More recently, it has been reported that novel *erbB2* mutations at kinase domain were found in 4% of European-derived NSCLC patients.[17]

To determine the *EGFR* and *erbB2* mutation status in Japanese lung carcinoma for screening purposes, we investigated *EGFR* and *erbB2* mutation status by the RT-PCR amplifications and direct sequences. The findings were compared to the clinicopathologic features of lung cancer.

Material and methods

Study subjects

The study group included 95 lung cancer patients who had undergone surgery (but did not receive gefitinib) at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2002. We have also investigated erbB2 mutation status for 27 NSCLC patients who had undergone surgery followed by treatment with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center. The lung tumors were classified according to the general rule for clinical and pathologic record of lung cancer in Japan.[18] All tumor samples were immediately frozen and stored at −80°C until assayed.

The clinical and pathologic characteristics of the 95 lung cancer patients are as follows: 52 cases at stage I, 9 at stage II and 34 at stage III–IV. The mean age was 64.9 years (range, 42–82). Among the 95 lung cancer patients, 71 (74.7%) were diagnosed as adeno-

Grant sponsor: Grant-in-Aid for Cancer Research (16-1) from the Ministry of Health, Labour and Welfare; Grant sponsor: AstraZeneca Research Grant 2004.
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Received 11 March 2005; Accepted after revision 18 April 2005
DOI 10.1002/ijc.21301
Published online 7 July 2005 in Wiley InterScience (www.interscience.wiley.com).

UICC Publication of the International Union Against Cancer
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carcinoma, 17 (17.9%) were squamous cell carcinoma and 4 (4.2%) were adenosquamous cell carcinoma. The samples from these patients had never been sequenced for *EGFR* before.

PCR assays for EGFR and erbB2

Total RNA was extracted from lung cancer tissues and adjacent nonmalignant lung tissues using Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 200 ng/ml. About 10 cases were excluded because tumor cells were too few to sufficiently extract tumor RNA. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD) with 0.5 μg oligo $(dT)_{12-16}$ (Amersham Pharmacia Biotech, Piscataway, NJ). The reaction mixture was incubated at 42°C for 50 min and then at 72°C for 15 min. We then used 1 μl of each DNA for PCR analyses. The PCR reactions were performed using LA-Taq kit (Takara Bio, Shiga, Japan) in a 25 μl reaction volume. The primer sequences for *EGFR* gene for kinase domain (exons 18–21) were as follows: the forward primer, 5-CTCTTACACCCAGTGGAGAA-3 and the reverse primer, 5-CATCCACTTGATAGGCACTT-3 (572 bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 40 sec, 60°C for 40 sec, 72°C for 45 sec The primer sequences for *erbB2* gene for kinase domain (exons 19–22) were as follows: the forward primer, 5-CGCTTTTGGCACAGTCTACA-3 and the reverse primer, 5-GGGATCCCATCGTAAGGTTT-3 (594bp). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 40 sec, 60°C for 40 sec, 72°C for 45 sec. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA). Amplified cDNAs were separated on 1% agarose gels, and the bands were visualized by ethidium bromide and photographed under ultraviolet transillumination. These samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan, Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review.

Immunohistochemistry

Tissue blocks were cut into 4 mm sections and mounted on silane-coated slides. The slides were then deparaffinized in xylene, dehydrated in a grade alcohol series and blocked for endogenous peroxidase with 3% H_2O_2 in absolute methanol. After microwave pretreatment in Blockace solution, immunostaining was done at 4°C overnight with a rabbit polyclonal c-erbB2 oncoprotein antibody (A04085, DakoCytomation, Glostrup, Denmark) at a 1:200 dilution. The expression of erbB2 was scored as follows: −, no discernible staining or <10% of cell stained; 1+, >10% of cytoplasmic staining or plasma membrane staining with weak intensity; 2+, >10% of plasma membrane staining with moderate intensity; and 3+, >10% of plasma membrane staining with strong intensity.

Statistical methods

Statistical analyses were done using the Mann-Whitney U-test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and χ^2test. The overall survival of lung cancer patients was examined by the Kaplan-Meier methods, and differences were examined by the log-rank test. All analysis was done using the Stat-View software package (Abacus Concepts, Berkeley, CA) and was considered significant when the *p*-value was less than 0.05.

