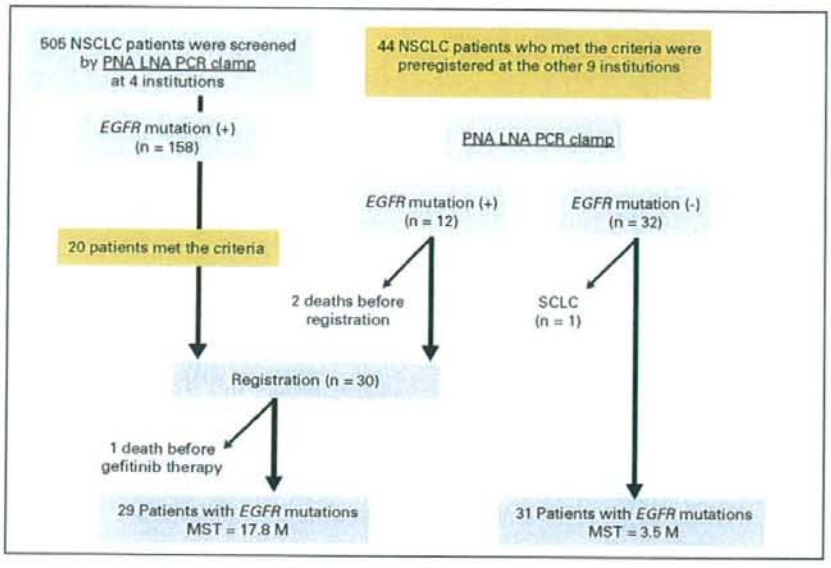

Fig 1. Flowchart of patient accumulation. Epidermal growth factor receptor (*EGFR*) mutations were routinely examined by the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp in four institutions, and the other nine institutions sent materials to the same laboratory after informed consent was obtained. From the four screening institutions, 20 patients with *EGFR* mutations were entered onto the study. From the other institutions, 12 of 44 patients tested were found to have *EGFR* mutations; of these 12 patients, 10 were enrolled and two died before they could be enrolled. Of the 30 eligible patients, 29 patients, except for one patient who died as a result of rapid disease progression, received gefitinib. NSCLC, non–small-cell lung cancer; pts, patients; SCLC, small-cell lung cancer; MST, median survival time.

RESULTS

Patient Characteristics

Two groups of institutions participated in this study. The first group included four institutions where *EGFR* mutation was tested routinely by the PNA-LNA PCR clamp test, and information on *EGFR* mutation was available at the time of diagnosis. During the study period, the four institutions screened 505 patients with NSCLC, and 158 patients (31%) were found to have sensitive *EGFR* mutations, regardless of their PS. Among them, 20 patients with poor PS entered this study (Fig 1). The second group included nine institutions where *EGFR* mutation testing was not routine. Informed consent for both testing *EGFR* mutations and for the possibility to enter this study was obtained from 44 patients in this second group. *EGFR* mutation was positive in 12 of the 44 patients, and 10 patients entered onto this study (two patients died before enrollment). In contrast, except for one patient whose diagnosis was changed to be small-cell lung cancer, 31 patients with NSCLC and poor PS and without *EGFR* mutation from these institutions were considered to be free from selection bias (Fig 1). Among these 31 patients (21 men and 10 women, with average age of 72.6 years), eight patients received a carboplatin-based doublet, two patients received mono-chemotherapy, three patients received palliative irradiation, and 18 patients were treated with BSC alone.

Between February 2006 and May 2007, a total of 30 patients with NSCLC and *EGFR* mutations were enrolled: 20 from group 1 and 10 from group 2 (Table 1). Of the 30 eligible patients, one patient did not receive gefitinib as a result of bacterial pneumonia and rapid disease progression after registration. Most patients had stage IV disease, and 16 patients had multiple sites of distant metastasis. Twenty-two patients had PS 3 or 4 because of various cancer-related conditions, as follows: respiratory failure owing to multiple pulmonary metastasis, carcinomatous lymphangiosis, malignant pleural effusion, superior vena cava stenosis owing to mediastinal lymph node invasion, carcinomatous pericarditis, symptomatic brain metastasis, carcinomatous meningitis, symptomatic vertebral metastasis, uncontrollable pain because of bone metastasis, and other conditions. Eight patients were oxygen dependent. Three patients with PS 1 and four patients with PS 2 were all ≥ 80 years of age. Because smoking status and adenocarcinoma histology were not included in the selection criteria, heavy smokers (defined as > 10 pack-years) and patients with nonadenocarcinomas were also enrolled, although we found the majority of patients were nonsmokers or light smokers and had adenocarcinomas. The mutations were mainly exon 19 deletions or L858R, which is similar to the results of previous reports.[9-19]

Response and Survival

The objective tumor responses are summarized in Table 2. The ORR and disease control rates were 66% (90% CI, 51% to 80%) and 90% (90% CI, 80% to 99%), respectively. Posthoc subset analyses performed to examine the difference in response rate between certain clinical factors (sex, smoking status, PS) and the type of *EGFR* mutation revealed no relationship between these factors and gefitinib response.

The median follow-up time was 17.8 months (range, 12 to 26 months), and 12 patients were alive at the data cutoff point. The median PFS was 6.5 months (Fig 2A). The median survival time (MST) was 17.8 months, and 1-year survival rate was 63% (Fig 2B). There were no statistical differences in OS and PFS between patients with PS 1 to 2 (frail older adult patients) and patients with PS 3 to 4.

Of the 29 patients treated with gefitinib, five patients received postgefitinib chemotherapy. Although an ORR of their second-line chemotherapy was not reviewed, the MST of the five patients from the time of gefitinib failure was 11.7 months. Conversely, MST of the other 24 patients with BSC alone after gefitinib failure was 8.2 months. There was no significant difference of MST after gefitinib failure between patients treated with second-line chemotherapy and those treated with BSC alone ($P = .122$).

Table 1. Patient Characteristics

Characteristic	No. of Patients
Enrolled patients	30
Treated patients	29*
Sex	
Male	6
Female	23
Age, years	
Median	72
Range	50-84
Performance status	
1	3†
2	4†
3	17
4	5
Smoking status, pack-years	
0	22
1-19	2
≥ 20	5
Histology	
Adenocarcinoma	27
Adenosquamous	1
Undifferentiated	1
Stage	
IV	27
Others	2‡
Metastatic site	
Brain	11
Meninges	1
Lung	18
Bone	12
Liver	2
Others	2
Malignant effusion	
Malignant pleural effusion	10
Malignant pericardial effusion	4
Sample used to detect mutations	
Sputum	4
Pleural effusion	11
Bronchial washing	7
Paraffin embedded tissue	7
Type of mutations	
Deletion	18
L858R	10
L861Q	1

*One patient did not receive gefitinib due to pneumonia and rapid disease progression.
†All ≥ 80 years of age.
‡Includes one patient with stage IIIA disease and one patient with postoperative recurrence.

Improvement of PS

F3 Figure 3 shows the change in PS for each patient during this study. Twenty-three (79%) of 29 patients (90% CI, 67% to 92%) had improved PS after gefitinib treatment. This improvement was highly significant by Wilcoxon signed rank test ($P < .00005$). In particular, an improvement from PS 3 to 4 at baseline to PS 0 to 1, which we considered clinically valuable, was observed in 68% of patients. Some patients with PS 4 experienced a dramatic improvement in systemic advanced disease shortly after the initiation of gefitinib treatment.

Table 2. Response to Treatment

Response	No. of Patients	Response Rate (%)	90% CI
Complete response	1	3	—
Partial response	18	62	—
Stable disease	7	24	—
Progressive disease	2	7	—
Not assessable	1	3	—
Overall response	19	66	51 to 80
Disease control rate	26	90	80 to 99

Toxicity

The most frequent adverse event with severity of grade 2 or worse was AST/ALT elevation (Table 3), and all affected patients improved T3 within a few months of modification of the treatment. One patient

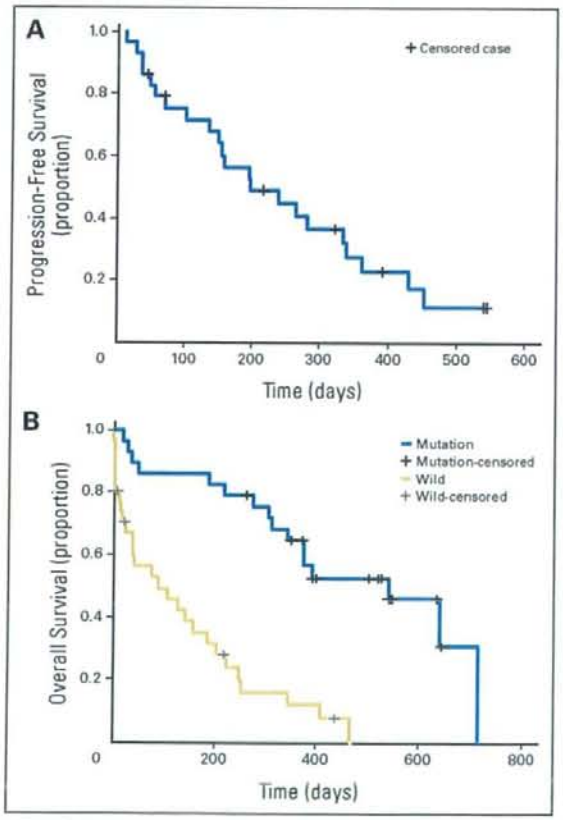

Fig 2. Progression-free survival (PFS) and overall survival (OS) of patients. (A) PFS of all patients in the study. (B) OS for all the patients with sensitive epidermal growth factor receptor (*EGFR*) mutations (green line). Vertical bars indicate censored cases at the data cutoff point. The median PFS, median survival time, and 1-year survival rate of the patients with sensitive *EGFR* mutations were 6.5 months, 17.8 months, and 63%, respectively. B also shows a survival curve of the 31 patients without *EGFR* mutations (red line; Fig 1). Their median survival time was 3.5 months.

