Increases of Amphiregulin and Transforming Growth Factor-α in Serum as Predictors of Poor Response to Gefitinib among Patients with Advanced Non–Small Cell Lung Cancers

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Abstract

Serum levels of amphiregulin and transforming growth factor-α (TGF-α), which were identified previously to be expressed at high levels in non–small cell lung cancer (NSCLC) with poor response to gefitinib, were examined by ELISA using blood samples taken from 50 patients with advanced NSCLCs. Of 14 cases that revealed above the cutoff line for amphiregulin in serum, 12 responded poorly to gefitinib, whereas 18 of the 36 cases showing below the cutoff revealed partial response (PR) or stable disease (SD; *P* = 0.026). Thirteen of 15 patients who were positive for TGF-α responded poorly to gefitinib, whereas 18 of the 35 patients with negative TGF-α levels turned out to be relatively good responders (*P* = 0.014). Of 22 patients with positive values for either or both markers, 19 were poor responders. On the other hand, among 28 patients negative for both markers, 17 were classified into the PR or SD groups (*P* = 0.001). Gefitinib-treated NSCLC patients whose serum amphiregulin or TGF-α was positive showed a poorer tumor-specific survival (*P* = 0.037 and 0.002, respectively, by univariate analysis) compared with those whose serum amphiregulin or TGF-α concentrations were negative. Multivariate analysis showed an independent association between positivity for TGF-α and shorter survival times among NSCLC patients treated with gefitinib (*P* = 0.034). Amphiregulin or TGF-α positivity in NSCLC tissues was significantly higher in male, nonadenocarcinomas, and smokers. Our data suggest that the status of amphiregulin and TGF-α in serum can be an important predictor of the resistance to gefitinib among patients with advanced NSCLC. (Cancer Res 2005; 65(20): 9176-84)

Introduction

Epidermal growth factor receptor (EGFR) plays an important role in the growth of solid tumors originating from various tissues and is overexpressed in 40% to 80% of non–small cell lung cancers (NSCLC) examined (1, 2). Overexpression of this receptor is also associated with poor prognosis of patients with lung cancer (3). Gefitinib (Iressa, ZD1839) is an orally administered inhibitor of EGFR tyrosine kinase, a key enzyme in the EGFR signaling pathway involved in proliferation, invasion, and survival of cancer cells (4). Potent antitumor effects have been observed in clinical trials that enrolled patients with advanced NSCLC who had not responded to platinum-based chemotherapy (5, 6). Gefitinib has been used in several countries, including Japan, Australia, and the United States, for the treatment of advanced NSCLC.

Approximately 37,000 patients with advanced NSCLC have been treated with this drug in Japan since its approval (7). Although gefitinib has been effective for many of those Japanese patients, improving their prognosis and quality of life, ~60% of them have shown no improvement in symptoms. Furthermore, the incidence of severe gefitinib-induced acute interstitial pneumonia can be as high as 5.4% (8). Obviously, a method that would allow physicians to select patients likely to respond well to gefitinib would be highly desirable. Unfortunately, no single factor examined thus far, including somatic mutations of *EGFR*, has been able to perfectly determine the susceptibility of patients to gefitinib treatment from the viewpoint of disease control or survival benefit [i.e., they have failed to discriminate certain proportion of potential long-term survivors, including stable disease (SD) patients from short-term survivors]. The presence of *EGFR* mutations can usually predict patients likely to show partial response (PR) to gefitinib, but this approach has no power to indicate patients keeping stable condition who could receive a survival benefit (9–13).

We recently identified dozens of genes associated with sensitivity to gefitinib through statistical analysis of gene expression profiles of advanced NSCLCs and introduced a gefitinib response scoring system based on expression of selected genes that had shown the most significant differences in expression levels between PR and progressive disease (PD) groups (14). The gefitinib response scoring system successfully predicted all of additional "test" cases (PR and PD) in accordance with their clinical responses to gefitinib. Moreover, this system was able to separate SD into two groups, one representing patients who succeeded in maintaining the tumor-static effect for a long period and the other representing patients who failed to do so.