Results

EGFR gene mutation status in Japanese lung cancer patients

Using the primer sets for *EGFR* kinase domain, a PCR product of 572 bp was obtained. When we visualized the PCR products with 1% agarose gel, these samples were further studied. In exon 18, there was no G719S mutation found from this study. In exon 19, 9 patients had the del 1a type mutation, 6 patients had the deletion 4 type mutation and 1 patient had the del 1b type mutation. Seven were male and 10 were female. Thirteen were nonsmokers and 4 were smokers. Fifteen patients had adenocarcinoma, 1 had squamous cell carcinoma and 1 had adenosquamous cell carcinoma. Three of the tumors were moderately differentiated, 2 were poorly differentiated and 11 were well differentiated. Five of 15 adenocarcinomas showed bronchioloalveolar carcinoma (BAC) pattern at the edge of tumor. Thus *EGFR* mutation status at exon 19 was significantly correlated with gender (p = 0.0172) and tobacco-smoking (p = 0.0008) but not with pathologic stages (stage I *vs.* II–IV, p = 0.9144), subtypes (adenocarcinoma *vs.* nonadenocarcinoma, p = 0.2675) and differentiation of lung cancer (well *vs.* moderately or poorly differentiated, p = 0.3812).

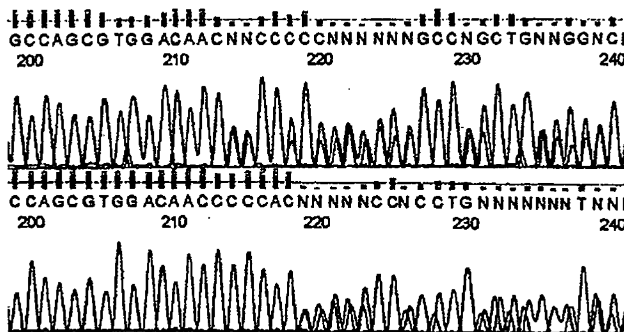

FIGURE 1 – Novel EGFR mutation at exon 20. Top: a male well-differentiated adenocarcinoma patient had the novel 2312–2313 insertion CAA. Bottom: a female, well-differentiated adenocarcinoma patient had the novel 2319–2320 insertion AACCCCCAC.

In exon 20, 3 patients had theheterozygous in-frame insertion mutations. Two were male and 1 was female. All 3 were smokers. A female, well-differentiated adenocarcinoma patient had the novel 2319-2320 insertion AACCCCCAC. A male well-differentiated adenocarcinoma patient had the novel 2312-2313 insertion CAA (Fig. 1). We have found one point mutation, C2369T (T790M). This patient also has the predominant L858R mutation (Fig. 2).

For exon 21, 14 patients had the L858R mutation and 1 patient had the L861Q mutation. Four were male and 11 were female. Twelve were nonsmokers and 3 were smokers. All 15 patients had adenocarcinoma, 1 was moderately differentiated and 14 were well differentiated. Six of 15 adenocarcinomas exhibited the BAC pattern at the edge of the tumor. Thus, exon 21 mutation status was significantly correlated with gender (p = 0.0005), smoking status (p = 0.0007), pathologic stages (p = 0.0152), the pathologic subtypes (p = 0.0329) and differentiation of lung cancer (p = 0.0033).

The mutations detected in lung cancer specimens from 95 lung cancer patients are summarized in Table I. Taken together, 36 mutations were found from 35 lung cancer samples in our analysis. Total *EGFR* mutations were present in 35 patients (36.8%). These mutation statuses were significantly correlated with gender (women 73.3% *vs.* men 20%, $p < 0.0001$), smoking status (never smoker 69.4% *vs.* smoker 16.9%, $p < 0.0001$), pathologic subtypes (adenocarcinoma 45.1% *vs.* nonadenocarcinoma 12.5%, p = 0.0089) and differentiation status of the lung cancers (well 51% *vs.* moderately or poorly 18.4%, p = 0.0021).