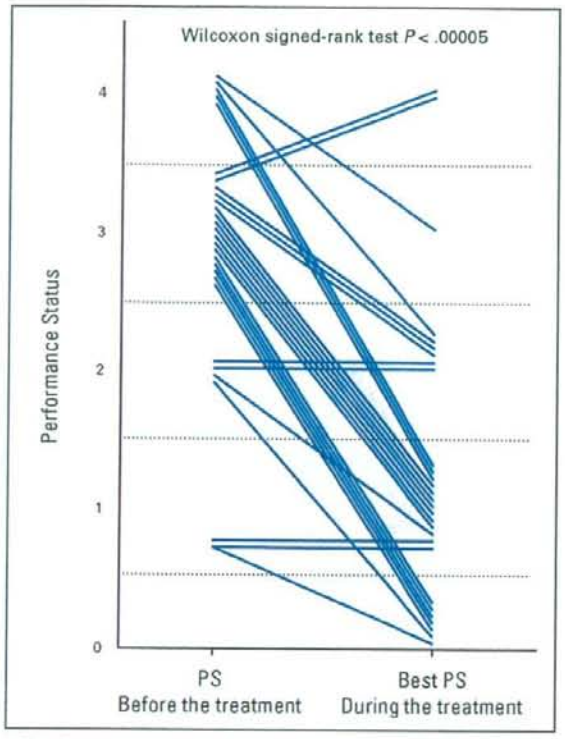

Fig 3. Change of performance status of each patient during treatment. Each line shows the change of performance status (PS) of a patient from baseline to best status during the treatment. A clinically valuable improvement in 68% of patients was observed (ie, improvement from PS 3 to 4 at baseline to PS 0 to 1).

Table 3. Grade 2 or Worse Adverse Events

Toxicity	No. of Patients			Total With ≥ Grade 3 Toxicity	
	Grade 2	Grade 3	Grade 4	No.	%
Hematologic					
Anemia	3	2	0	2	7
Neutropenia	2	0	0	0	
Nonhematologic					
Pneumonitis	0	0	1	1	3
AST/ALT	4	3	0	3	10
Anorexia	0	1	0	1	3
Rash	4	0	0	0	
Diarrhea	2	0	0	0	
Hypoalbuminemia	2	0	0	0	
Vomiting	1	0	0	0	
Pain	1	0	0	0	
Hyperkalemia	1	0	0	0	

experienced grade 4 interstitial lung disease that resolved after corticosteroid pulse therapy (methylprednisolone 1 g/d for 3 days). Most of the other toxicities observed were mild or moderate. There was no treatment-related death. Two patients died within 30 days after initiation of gefitinib as a result of rapid disease progression. Toxicity was comparable to that observed in patients with PS 0 to 2 in the previous studies and was considered acceptable.

DISCUSSION

There have been few previous reports of successfully treating patients with advanced NSCLC and extremely poor PS (especially PS 3 to 4). A recent randomized trial comparing gefitinib with BSC for unselected patients with NSCLC having poor PS also did not show a clinical benefit.[25] By selecting patients with sensitive *EGFR* mutations, the present phase II study of first-line gefitinib for patients with NSCLC having poor PS could be the first to demonstrate promising efficacy.

The ORR (66%) was comparable to that observed when gefitinib was given to *EGFR* mutation-positive patients with PS 0 to 2.[14-19] Median PFS (6.5 months) was markedly better than that expected in patients managed with only BSC.[25] The PFS in this study was even better than that reported previously for standard chemotherapy in patients with PS 2, and that observed in unselected patients with PS 2 treated with erlotinib (median PFS, 1.9 to 2.9 months).[26,27,30]

The PS results were also remarkable because they met the important study end point for improvement of PS, which suggests the clinical usefulness of gefitinib for this population. A clinically valuable improvement in 68% of patients was observed (ie, they improved from PS 3 to 4 at baseline to PS 0 to 1). Namely, these patients enjoyed the rest of their lives with a good quality of life instead of spending the end of their lives in bed. As for other secondary end points, MST of patients who had sensitive *EGFR* mutations and were treated with gefitinib (17.8 months) was markedly longer than that of 31 patients without *EGFR* mutations (3.5 months; Fig 2B). The results also indicated that the MST of 17.8 months was much longer than that of patients managed with BSC alone,[25] as well as that of patients with PS 2 treated with standard chemotherapy (MST, 8.0 months; 1-year survival rate, 31%).[26,27] Although there is little evidence on prognosis of untreated patients with NSCLC having *EGFR* mutations, we consider that even if *EGFR* mutated tumors have somewhat indolent behavior, MST of more than 1 year is a rare and represents a promising result for patients with advanced NSCLC having poor PS.

Although some studies have indicated that clinical factors, including adenocarcinoma and/or nonsmoker status, are enough to identify a responder to gefitinib,[28] there is much evidence that a number of patients with nonadenocarcinomas or who are heavy smokers also have *EGFR* mutations and show a response to gefitinib.[11-13] In the present study, if these clinical factors had been used in the eligibility criteria, 11 (38%) of 29 patients would not have been treated with gefitinib. Assessment of *EGFR* mutation status before treatment is thus a reasonable strategy to increase the accuracy of predicting a response. Stressful examination procedures such as open-lung biopsy are often contraindicated in patients with poor PS or who are frail and older in age. The present study found that the PNA-LNA PCR clamp method, which has been commercially available in Japan for more than 2 years (US$200), could detect both sensitive and insensitive *EGFR* mutations from patients with poor PS (Table 1). With this test, the strategy of first-line gefitinib is readily applicable to the clinical setting.

Currently, patients ≥ 80 years of age with advanced NSCLC are often treated only with BSC, because no standard chemotherapy regimen has been established for such older patients. Although the number of ≥ 80-year-old patients treated with first-line gefitinib was small in this study, the efficacy observed was at least comparable to that achieved with standard chemotherapy regimens given to younger patients, which indicates that a large-scale study of first-line gefitinib for *EGFR* mutation-positive older patients is warranted. In contrast, we are conducting a phase III trial to compare first-line gefitinib with standard chemotherapy, carboplatin and paclitaxel, for patients with NSCLC and PS 0 to 2 with *EGFR* mutation. However, in patients with sensitive *EGFR* mutations with extremely poor PS, the difference in benefit with or without gefitinib treatment is so marked that a randomized phase III study to compare gefitinib with BSC alone may not be justified. Additional studies to confirm our results are considered to be appropriate.

In conclusion, *EGFR* mutation-positive patients with extremely poor PS benefit from first-line gefitinib. Previously, these patients have usually been treated with BSC alone because no standard treatment has been established. Examination of *EGFR* mutations as a biomarker is strongly recommended for these patients, because it identifies patients for whom salvage therapy with gefitinib may be beneficial, which is especially relevant to Asian and South European patients with advanced NSCLC who have high possibilities of harboring *EGFR* mutations.[29]

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest. AQ: K

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Acknowledgment

We thank all patients, Drs Nagao, Nakai, Shibuya, Kanazawa, Kudoh, Yoshimori, Hasegawa, Suzuki, Sugawara, and Abe; and TCOG for supporting this study.

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A—AUTHOR: No affiliations were included with the manuscript. I have added the affiliation for the corresponding author, but if other authors are located at other institutions, please add those institutions to the footnote, including department, institution, city, and country.

B—AUTHOR: Per Journal style, information about the group was removed from the byline and added as a footnote.

C—AUTHOR: Correct to italicize EGFR throughout as a gene? (Per Journal style, all genes, oncogenes, and proto-oncogenes should be italicized. Proteins, antigens, and receptors should not be italicized.)

D—AUTHOR: Manufacturer and location correct as added for Iressa?

E—AUTHOR: Should EGFR-TKI also be italicized? Please confirm and correct throughout if necessary.

F—AUTHOR: Please note that any figure changes made at this advanced stage of publication should be to correct data/factual errors, or errors that severely compromise clarity or meaning.

G—AUTHOR: Sentence ("...and 10 patients entered onto this study [two patients died before enrollment])" correct as edited?

H—AUTHOR: "oxygen dependent" as meant (for "O2 dependent")?

I—AUTHOR: "unselected" as meant (instead of "gunselected")?

J—AUTHOR: Please again confirm substitution of "unselected" (this time for "non-selective").

K—AUTHOR: Please verify that the conflicts of interest information is correct and accurate as of October 9, 2008.

L—AUTHOR: Please verify that all contribution information is correct for each author.

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An Algorithm to Tackle Acute Exacerbations in Idiopathic Pulmonary Fibrosis

To the Editor:

The pulmonary perspective by Dr. Collard and colleagues (1) provides an overview of the current understanding about acute exacerbations in idiopathic pulmonary fibrosis (IPF). To tackle this challenging problem, we developed an algorithm (Figure 1). As a first step, we test for the presence of D-dimer and clinical probability of pulmonary embolism. If the test is positive, a specific computed tomography (CT) scan is performed. The CT scan is an accurate modality (2) with which to detect pulmonary embolism; moreover, images can be compared with previous CT scans to evaluate reticular shadowing, honeycombing, and ground-glass appearances. Echocardiography is performed to rule out left heart failure. At the same time, (increasing) pulmonary hypertension can be ruled out. If diffusion capacity is greater than 30%, and if hypoxemia can be corrected to a Po_2 of 75 mm Hg with supplemental oxygen (3), then bronchoalveolar lavage (BAL) is performed to rule out infection (bacterial, viral, opportunistic infections, and fungi).

BAL must be performed soon after admission, and broad-spectrum antibiotics are started immediately after bronchoscopy. We usually start broad-spectrum antibiotics (such as piperacillin-tazobactam or third-generation cephalosporins intravenously). However, atypical bacilli (such as *Legionella* and *Mycoplasma*) must be covered by adding quinolones. As most of these patients with IPF are immunocompromised, *Pneumocystis jiroveci* pneumonia needs to be covered empirically with sulphametoxazole. If infection cannot be proven, corticosteroids are added to the treatment in a dose of 500–1,000 mg for 3 consecutive days. Antiviral agents should be considered when herpesvirus or cytomegalovirus (CMV) is found in BAL, and antifungal agents should be initiated especially when *Aspergillus* is found in an immunocompromised patient or if the *Aspergillus* antigen test in BAL is clearly positive (4).

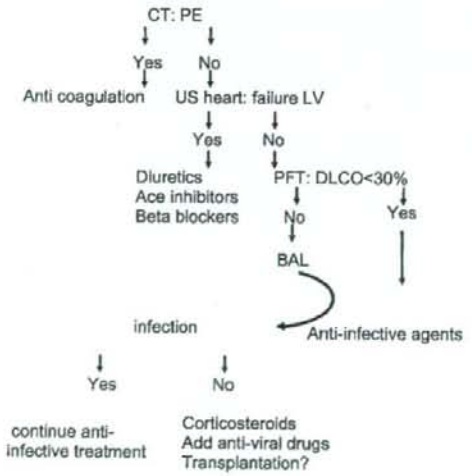

Figure 1. Algorithm of treatment for patients with idiopathic pulmonary fibrosis. BAL = bronchoalveolar lavage; CT = computed tomography; $D_{L_{CO}}$ = diffusion capacity for CO; LV = left ventricle; PE = pulmonary embolism; PFT = pulmonary function testing; US = ultrasound.

If there is no effect from these therapies, and in the absence of generalized infection, lung transplantation should be considered as a treatment for acute exacerbation in IPF. It is the only treatment that improves survival in patients with interstitial lung disease (5). The patient can only be put on the high-urgency list when a transplant workup has been completed before the acute exacerbation. This is another strong reason to refer patients with IPF in an early stage to a transplant center for transplant evaluation, as an acute exacerbation is not related to pulmonary function tests (1).

In conclusion, Collard and coworkers' article gives an excellent overview of the challenges in acute exacerbations of IPF. We need carefully chosen protocols to guide us in this challenge. We hope that the algorithm we propose will help physicians in dealing with this problem.

Conflict of Interest Statement: W.A.W. has attended an expert meeting organized by Actelion and received €1,000 as a consultancy fee. M.T. is a member of Intermune's steering committee (INSPIRE Study). L.J.D. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.M.V. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Basis of Acute Exacerbation of Idiopathic Pulmonary Fibrosis in Japanese Patients

To the Editor:

A pulmonary perspective by Dr. Collard and colleagues focused on acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF), trying to establish a worldwide consensus about acutely progressive respiratory failure during the course of IPF (1). Awareness of this serious phenomenon as a distinct pattern of IPF is extremely

important, and helps clinicians worldwide to share the same background information on the diagnosis and management of AE. However, Japanese patients with IPF seem to suffer from AE much more frequently than patients of other ethnic backgrounds. AE is often triggered by infections, drugs, radiotherapy, anticancer agents, and surgery. Epidemiological data suggest that Japanese more frequently suffer from diffuse alveolar damage (DAD) due to various causes such as drugs (2). These observations strongly suggest that genetic factor(s) predispose Japanese to DAD. Reports on AE-IPF are frequent from Japan (3, 4). A large-scale study showed that 5.8% of Japanese patients on gefitinib therapy developed interstitial lung disease, a frequency 10- to 100-fold higher than for patients of other genetic backgrounds (2).

Ethnic differences in the incidence of pulmonary disease have been observed before. For example, most Japanese clinicians have never seen α_1-antitrypsin deficiency or cystic fibrosis, because these diseases are extremely rare in the Japanese population. Population genetics indicates that a founder gene may predominate in a genetically isolated population through the bottleneck effect or random genetic drift (5).

Homo sapiens emerged in Africa around 200,000 years ago and reached Japan about 30,000 years ago, at the end of the Ice Age when the sea level was low and Japan was joined to the Eurasian continent. Subsequent elevation of the sea has genetically isolated Japan, so that the Japanese population may share disease genes that are not found or rarely found in other ethnic groups.

Although there is more movement of people in modern society, the world is still a mosaic of ethnic groups with different diseases. We believe that AE-IPF is actually a collection of diseases with different causes, among which a cause specific to Japanese patients accounts for many cases in Japan. Elucidation of the responsible genetic factor(s) would be important for public health in the world, as well as for understanding pulmonary physiology.

Conflict of Interest Statement: A.A. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.H. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.K. received 220,000 Japanese yen from AstraZeneca for advisory board activities.

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From the Authors:

We thank Dr. Wuyts and colleagues and Dr. Azuma and colleagues for their interest in our pulmonary perspective on acute exacerbations of idiopathic pulmonary fibrosis (IPF) (1). It was our hope in writing this perspective to stimulate thoughtful discussion, debate, and research on this important topic.

Dr. Wuyts and coworkers present an interesting algorithm for the diagnosis and management of acute exacerbations of IPF, and we agree with the authors' emphasis on aggressively looking for alternative etiologies (e.g., infection, pulmonary embolism). While the proposed approach to the diagnosis and management of acute exacerbations of IPF is a reasonable one, it is important to note that data supporting its specific recommendations are lacking. This is particularly true for the management recommendations, including the use of antibiotic, antifungal, and corticosteroid therapies. The authors' comments regarding the appropriateness of early referral for lung transplantation evaluation are strongly endorsed, as many patients with IPF may suffer acute worsening of their disease and death despite previously preserved pulmonary function (2, 3).

Nothing is known about susceptibility to acute exacerbation of IPF, but we agree with Dr. Azuma and coworkers that the Japanese preponderance of published cases may suggest an important genetic predisposition (4, 5). It is interesting to note that genetic and ethnic disparities exist in acute lung injury, a condition clinically and histopathologically similar to acute exacerbation of IPF (6). Better epidemiological data are sorely needed to further investigate Dr. Azuma's hypothesis.

Conflict of Interest Statement: H.R.C. has served as a consultant for Intermune, Genzyme, and Gilead. F.J.M. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Oncogene (2008) 27, 6581–6589

www.nature.com/onc

ORIGINAL ARTICLE

Identification of IGFBP-6 as an effector of the tumor suppressor activity of SEMA3B

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SEMA3B, a member of class 3 semaphorins, is a tumor suppressor. Competition with vascular endothelial growth factor $(VEGF)_{165}$ explains a portion of the activity, whereas the VEGF-independent mechanism was not elucidated. We employed a microarray and screened for the genes whose expression was increased by SEMA3B in NCI-H1299 cells. Insulin-like growth factor-binding protein-6 (IGFBP-6), a tumor suppressor, showed greatest difference in the expression level. Introduction of *IGFBP-6* cDNA reduced colony formation both on the dish surface and in soft agar. Insulin-like growth factor II, which antagonizes IGFBP-6, partly abrogated the effect. Inhibition of IGFBP-6 by small interfering RNA diminished the sub-G0/G1 population that was induced by SEMA3B and abrogated the growth suppressive effect of SEMA3B. We concluded that IGFBP-6 is the effector of tumor suppressor activity of SEMA3B in NCI-H1299 cells. It has been reported that β-catenin suppresses the expression of IGFBP-6. Introduction of β-catenin into the cells partly abrogated the growth suppressive effect of SEMA3B. Our result indicates that semaphorin signaling and β-catenin signaling converge on IGFBP-6 and antithetically affect their functions.
Oncogene (2008) **27,** 6581–6589; doi:10.1038/onc.2008.263; published online 11 August 2008

Keywords: semaphorin 3B; insulin-like growth factor-binding protein-6; β-catenin; microarray; tumor suppression

Introduction

The semaphorins are a family of proteins involved in cell migration, and so are implicated in nerve regeneration (Pasterkamp *et al.*, 1998), tumorigenesis (Christensen *et al.*, 1998), cardiovascular morphogenesis (Behar *et al.*, 1996; Miao *et al.*, 1999) and immune response (Hall *et al.*, 1996). The class 3 semaphorins (SEMA3s) are secreted proteins consisting of seven members, SEMA3A through -3G. Neuropilins (NRP1 and NRP2) and plexins form functional semaphorin receptors that transfer semaphorin signaling into the cells (Kolodkin *et al.*, 1993; He and Tessier-Lavigne, 1997). SEMA3E is the only exception to the above scheme in that it directly binds to a plexin (Gu *et al.*, 2005). An isoform of vascular endothelial growth factor (VEGF), $VEGF_{165}$, is a product of alternative mRNA splicing and also binds to NRPs. The $VEGF_{165}$–NRP complex then binds to VEGFR2 and transmits its signal (Soker *et al.*, 1996, 1998). Therefore, the NRPs are a component of both the SEMA3–NRP–plexin complexes and the $VEGF_{165}$–NRP–VEGFR2 complexes, and are located at the crossroads of SEMA3 and VEGF signaling. SEMA3s and $VEGF_{165}$ inhibit each other by competing for available molecules of NRP (Mac Gabhann and Popel, 2006).