The overall survival of 95 lung cancer patients from Nagoya City University, with follow-up through December 30, 2003, was studied in reference to the *EGFR* mutation status. The patient with the mutation in the *EGFR* gene (n = 35, 4 were dead) had a significantly better prognosis than the patient with wild-type *EGFR* (n = 60, 20 were dead; log-rank test p = 0.0143, Breslow-Gehan-Wilcoxon test p = 0.0220), although the observation period was short (Fig. 3). But a multivariate analysis revealed that pathologic

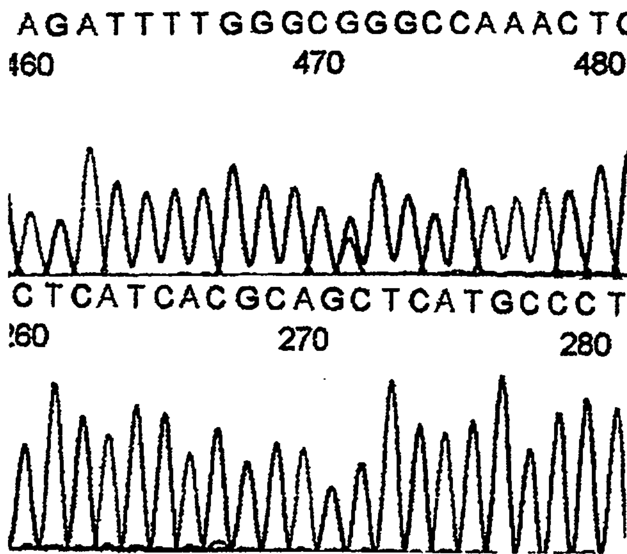

FIGURE 2 – The premoninant L858R (2573 T to G) mutation in exon 21 (top) and T790M (2369 C to T) mutation at exon 20 (bottom) within the EGFR kinase domain.

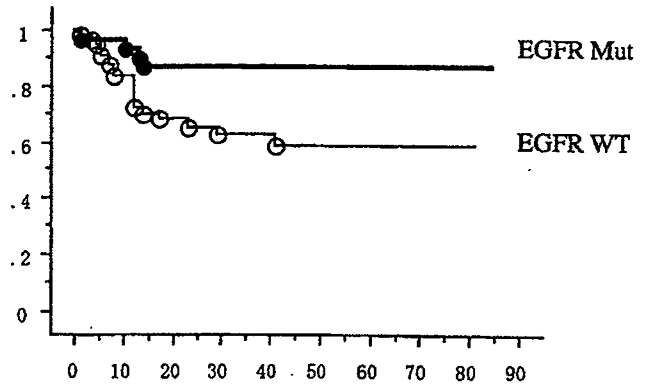

FIGURE 3 – The patient with a mutation in the *EGFR* gene (n = 35, 4 were dead) had a significantly better prognosis than the patient with wild-type *EGFR* (n = 60, 20 were dead) (log-rank test, $p = 0.0143$; Breslow-Gehan-Wilcoxon test, $p = 0.0220$), although the observation period was short.

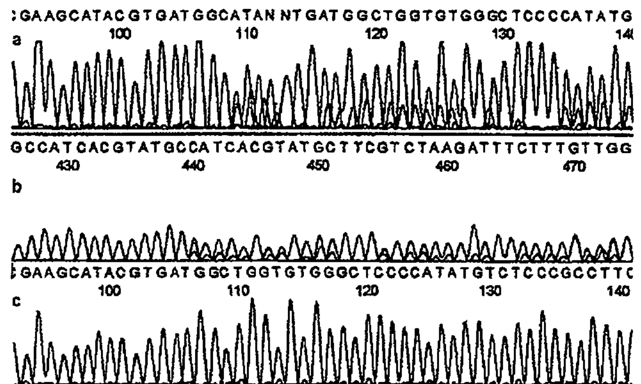

FIGURE 4 – Detection of the insertion mutation in the *erbB2* gene in genomic DNA extracted from lung cancer. *(a)* The 12 nucleotide insertion mutation (2324–2325 ins ATACGTGATGGC) was located in the exon 20 at kinase domain (775–776 ins YVMA). *(b)* Reverse sequence was performed and confirmed. *(c)* Adjacent normal lung tissue showed a wild-type sequence for the *erbB2* gene.