SEMA3B has a tumor suppressor activity. Addition of SEMA3B protein to the medium or introduction of SEMA3B expression plasmid into the cells induces apoptosis and decreases colony-forming activity in NCI-H1299 cells (Sekido *et al.*, 1996; Tomizawa *et al.*, 2001). *SEMA3B* expression is induced by p53 mediated by a p53 responsive element located in the promoter (Ochi *et al.*, 2002). *SEMA3B* expression is frequently suppressed by the methylation of the promoter in lung cancers (Tomizawa *et al.*, 2001). The *SEMA3B* gene is located at 3p21.3 where chromosomal deletions have frequently been observed in lung cancers (Sekido *et al.*, 1996; Tomizawa *et al.*, 2001). These data suggest that SEMA3B may have a tumor suppressor activity that is attributable to the competitive inhibition of $VEGF_{165}$ working as an autocrine survival factor in some cancer cells (Castro-Rivera *et al.*, 2004).

Recent studies on two other members of the SEMA3 family have demonstrated complexity in their signaling pathways. SEMA3A inhibits VEGF activity by mechanisms that are both dependent on and independent of the competition for receptor binding (Guttmann-Raviv *et al.*, 2006). β-catenin/T-cell factor (TCF) signaling has been implicated in the growth inhibitory effects of SEMA3F (Nasarre *et al.*, 2005). These findings suggest that, in addition to the competition with $VEGF_{165}$, SEMA3B may employ an as yet unidentified mechanism for its tumor suppressor activity.

In this study, we have investigated whether SEMA3B activity is mediated by a mechanism other than

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Received 20 December 2007; revised 20 June 2008; accepted 29 June 2008; published online 11 August 2008

competition with $VEGF_{165}$. Some tumor suppressors function by inducing the expression of their effector genes (Yu *et al*., 2001; Barz *et al*., 2006; Chow *et al*., 2006; Feng *et al*., 2006). We hypothesized that SEMA3B might be such a tumor suppressor. To test this hypothesis, we employed microarray technology and screened for those genes with increased expression following SEMA3B transfection. We then investigated whether and by what mechanism the identified gene, *insulin-like growth factor-binding protein-6* (*IGFBP-6*), acted as an effector of SEMA3B.

Results

Establishment of stable SEMA3B transfectants

Cell lines (stSEMA3B and stSEMA3B-FLAG) that stably express the native form of SEMA3B or a FLAG-tagged SEMA3B were established by introducing their expression plasmids into NCI-H1299 cells that do not express SEMA3B. This established an experimental system where SEMA3B expressed from the plasmid was secreted and acted on the cells in an autocrine manner. As SEMA3B function may be post-translationally modified, this system was considered more suitable for investigating SEMA3B function than what employs recombinant proteins expressed in prokaryotes. SEMA3B was detected both in the conditioned medium (CM; Figure 1a) and in the cytoplasm (cell lysate, CL; Figure 1b). Expression was not observed in the cell line (stpcDNA) that was established by transfecting an empty plasmid.

Identification of IGFBP-6

We employed a microarray to search for mRNAs that were more highly expressed in stSEMA3B than in stpcDNA in an attempt to find a gene that is an effector of SEMA3B (Figure 2a, also see Supplementary information). Reverse transcription PCR (RT–PCR) was performed on several growth-related genes to confirm the microarray data (Figure 2b). Among the cell growth-related genes studied, *IGFBP-6* showed the greatest increase. As expected, IGFBP-6 protein was increased in CM of stSEMA3B, as was in CM of trSEMA3B, stSEMA3B-FLAG and trSEMA3B-FLAG (Figure 2c). IGFBP-6 has been shown to suppress the growth of cancer cells (Sueoka *et al*., 2000; Leng *et al*., 2001; Koike *et al*., 2005) and thus may be an effector of tumor suppressor activity for SEMA3B. Next, we semiquantified the expression levels of *SEMA3B* and *IGFBP-6* in lung cancer cell lines (Figure 2d). The expression of *IGFBP-6* positively correlated with that of *SEMA3B* and was lower than that of normal lung tissue. These observations were consistent with the hypothesis that IGFBP-6 is an effector of SEMA3B and so we decided to further investigate the functions of IGFBP-6.

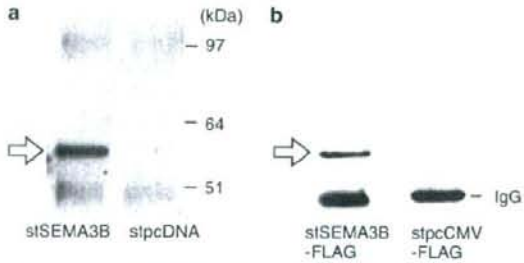

Figure 1 Expression of SEMA3B protein in the stSEMA3B cell line. (**a**) Western blot. SEMA3B protein (60 kDa) in CM of stSEMA3B is indicated by a white arrow. (**b**) Immunoprecipitation. FLAG-tagged SEMA3B protein in CL of stSEMA3B is indicated by a white arrow. IgG that bound nonspecifically to the ANTI-FLAG M2 Affinity Gel serves as a control and confirms the equal loading of the samples. CL, cell lysate; CM, conditioned medium.

Suppression of colony formation by IGFBP-6

IGFBP-6 binds to and sequesters insulin-like growth factor II (IGF-II) (Bach *et al*., 1994). IGF-II often acts as an autocrine growth factor stimulating the growth of some cancer cells, whereas IGFBP-6 suppresses their growth (Kato *et al*., 1995). Therefore, IGFBP-6 acts as a tumor suppressor for such cancer cells. In addition to this IGF-II-dependent mechanism, IGFBP-6 may act by an IGF-II-independent mechanism to exert its tumor suppressor activity. This mechanism is as yet vaguely understood (Grellier *et al*., 1998; Sueoka *et al*., 2000). The effect of IGFBP-6 on the growth of NCI-H1299 cells was studied by measuring colony formation on the dish surface and in soft agar (Figures 3a and b). In both assays, the introduction of an IGFBP-6 expression plasmid significantly reduced the number of colonies, demonstrating that IGFBP-6 exerted a tumor suppressor activity on NCI-H1299 cells.

Inhibition of IGFBP-6 suppressed SEMA3B effects

Results obtained so far suggest that SEMA3B exerts a tumor suppressor activity by inducing the expression of IGFBP-6 as IGFBP-6 is an immediate cause of the activity. To confirm this, we investigated whether the inhibition of IGFBP-6 could abrogate the tumor suppressor activity of SEMA3B.

First, we used small interfering RNA (siRNA) to inhibit IGFBP-6. RT–PCR and western blot analyses showed that 10 nM of *IGFBP-6* siRNA efficiently inhibited the expression of IGFBP-6 (Figure 4a); so this concentration was used. Introduction of *SEMA3B* cDNA into NCI-H1299 cells increased the sub-G0/G1 population, reflecting the tumor suppressor activity of SEMA3B (Figure 4b). Transfection of *IGFBP-6* siRNA canceled this effect (Figure 4b(iii)), whereas that of scrambled siRNA did not (Figure 4b). Transfection of *IGFBP-6* siRNA but not scrambled siRNA also abrogated the growth inhibition by SEMA3B (Figure 4c). Second, we used IGF-II to inhibit IGFBP-6, as the amount of IGF-II higher than that of IGFBP-6 is able to sequester the growth suppressor activity of IGFBP-6 (Bach *et al*., 1995). In the presence of 50 ng/ml of human IGF-II, the effect of IGFBP-6 was partially abrogated (Figure 4d). These results demonstrate that

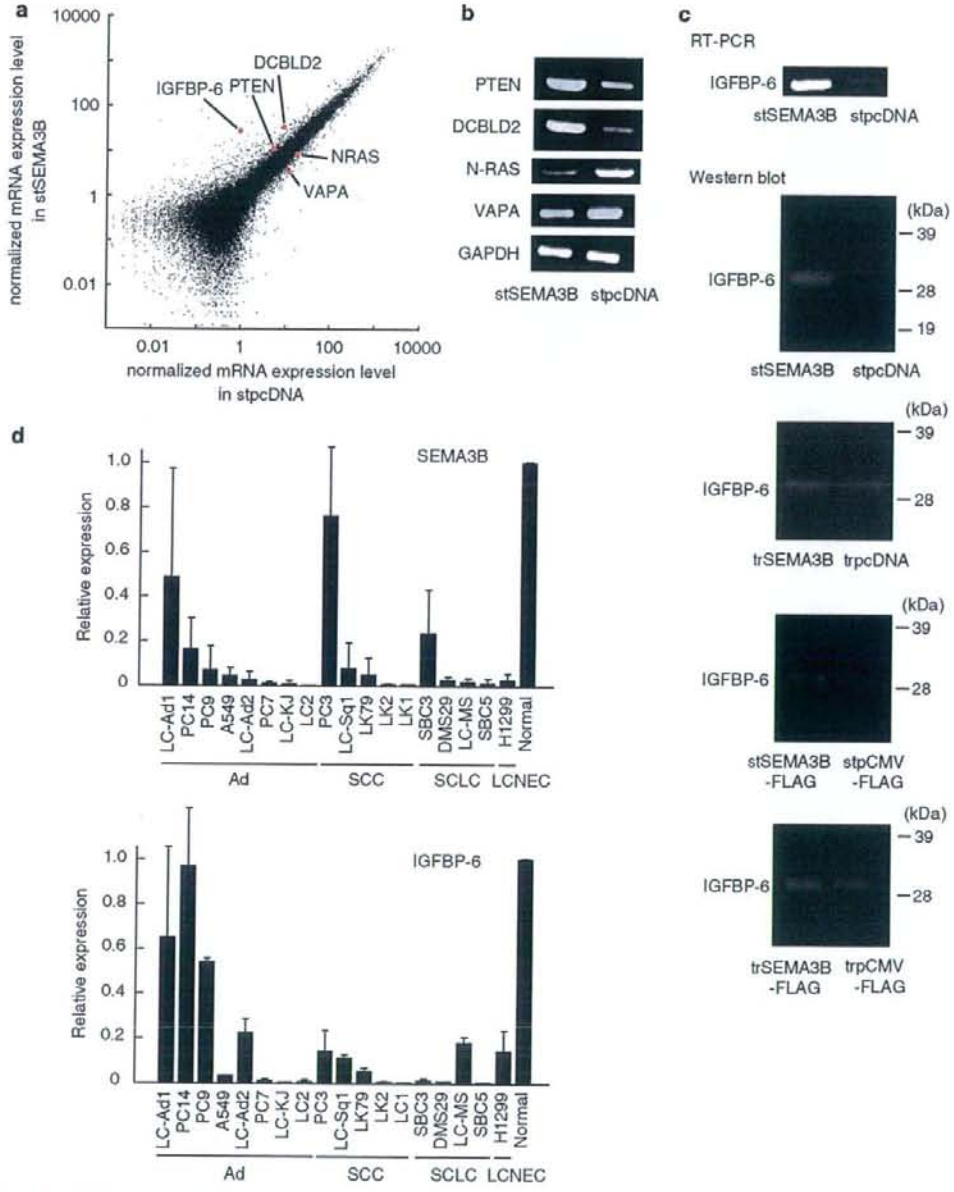

Figure 2 Identification of IGFBP-6. (**a**) A microarray analysis comparing the levels of each mRNA in stSEMA3B and stpcDNA. The spots for *IGFBP-6*, *DCBLD2*, *PTEN*, *NRAS* and *VAPA* are colored red. (**b**) Confirmation of the microarray data by RT–PCR. The results reproduce the microarray data. *GAPDH* mRNA is used as a control. (**c**) *IGFBP-6* expression by RT–PCR and that in CM observed by western blot. (**d**) Expression of *SEMA3B* and *IGFBP-6* in lung cancer cell lines. The expression level was semiquantitatively measured by real-time PCR and normalized to the level of *GAPDH*. The value for the normal lung tissue was set to 1.0. Error bars indicate standard deviations. Ad, adenocarcinoma; AdSq, adenosquamous cell carcinoma; CM, conditioned medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGFBP-6, insulin-like growth factor-binding protein-6; LCNEC, large-cell neuroendocrine carcinoma; RT–PCR, reverse transcription PCR; SCC, squamous cell carcinoma; SCLC, small-cell lung cancer.

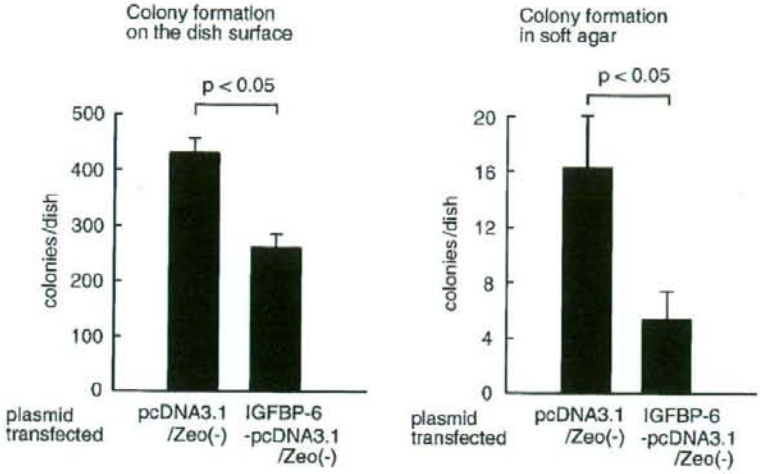

Figure 3 Colony formation on the dish surface and in soft agar. Numbers of colonies formed after the transfection of either the IGFBP-6 expression vector (IGFBP-6-pcDNA3.1/Zeo(−)) or an empty vector (pcDNA3.1/Zeo(−)) are shown. The medium contained 400 μg/ml Zeocin. Experiments were done in triplicates. Error bars indicate standard deviations. IGFBP-6, insulin-like growth factor-binding protein-6.

IGFBP-6 does mediate the tumor suppressor activity of SEMA3B.

Interaction of SEMA3B and β-catenin/TCF signaling

β-catenin is a key component of Wnt/TCF signaling. An accumulation of β-catenin in the cytoplasm activates TCFs and modifies the expression of downstream genes. Excessive activity in the β-catenin/TCF pathway is often observed in cancer cells (Nelson and Nusse, 2004; Clevers, 2006). In NCI-H1299 cells, TCF activity is induced by introducing *β-catenin* cDNA (Usami *et al.*, 2003). The *IGFBP-6* promoter has two copies of TCF-binding motifs, and β-catenin suppresses the expression of IGFBP-6 through these motifs (Denys *et al.*, 2004). These results suggest that, in NCI-H1299 cells, β-catenin may antagonize the tumor suppressor activity of SEMA3B by inhibiting the expression of IGFBP-6. To confirm this, we introduced *β-catenin* cDNA into stSEMA3B to establish stSEMA3B-β-catenin and investigated its effect. The introduction of *β-catenin* cDNA reduced the expression of IGFBP-6, which had been increased by the introduction of *SEMA3B* cDNA (Figure 5a). Moreover, the growth-suppressing activity of SEMA3B was partially abrogated (Figure 5b). These observations are consistent with the inference that β-catenin may antagonize SEMA3B, and indicate that SEMA3B signaling and β-catenin/TCF signaling converge at IGFBP-6 to affect the growth of NCI-H1299 cells.

Discussion

The aim of our study was to find out the as yet unidentified mechanism for the growth inhibition of lung cancer cells by SEMA3B. We have demonstrated that SEMA3B exerted its growth inhibitory effect through the induction of IGFBP-6 expression, and the effect was antagonized by β-catenin.

SEMA3B is an extracellular factor that binds to its receptor, transmits the signal into the cell and affects the cell growth. Signaling that is mediated by a membrane receptor often induces activation or inactivation of a specific transcription factor that changes the transcription of a variety of genes. The transcribed gene in turn exerts its function on cell growth. We speculate that a similar mechanism is at work for SEMA3B because SEMA3B increases the amount of *IGFBP-6* mRNA. Whatever the mechanism, the identification of the effector would have been a tremendous challenge without microarray technology. We focused only on growth-related genes to narrow the number of candidates to identify the effector. Similar approaches are found in the literature where the researchers employed a microarray and showed that peroxisome proliferator-activated receptor γ is a Zac target gene mediating Zac antiproliferation, and that heat shock protein 70 induced by the inhibitor of growth 1 sensitized cells to tumor necrosis factor-α receptor-mediated apoptosis (Barz *et al.*, 2006; Feng *et al.*, 2006). With an enrichment of functional information on individual genes, the strategy taken in this study and others will become much easier and more promising.