TABLE I – CLINICOPATHOLOGIC DATA OF 95 LUNG CANCER PATIENTS

Factors	*EGFR* gene status		
	Mutation patients	Wild-type patients	*p*-value
Mean age (years)			
64.9 ± 9.0	35	60	
Stage			
I	25 (72.4%)	27 (45.8%)	0.0274
II–IV	10 (28.6%)	32 (54.2%)	
Lymph node metastasis			
N0	8 (22.9%)	21 (35.0%)	0.3119
N+	27 (77.1%)	39 (65.0%)	
BI			
Never smoker	25 (71.4%)	11 (34.0%)	0.001
Smoker	10 (28.6%)	49 (66.0%)	
Differentiation			
Well	26 (78.8%)	23 (42.6%)	0.0021
Moderately or poorly	7 (21.2%)	31 (57.4%)	
Pathologic subtypes			
Adeno	32 (91.4%)	39 (74.7%)	0.0089
Nonadeno	3 (8.6%)	21 (25.3%)	
Age			
≤ 65	19 (54.3%)	29 (48.3%)	0.7269
> 65	16 (45.7%)	31 (51.7%)	
Gender			
Male	13 (37.1%)	52 (86.7%)	< 0.0001
Female	22 (62.9%)	8 (13.3%)	

N+, lymph node metastasis positive; Adeno, adenocarcinoma.

stage ($p = 0.0006$) was the only significant factor but not *EGFR* mutation ($p = 0.1824$).

ErbB2 *gene mutation status in Japanese lung cancer patients*

We identified only one *erbB2* mutation from 95 NSCLC patients. This 12-nucleotide insertion mutation (2324-2325 ins ATACGTGATGGC) was located in the exon 20 at kinase domain (775-776 ins YVMA) (Fig. 4). This patient was a female non-smoker with well-differentiated adenocarcinoma, without *EGFR* mutation. Adjacent normal lung tissue exhibited a wild-type sequence for the *erbB2* gene, suggesting that this mutation was somatic. We have also done sequencing for 27 gefitinib-treated NSCLC patients. Among 27 patients, 9 patients had *EGFR* mutations (data not shown). However, no *erbB2* mutation was found within the kinase domain. Totally, we have found only 1 *erbB2* mutation from 122 (0.8%) Japanese NSCLC patients.

Immunohistochemistry

The immunohistochemical evaluation was done according to the scoring system described in Material and Methods. Immunohistochemistry was done only for 87 patients because the tissue blocks were not available for other patients. The erbB2-positive (2+/3+) ratio was 26.4% (23/87). There was a significantly higher erbB2-positive ratio in *EGFR*-mutant patients (13/25, 52.0%) compared to *EGFR* wild-type patients (10/62, 16.1%) ($p = 0.0247$). The patient with *erbB2* mutation exhibited 1+ immunoreactivity (Fig. 5).

Discussion

We obtained findings that *EGFR* mutation status was significantly correlated with gender and smoking history of lung cancers. This was in agreement with the recent reports that *EGFR* gene mutations are common in lung cancers from never smokers[13,14] and females with adenocarcinoma.[11,14] However, our analysis also suggested that *erbB2* mutation might be less common in Japanese NSCLC patients.