Members of the IGFBP family act on cell migration and growth in both IGF-dependent and IGF-independent mechanisms (Firth and Baxter, 2002). IGFBP-6 is unique among the family members as it provides a 20- to 100-fold higher affinity to IGF-II than to IGF-I (Martin *et al.*, 1990; Bach, 1999). The affinity is not so much differentiated in other members of IGFBPs. IGFBP-6 sequesters IGF-II, and thus inhibits growth in lung

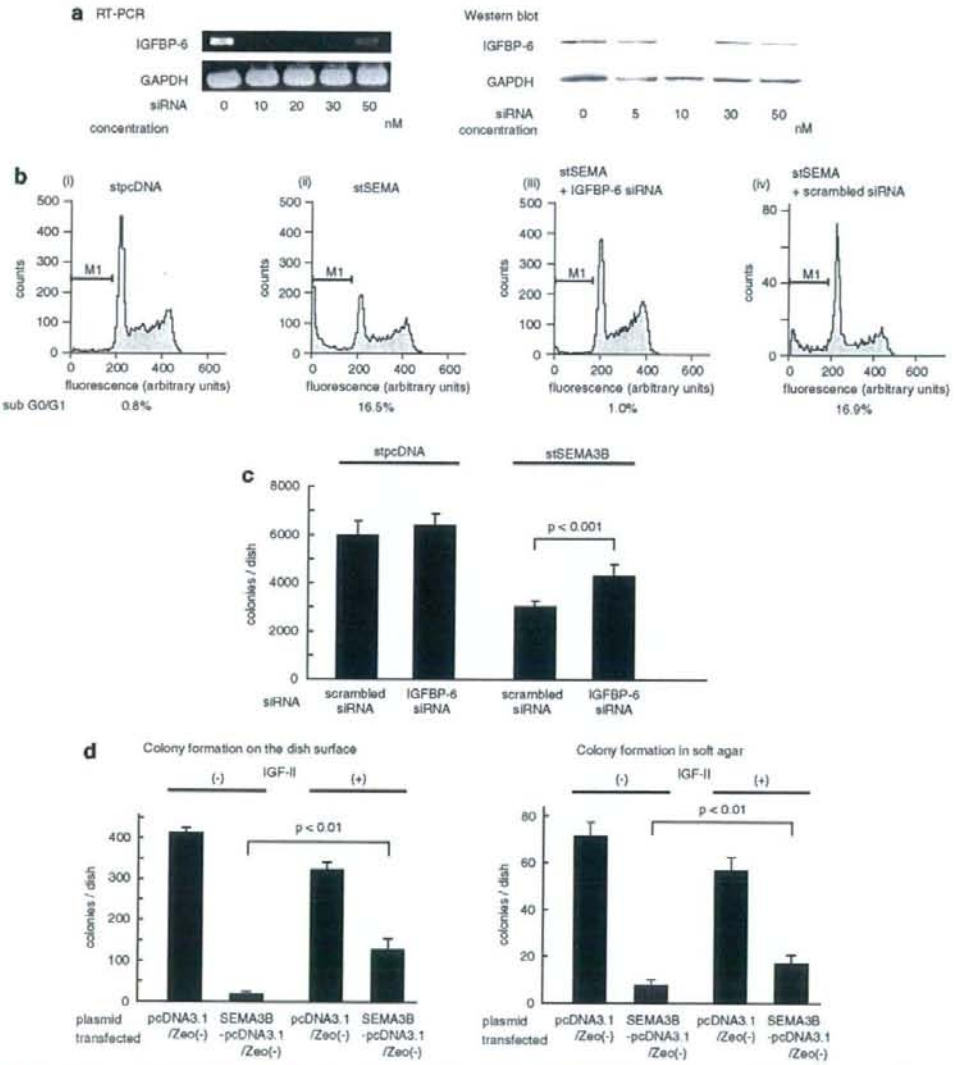

Figure 4 Effects of IGFBP-6 inhibition. (**a**) Optimization of the amount of siRNA monitored either by RT–PCR or by western blot. (**b**) The effect on cell cycle investigated by flow cytometry. The percent of cells that represent sub-G0/G1 fluorescence are indicated. (**c**) The effect on cell proliferation investigated by the cell growth assay. Cell growth suppressed by the introduction of SEMA3B is restored by *IGFBP-6* siRNA. (**d**) The effect on colony formation. Numbers of colonies formed on the dish surface or in soft agar after the transfection of either the SEMA3B expression vector (SEMA3B-pcDNA3.1/Zeo(−)) or an empty vector (pcDNA3.1/Zeo(−)). The medium contained 400 μg/ml of Zeocin together with either 0 or 50 ng/ml of human IGF-II. Experiments were done in triplicates. Error bars indicate standard deviations. IGF-II, insulin-like growth factor II; IGFBP-6, insulin-like growth factor-binding protein-6; RT–PCR, reverse transcription PCR; siRNA, small interfering RNA.

cancer (Sueoka *et al*., 2000), colorectal cancer (Leng *et al*., 2001), prostate cancer (Koike *et al*., 2005), neuroblastoma (Grellier *et al*., 1998) and rhabdomyosarcoma (Gallicchio *et al*., 2001), whereas its expression is lower in malignant or metastatic tumors than in benign or non-metastatic tumors (Scholl *et al*., 2000; Yao *et al*., 2002). Moreover, the p53 tumor suppressor increases *IGFBP-6* expression (Kannan *et al*., 2001). These results indicate that IGFBP-6 is a tumor suppressor for a variety of cancers. In our experiment, inhibition of IGFBP-6 by siRNA abolished the effect of SEMA3B on NCI-H1299 cells, indicating that the

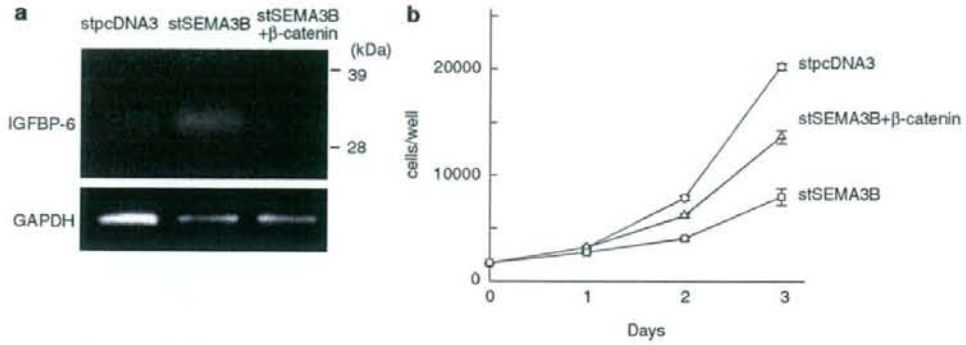

Figure 5 Inhibition of SEMA3B activity by β-catenin. (**a**) Change in the IGFBP-6 expression, western blot. IGFBP-6 expression increases after *SEMA3B* cDNA introduction is reduced by the introduction of *β-catenin* cDNA. (**b**) Change in cell growth. Cell growth suppressed by the introduction of SEMA3B is increased by the introduction of *β-catenin* cDNA. IGFBP-6, insulin-like growth factor-binding protein-6.

growth inhibitory effect of SEMA3B is mediated by IGFBP-6 and links the effects of these two tumor suppressors.

Pathways that transmit growth-related signals often converge on key molecules to antithetically affect their functions. We showed that IGFBP-6 is one of such key molecules where the signaling from SEMA3B and β-catenin converges. Semaphorin increases the expression of phosphatase protein homolog to tensin (PTEN) and activates glycogen synthase kinase-3β, resulting in the inhibition of integrin-mediated adhesion to the extracellular matrix (Tran *et al*., 2007). These results suggest that semaphorin signaling and β-catenin/TCF signaling may also converge at other points, which we have shown in this study.

Carcinogenesis requires both the activation and the inactivation of multiple signaling pathways, leading to the concept of multistep carcinogenesis. Further study into the interrelationship of SEMA3B signaling with other signaling pathways will contribute to our overall understanding of cancers.

Materials and methods

Cell lines

Lung cancer cell lines were obtained as follows. NCI-H1299 (large cell neuroendocrine carcinoma) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). PC-7 and PC-9 (adenocarcinomas) were purchased from IBL (Takasaki, Japan). RERF-LC-Ad1, RERF-LC-Ad2, RERF-LC-MS and PC-3 (adenosquamous carcinomas), as well as RERF-LC Sq-1 (squamous cell carcinomas) were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan). RERF-LC-KJ, LC2/ad and PC-14 (adenocarcinomas), and RERF-LC-A1 (squamous cell carcinomas) were purchased from the Riken Bioresource Center (Tsukuba, Japan). SBC3 and SBC5 (small cell carcinomas) were purchased from the Health Science Research Resources Bank (Osaka, Japan). A549 (adenocarcinoma), LK79 and LK2 (squamous cell carcinomas) were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). Cells were grown in RPMI medium 1640 (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (CELLect GOLD; MP Biomedicals, Eschwege, Germany).