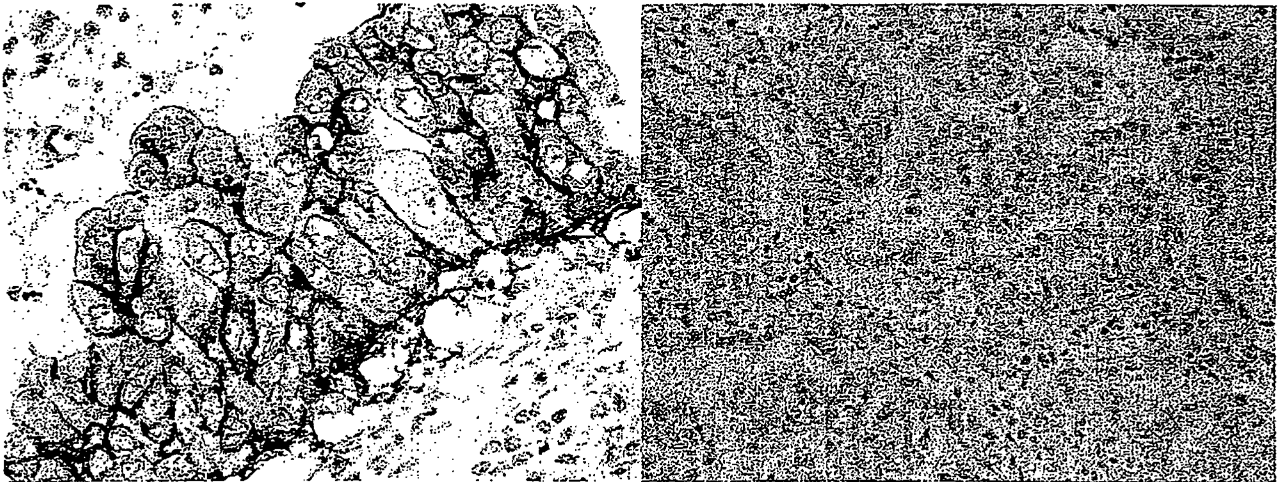

FIGURE 5 – Immunohistochemistry for ErbB2. Left: erbB2-positive (3+) section. Right: the NSCLC specimen with *erbB2* mutation exhibited 1+ immunoreactivity.

Overexpression of EGFR/ErbB2 and ErbB ligands is correlated with advanced diseases and poor patient prognosis.[19] Although *EGFR* is more abundantly expressed in lung carcinoma,[20,21] *erbB2* overexpression is less common; it is found in <35% of patients with nonsmall cell lung cancers, mainly in those with adenocarcinoma.[21] Amplification of *EGFR* and *erbB2* mRNA[22] or overexpression of their proteins[23] has been found to relate to survival in patients with NSCLC, although contradictory results have also been reported.[24,25] The drug trastuzumab, a humanized antibody against the extracellular domain of erbB2, has been approved for treatment of metastatic breast cancer and is most effective in breast cancer with *erbB2* amplification. Preliminary results suggested that the combination of chemotherapy and trastuzumab is well tolerated for NSCLC.[21] However, results from phase II trials of trastuzumab as a treatment for NSCLC have not shown any advantage for most patients[22] and have provided insufficient evidence to proceed to phase III trials.[23] Because the presence of a mutation appears to be a determination of response to therapy, as is the case with gefitinib and *EGFR* mutations, we therefore investigated the *erbB2* and *EGFR* gene mutation status. However, we have found only 1 *erbB2* mutation from 122 Japanese lung cancer patients. More recently, Shigematsu *et al.* reported that *erbB2* mutations were found in 3% (8/269) of Japanese NSCLC.[26] The single *erbB2* mutation we have found was the same as the one repeatedly found by Shigematsu *et al.*[26] Because very few NSCLC patients have gene amplification of *erbB2*, trastuzumab in the treatment of NSCLC might have a limited role.[9] Lung cancers that coexpress both EGFR and erbB2 appear to have more virulent behavior.[27] In addition, EGFR-erbB2 heterodimers are associated with a stronger and more sustained proliferative signal than EGFR homodimers.[22,28] Blockade of a signaling pathway may in theory be overcome by compensatory activation of a separate pathway in the same tumor cell. Because there was a significantly higher erbB2-positive ratio in *EGFR*-mutant patients, blockade of both may ultimately yield superior results.

Because so many *EGFR* mutation phenotypes were discovered, it would be of interest to determine whether resistance to *EGFR* inhibition emerges through secondary mutation as is the case in imatinib-treated chronic myelogenous leukemia.[29] In our analysis, a female never smoker adenocarcinoma patient had the predominant L858R mutation as well as T790M mutation. Actually, this case was untreated with EGFR kinase inhibitors. Threonine 315 to isoleucine substitution in the Abl kinase domain was a critical structural determinant controlling inhibitor sensitivity of STI571.[29] Introduction of bulkier hydrophobic side chains at the Thr-790 position fully preserved the cellular kinase activity of the *EGFR* in the presence of selective kinase inhibitors, indicating potential mechanisms of molecular resistance formation as previously found for BCR-Abl at T315I. Previous *in vitro* study showed that mutation of T790M in the *EGFR* revealed a hotspot for resistance formation against gefitinib,[30] also *in vivo*.[31]

Over the decades, the incidence of lung adenocarcinoma has increased worldwide. Most individuals with lung adenocarcinoma (especially women) are nonsmokers,[32] who are corresponding with the sensitive population to gefitinib. In Taiwan, *EGFR* mutation ratio from adenocarcinoma was also high (55%, 38 of 69), and all of the adenocarcinomas with *EGFR* mutation were well to moderately differentiated.[33] These data were compatible for our results. Because well-differentiated adenocarcinoma patients had a better prognosis,[34] *EGFR* mutant patients showed better prognosis in our univariant analysis. The reason why many mutations were especially found in Asian, female nonsmoker adenocarcinoma remains unknown. Human papilloma virus type 16/18 infections,[35] cooking oil fume,[36] nutritional status, genetic susceptibility, immunologic infection, tuberculosis and asthma[32] have been investigated as causes of lung cancer occurring in nonsmoking women.

The findings of the breakdown of *EGFR* mutations among the 3 exons were interesting. The exon 21 mutations correlated with pathologic stage and subtype, unlike mutations in exon19. Since exon 21 mutations are more closed to the activation loop of *EGFR*, these may be more correlated with gefitinib sensitivity. Especially since 3 patients with exon 20 mutations were smokers, all of the mutations might not be equally correlated with sensitivity for gefitinib.

Acknowledgements

The authors thank Mr. N. Hosono, Mr. R. DeBiasi and Dr. J. Lee for their excellent technical assistance.

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EGFR mutation in gefitinib-responsive small-cell lung cancer

Activating mutations within the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) underlie responsiveness to gefitinib in non-small-cell lung cancer (NSCLC) [1–3]. To date, however, only a few EGFR mutations have been detected in other solid tumors [4]. We now describe a patient with gefitinib-responsive small-cell lung cancer (SCLC) who harbors a deletion in exon 19 of EGFR.

A 72-year-old woman with no history of smoking presented with a 2-week history of cough, dyspnea and intermittent hemoptysis. Computed tomography (CT) revealed a mass in the upper lobe of the right lung and a large metastatic mass in the liver. Bronchoscopic examination revealed a tumor occluding the right upper bronchus and a bronchoscopic biopsy was performed. Treatment with 250 mg of gefitinib once daily was initiated at the patient's request. Her symptoms improved rapidly, with CT performed 3 weeks after the initiation of gefitinib treatment revealing marked regression of both the primary lung tumor and the metastatic liver tumor. Histological examination of the transbronchial biopsy specimens showed that the tumor comprised small cells with round or oval nuclei (Figure 1A). The final pathological diagnosis was thus SCLC and was confirmed independently by three additional pathologists. Positive staining of the tumor cells for neural cell adhesion molecule (CD56), a sensitive and specific marker of neuroendocrine differentiation, supported the pathological diagnosis. Further immunohistochemical analysis revealed expression of EGFR in the tumor cells (Figure 1B). Direct sequencing of the region of EGFR that encodes the kinase domain (exons 18 to 21) in DNA isolated from tumor biopsy specimens identified a heterozygous in-frame 15-base pair deletion that resulted in the loss of amino acids 746 to 750 (delE746-A750) (Figure 1C). This mutation is identical to a previously described deletion in exon 19 of EGFR in NSCLC [1–3]. The mutation in the proband was detected in both sense and antisense sequences of the products of two independent polymerase chain reactions.

In contrast to NSCLC, EGFR expression has been reported to be low in SCLC. Gefitinib was recently shown to inhibit EGFR signaling in SCLC cell lines that express the receptor even at a low level [5], however, suggesting the presence of functional EGFRs in SCLC. As far as we are aware, ours is the first report of an EGFR mutation in a patient with SCLC, a finding that suggests that EGFR tyrosine kinase inhibitors may be a treatment option for a subset of SCLC tumors that express functional EGFRs.