Plasmid construction and transfection

A plasmid, SEMA3B-pcDNA3.1/Zeo(−), that expresses wild-type SEMA3B protein was constructed by inserting human *SEMA3B* full-length cDNA into pcDNA3.1/Zeo(−) (Invitrogen, Carlsbad, CA, USA). A plasmid, SEMA3B-pCMV-Tag2A, that expresses N-terminal FLAG-tagged SEMA3B protein was constructed by inserting human *SEMA3B* full-length cDNA into pCMV-Tag2A (Stratagene, La Jolla, CA, USA). A plasmid, IGFBP-6-pcDNA3.1/Zeo(−), that expresses IGFBP-6 protein was constructed by inserting human *IGFBP-6* full-length cDNA into pcDNA3.1/Zeo(−). A plasmid, β-catenin-pcDNA3.1(+), that expresses β-catenin protein was constructed by inserting human *β-catenin* full-length cDNA into pcDNA3.1(+) (Invitrogen). These plasmids and the three appropriate negative control plasmids, pcDNA3.1/Zeo(−), pCMV-Tag2A and pcDNA3.1(+), were transfected into NCI-H1299 cells using FuGENE 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. In brief, a plasmid (1 μg) and FuGENE 6 (3 μl) were complexed in 100 μl Opti-MEM (Invitrogen) for 15 min. The complex was added to 3×10^5 NCI-H1299 cells seeded in a 35-mm dish and the cells were incubated for 48 h (transient transfectants). To establish the stable transfectants, transient transfectants were incubated in the medium containing 400 μg/ml Zeocin (Invitrogen) or 1 mg/ml Geneticin (Invitrogen) for 14 days. The drug-resistant colonies were cloned, and the clones were expanded. The names of the plasmids transfected and the names of the stable transfectants are paired below and are separated by commas: SEMA3B-pcDNA3.1/Zeo(−), stSEMA3B; SEMA3B-pCMV-Tag2A, stSEMA3B-FLAG; SEMA3B-pcDNA3.1/Zeo(−) and β-catenin-pcDNA3.1(+), stSEMA3B-β-catenin; pcDNA3.1/Zeo(−), stpcDNA; pCMV-Tag2A, stpCMV-FLAG. The names of the plasmids transfected and the names of the transient transfectants are SEMA3B-pcDNA3.1/Zeo(−), trSEMA3B; SEMA3B-pCMV-Tag2A, trSEMA3B-FLAG; SEMA3B-pcDNA3.1/Zeo(−) and β-catenin-pcDNA3.1(+), trSEMA3B-β-catenin; pcDNA3.1/Zeo(−), trpcDNA; pCMV-Tag2A, trpCMV-FLAG.

Western blot analysis
Cells (1×10^7) were plated in a 100-mm dish and incubated in 10 ml RPMI-1640 for 24 h. Cell lysate was prepared using mammalian cell lysis/extraction reagent (Sigma). Both the cell lysate and the cryoconcentrated conditioned medium were subjected to western blot analysis. Samples were dissolved in LDS sample buffer (Invitrogen), heated at 99 °C for 5 min, electrophoresed in NuPAGE 4–12% bis-Tris gel (Invitrogen) and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was immersed in 5% skim milk, 0.1% Tween 20 and dissolved in Tris-buffered saline to block the nonspecific binding. The membranes were incubated overnight at 4 °C with a primary antibody that included goat anti-human SEMA3B polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse anti-human IGFBP-6 monoclonal antibody (R & D Systems, Minneapolis, MN, USA) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Chemicon International Inc., Billerica, CA, USA). The membranes were then incubated for 1 h at 25 °C with a secondary antibody that included horseradish peroxidase-conjugated anti-goat, anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology). The specific signals were visualized by ECL plus (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Immunoprecipitation
Cells were harvested and sonicated in lysis buffer that contained Tris–HCl (50 mM), NaCl (150 mM), EDTA (0.1 mM), 1% Triton-X, 1% NP-40 and Complete Protease Inhibitor Cocktail Tablets (Roche). The FLAG-tagged recombinant protein was captured to the EZview Red ANTI-FLAG M2 Affinity Gel (Sigma) for 24 h at 4 °C. Bound proteins were eluted in an extraction buffer by boiling the gel at 100 °C for 5 min, electrophoresed in an SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The protein was detected as described in the section 'Western blot analysis' except that a mouse monoclonal ANTI-FLAG M2 (Sigma) was used as the primary antibody.

Microarray analysis
stSEMA3B was used as the reference cell line and stpcDNA was used as the control cell line. Total RNA was extracted from the cells by use of the Trizol reagent (Invitrogen). DNA from the RNA sample was completely removed by digesting with RQ1 RNase-free DNase (Promega, Madison, WI, USA), followed by phenol–chloroform extraction. Microarray analysis was performed using the Amersham CodeLink Bioarray Systems (GE Healthcare Bio-Sciences).

RT–PCR
IGFBP-6, *PTEN* and *discoidin, CUB and the LCCL domain containing 2* (*DCBLD2*), *neuroblastoma RAS viral (v-ras) oncogene homolog* (*NRAS*) and *vesicle-associated membrane protein-associated protein A* (*VAPA*) were used as probe genes and *GAPDH* as a control. Total RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). One microgram of cDNA was subjected to PCR (1 cycle of 95 °C for 3 min; 20–35 cycles of 95 °C for 30 s, 60 °C for 15 s and 72 °C for 1 min; 1 cycle of 72 °C for 7 min). The primers used were *IGFBP-6*-F, 5′-GCAGAGGAGAA TCCTCCTAAGGAGAG-3′ and *IGFBP-6*-B, 5′-CAGCCAA CACCAACACTCTTTC-3′; *PTEN*-F, 5′-CTGACACCAC TGACTCTGATCC-3′ and *PTEN*-B, 5′-ACCCTTCGGAA ACCTCTCTTAG-3′; *DCBLD2*-F, 5′-GCTGATGTAAACG GTTCGAGTG-3′ and *DCBLD2*-B, 5′-GTGACTACAGTA CATGCCGAGGTA-3′; *NRAS*-F, 5′-CAGAGAACCAAA CCGCAAAC-3′ and *NRAS*-B, 5′-AAACAGGCCTCTG GAAAACC-3′; *VAPA*-F, 5′-CTGACACCCAGTGTACCT ACCTACC-3′ and *VAPA*-B, 5′-CACGGACTGAGATTGTT AGTGG-3′; and *GAPDH*-F, 5′-CCTCAACGACCACTTT GTCA-3′ and *GAPDH*-B, 5′-TTACTCCTTGGAGGCCA TGT-3′. The ratios of the amounts of mRNAs were calculated by comparing the intensities of the specific bands measured by a densitometer.

Semiquantification of SEMA3B and IGFBP-6 mRNA by real-time PCR
Total RNAs from 17 human lung cancer cell lines and from normal human lung tissue (negative control; Clontech, Mountainview, CA, USA) were reverse-transcribed, mixed with SYBR Premix Ex Taq (Takara Inc., Shiga, Japan) and amplified by PCR (1 cycle of 95 °C for 120 s; 45cycles of 95 °C for 5 s and 62 °C for 20 s) using the Smart Cycler (Cepheid Innovation, Sunnyvale, CA, USA). The primers used were *SEMA3B*, 5′-GCGTGGAGTGGACTTTCCAG-3′ (forward) and 5′-CAGCCTGCGCAGCAGTAGTC-3′ (reverse); *IGFBP-6*, 5′-CGAGGGGCTCAAACACTCTA-3′ (forward) and 5′-CATCCGATCCACACACCAG-3′ (reverse); and *GAPDH*, 5′-CCTCAACGACCACTTTGTCA-3′ (forward) and 5′-TTACTCCTTGGAGGCCATGT-3′ (reverse). Each mRNA was quantified using its amplification data and then normalized using the amount of *GAPDH* mRNA.

Colony formation on the dish surface
Cells transfected with a plasmid and incubated for 48 h (see 'Plasmid construction and transfection' above) were replated into a 10-cm dish and cultured for 14 days in RPMI-1640 containing 10% fetal bovine serum and 400 μg/ml Zeocin. In the experiment investigating the effects of IGF-II on the growth inhibition through IGFBP-6, the medium also contained 0 or 50 ng/ml of human IGF-II (Strathmann Biotech Gmbh, Hamburg, Germany).

Colony formation in soft agar
NCI-H1299 cells (15 000 cells) transfected with either pcDNA3.1/Zeo(−) or SEMA3B-pcDNA3.1/Zeo(−) and incubated for 48 h (see 'Plasmid construction and transfection' above) were suspended in medium (RPMI-1640, 10% fetal bovine serum, 400 μg/ml Zeocin and 0 or 50 ng/ml of human IGF-II) containing 0.33% melted agar. The suspension was solidified on a presolidified medium containing 0.5% agar. The same medium was placed on top of the double agar layer and was renewed every 2 days. The cultures were incubated for 14 days and then the numbers of colonies were counted.

Transfection of small interfering RNA
The siRNAs for *IGFBP-6* were sense, 5′-CCCGCCCACAG GAUGUGAACC-3′ and antisense, 5′-UUCACAUCCU GUGGGCGGGCA-3′. These sequences were rearranged and used to design negative control siRNAs that were sense, 5′-CGCAUGCGGACUGCCAACACC-3′ and antisense, 5′-UGUUGGCAGUCCGCAUGCGCA-3′. Different concentrations of siRNA were transfected into stSEMA3B by Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Total RNA or cellular protein was extracted 48 or 72 h after transfection, respectively.

Flow cytometry analysis
Seventy-two hours after the siRNA transfection, cells were harvested, fixed in 70% ethanol and suspended in phosphate-

buffered saline containing 20 μg/ml Ribonulease A (Sigma), 50 μg/ml propidium iodide, 0.1% sodium citrate and 0.3% NP-40. Flow cytometry was performed using the FACSCalibur equipped with CELLQUEST software (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell growth assay

One thousand cells per well of stpcDNA, stSEMA3B or stSEMA3B-β-catenin were seeded into 96-well dishes containing 100 μl of RPMI-1640 with 2% fetal bovine serum (day 0). On days 1, 2 and 3, 10 μl of Cell Counting Kit-8 reagent (DOJINDO, Kumamoto, Japan) was added to each well. Cells were then incubated for 3 h, and the absorbance at 450 nm/620 nm was measured. Five samples were analysed for each cell group. For the assay treated with siRNA, *IGFBP-6* or scrambled siRNA was transfected into 3000 cells per well of stpcDNA or stSEMA3B on day 1. The medium was changed to remove the siRNAs 6 h after transfection, and the cells were incubated for 72 h. On day 4, the absorbance was measured.

Acknowledgements

We thank Ms Akemi Yokote for technical assistance. This study was supported in part by the following grants-in-aid: 1) for scientific research (nos. 18390242 and 16590738) from the Japan Society for the Promotion of Science and 2) for Comprehensive Research on Aging and Health from the Ministry of Health, Labor and Welfare, Japan.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

E-cadherin expression and epidermal growth factor receptor mutation status predict outcome in non-small cell lung cancer patients treated with gefitinib

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Received October 3, 2007; Accepted November 15, 2007

Abstract. It is known that an epidermal growth factor receptor (EGFR) gene mutation(s) is present in a percentage of non-small cell lung cancers (NSCLCs). Gefitinib, an inhibitor of the tyrosine kinase activity of EGFR, is effective on most of them. The EGFR mutation status alone cannot fully predict the response to gefitinib and the prognosis for the patients. We hypothesized that information on the expression levels of phosphorylated-EGFR and -Akt, and E-cadherin, alone or in combination with information on the EGFR mutation, may refine our ability of prediction. We investigated 24 NSCLCs that had recurred after surgery and were treated with gefitinib. Specimens resected by surgery were subjected to the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp reaction to determine the EGFR mutation status, and to immunohistochemical staining of phosphorylated-EGFR and -Akt, and E-cadherin to determine their expression levels. The EGFR mutation status was predictive of responsive disease (complete response: CR + partial response: PR) and controlled disease (CR + PR + stable disease: SD). Positive E-cadherin staining was predictive of longer time to progression (12.4 vs. 5.9 months, $p<0.05$) and overall survival (OS) (18.4 vs. 13.0 months, $p<0.05$). Together the patients with an EGFR mutation and the patients with positive E-cadherin staining defined a patient group with a median OS of 18.4 months and excluded the patient group with the median OS of 3.7 months. Neither p-Akt nor p-EGFR staining was associated with the response and survival. In patients with surgically resected NSCLC tumors, the EGFR mutation status and E-cadherin staining can select patients who will benefit from gefitinib therapy.

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Key words: non-small cell lung cancer, gefitinib, E-cadherin, epidermal growth factor receptor mutation

Introduction

Gefitinib (ZD1839, Iressa, AstraZeneca, Wilmington, DE) is a therapeutic reagent for non-small cell lung cancers (NSCLCs). It shows dramatic anti-tumor effects in some patients, but has no effect in others (1). The presence of an epidermal growth factor receptor (EGFR) gene mutation(s) (hereafter EGFR mutation) associates significantly with the gefitinib responsiveness (2,3) and serves as a marker in the choice of therapeutic regimens (4). Some tumors with an EGFR mutation do not respond to gefitinib therapy while those with wild-type gene do (5-7), so additional markers are required to more precisely select tumors that respond to gefitinib.

EGFR transmits signals that direct cell proliferation and survival. The wild-type EGFR preferentially transmits cell proliferation signals through Erk, while the mutant EGFR preferentially transmits cell survival signals through Akt or STAT (8). Gefitinib effectively inhibits the latter (2). This is why gefitinib selectively elicits an apoptotic response in cells with an EGFR mutation, thereby producing its clinical response (8). We hypothesized that molecules that interact with EGFR or are located downstream in the pathway modify the tumor cell response to gefitinib and therefore serve as markers that may help to more precisely predict their responsiveness to gefitinib.

In this study, three molecules were tested for their predictive ability, p-EGFR (phosphorylated at Tyr1173: pTyr1173), p-Akt (phosphorylated at Ser473: pSer473) and E-cadherin, in addition to the EGFR mutation status. p-EGFR(pTyr1173) transmits a signal that directs cell proliferation (9), p-Akt(pSer473) mediates signals that direct cell survival (10) and E-cadherin has been shown to interact with EGFR by modifying its activity (11). We investigated the expressions of these three molecules by immunohistochemistry

in 24 NSCLCs that had been resected by surgery, recurred afterward and were treated with gefitinib. The results enabled us to test their staining intensity, alone, or in combination with the EGFR mutation status. It also improved our ability to predict the responsiveness to gefitinib and patient outcome.

Materials and methods

Patients. This study was approved by the Tsuboi Cancer Center Hospital ethics board. After the written informed consent was obtained, we enrolled 24 Japanese patients who had suffered from lung cancers which were resected between 1996 and 2004 (Tsuboi Cancer Center Hospital, Fukushima, Japan) and then had recurred. The patient characteristics are summarized in Table I. Gefitinib, 250 mg per day, was initiated between July 2002 and October 2006 to treat the recurrent disease. The median time between the surgery and the start of the gefitinib treatment was 740 days (range: 113-2.012). Treatment was continued until the disease progressed, intolerable toxicity developed or a patient refused treatment for other reasons.

Evaluation of the response to gefitinib and patient outcome. Every 4 weeks chest X-rays or computed tomography (CT) scans were done to evaluate tumor response and lung toxicity, and blood tests were done to monitor systemic toxicity. Tumor response that remained stable for at least 30 days was graded according to the Response Evaluation Criteria in Solid Tumors (12). Time to progression (TTP) in these patients was defined as the interval from the start of gefitinib administration to disease progression or death. The outcomes were evaluated up to May 31, 2007, with an average follow-up time of 20.6 months (range: 1.1-50.0). Both mutation and immunohistochemical analyses were performed after completion of the response evaluation.

Table I. Patient characteristics.

Characteristic	No. of patients (n=24)	%
Gender		
Male	13	54.2
Female	11	45.8
Median age, years (range)	63.2	(44-84)
ECOG performance status		
0	6	25.0
1	18	75.0
Histology		
Adenocarcinoma	21	87.5
Squamous cell carcinoma	1	4.2
Adenosquamous cell carcinoma	2	8.4
Prior chemotherapy		
0-1 regimens	18	75.0
>2 regimens	6	25.0
Smoking history		
Never smoked	19	79.2
Smoker (current/former)	5	20.8
Stage		
I-II	19	79.2
III-IV	5	20.8

ECOG, Eastern Cooperative Oncology Group.

DNA extraction and mutation analysis. DNA was extracted from the paraffin-embedded tumor tissue (13-15). EGFR mutations were detected using the peptide nucleic acid-locked nucleic acid (PNA-LNA) polymerase chain reaction (PCR) clamp. This method, which has been described in detail elsewhere, is a rapid and sensitive detection system for EGFR gene mutations and can detect point mutations G719C, G719S, L858R and L861Q and deletions in exon 19 in the presence of a 100- to 1.000-fold background of wild-type EGFR (4,6,16).

Immunohistochemistry and scoring. Formalin-fixed, paraffin-embedded tumor tissue was tested for immunoreactivity to p-EGFR, p-Akt and E-cadherin. The primary antibodies used were: anti-p-EGFR that detects EGFR protein phosphorylated at Tyr1173 (Cell Signaling Technology Beverly, MA), anti-p-Akt that detects Akt protein phosphorylated at Ser473 (Cell Signaling Technology) and anti-E-cadherin (BD Biosciences, Beverly, MA). Tissue sections cut at a thickness of 5 μm were placed on glass slides, deparaffinized and then rehydrated. Antigen was quantified using the following procedure. The slides were incubated in citrate buffer in a steamer for 15 min. Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide for 5 min and non-specific background staining was blocked by incubation in a protein block for 5 min. Sections were then reacted with primary antibody dilutions (p-EGFR a 1/400 dilution at 37˚C for 15 min, p-Akt a 1/50 dilution at 4˚C for 16 h and E-cadherin a 1/100 dilution at 37˚C for 32 min). The bound antibody was detected by biotinylated secondary antibody and visualized using diaminobenzidine (DAB) chromogen. Sections were then counterstained with Mayer's hematoxylin and mounted using the resinous mounting medium.

The p-EGFR and p-Akt stainings were scored by their cytoplasmic and nuclear staining, while E-cadherin staining was scored by its membrane staining (17-19), all without the knowledge of clinical or laboratory information. The cytoplasmic and nuclear staining of the entire tumor was scored as follows: First, 500 randomly selected tumor cells (50 cells per randomly chosen microscopic field at x40 magnification) were scored as 0 (no staining), 1 (mild), 2 (moderate) or 3 (strong staining). Second, for p-EGFR, the most intense staining observed in >1% of the cells was the staining score for the tumor. For p-Akt, the mode of the cytoplasmic or nuclear staining score, whichever was greater, was the staining score for the tumor. Tumors with staining scores of 0 or 1 were ranked negative and scores of 2 or 3 were ranked positive. The membrane staining of the entire tumor was scored as 0 when no tumor cells were stained, 1 when <10%