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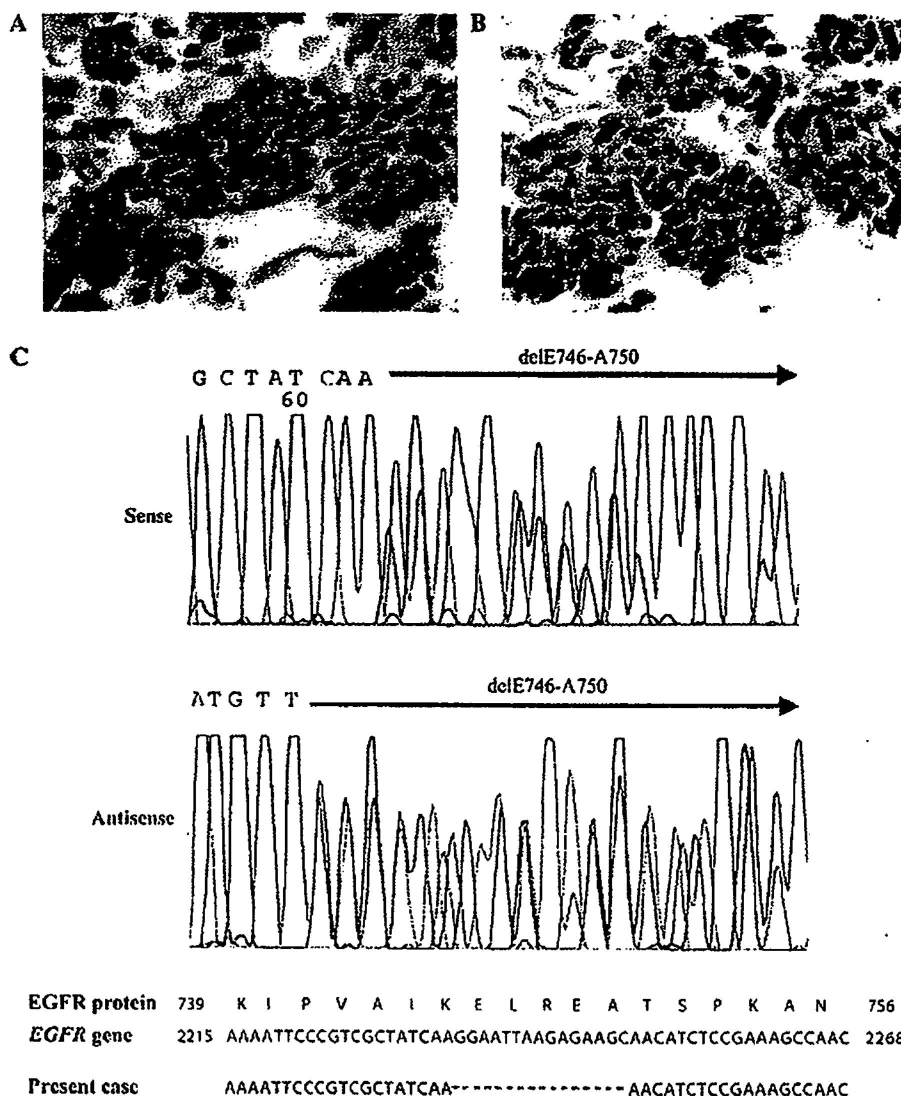

Figure 1. EGFR expression and mutation in tumor tissue at diagnosis of gefitinib-responsive SCLC. (A) Hematoxylin–eosin staining showed that the primary tumor was composed of small cells with round or oval nuclei and sparse cytoplasm. (B) Immunohistochemical analysis showed expression of EGFR in tumor cells. (C) Nucleotide sequencing of EGFR in tumor DNA revealed a heterozygous in-frame deletion within the region of the gene encoding the tyrosine kinase domain (double peaks).

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doi:10.1093/annonc/mdj114
Published online 15 December 2005