However, analysis of expression profiles or mutations requires acquisition of tissue specimens by surgery or biopsy, which is not routine in cases of advanced NSCLC, and sometimes these procedures themselves cause various complications. Hence, practical clinical tests using serologic markers that can predict the sensitivity or resistance of lung cancers to gefitinib therapy are urgently required. As a step toward that goal, the cDNA microarray analysis mentioned above revealed that genes encoding two EGFR ligands, amphiregulin and transforming growth factor-α (TGF-α),

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doi:10.1158/0008-5472.CAN-05-1556

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were among those overexpressed in tumor tissues obtained from nonresponders to gefitinib (14).

We report here our attempt to establish ELISA assays for these two protein markers in human serum. Our results suggest that circulating amphiregulin and TGF-α could be clinically applicable as indicators for an unfavorable response to gefitinib by identifying patients with a higher probability of resistance to the drug.

Materials and Methods

Patients. Between August 2002 and February 2005, 50 consecutive advanced NSCLC patients who had failed previous chemotherapy were treated with gefitinib (250 mg/day) at the Hiroshima University Hospital in Japan. Patients with locally advanced (stage IIIB), metastasized (stage IV), and postsurgically relapsed NSCLCs who were resistant to one or more regimens of conventional chemotherapy were enrolled in this experiment. Inclusion criteria were the same as described for our previous report (14). In brief, they were ages ≥20 years, had performance status (PS) of 0, 1, or 2, and had no significant abnormalities in liver or kidney functions. Treatment was continued until the patient was dropped from the study due to progression of disease, intolerable toxicity, or withdrawal of consent. Serum was obtained within 1 week before administration of gefitinib and stored at −80°C. Among the 50 patients whose serum samples were used for ELISA assays, we obtained formalin-fixed primary NSCLC tissue samples from 13 patients treated with gefitinib for their recurrent and chemoresistant diseases after they had undergone surgery at the initial treatment (nine adenocarcinomas, three squamous cell carcinomas, and one adenosquamous cell carcinoma). The use of all clinical materials obtained with written informed consent was approved by the Institutional Research Ethics Committee.

Objective tumor responses at every evaluable lesion were assessed every 4 weeks after the beginning of treatment according to criteria outlined elsewhere (14). After 4 months of treatment, the best overall response was evaluated for each patient based on the following definitions: complete response (CR), patients who qualified as CR at two sequential examination points with an interval of at least 28 days between them; PR, patients judged as PR or better at two sequential examination points with an interval of at least 28 days between them; SD, patients who were SD or better at two sequential examination points at least 28 days apart but who did not qualify as CR or PR. The first judgment of SD must be done at or after the first tumor assessment point 28 days after the start of gefitinib treatment; and PD, the patients determined as PD at or before the first tumor assessment point (28 days after the start of gefitinib treatment).

ELISA. TGF-α concentrations in serum were measured using a commercially available enzyme test (TGF-α ELISA kit, R&D Systems, Minneapolis, MN) as described previously (14, 15). For detection of soluble amphiregulin in serum, 96-well flexible microtiter plates (Nalge Nunc International, Rochester, NY) were coated with 1 ng/mL capturing antibody (anti-amphiregulin monoclonal antibody, R&D Systems) overnight. Wells were blocked with 300 μL PBS (pH 7.4) containing 1% bovine serum albumin, 5% sucrose, and 0.05% NaN_3 for 2 hours and then incubated for 2 hours with serum samples diluted 1:3 in PBS (pH 7.4) containing 1% bovine serum albumin. After washing with PBS (pH 7.4) containing 0.05% Tween 20, the wells were incubated for 2 hours with 100 ng/mL biotin-conjugated polyclonal anti-amphiregulin antibody (R&D Systems) followed by reaction with avidin-conjugated peroxidase (DakoCytomation, Glostrup, Denmark) using a substrate reagent (R&D Systems). The color reaction was stopped by addition of 2 N sulfuric acid. Color intensity was determined by a photometer at a wavelength of 450 nm with a reference wavelength of 630 nm. A standard curve was drawn for each plate using recombinant amphiregulin or TGF-α proteins for reference. Minimum detection limits of the assays for serum amphiregulin and TGF-α were 10.1 and 3.1 pg/mL, respectively. We verified by Western blot or immunoprecipitation assays that these commercial anti-amphiregulin and anti-TGF-α antibodies could specifically detect individual proteins using malignant pleural effusions obtained from five patients with advanced NSCLC whose serum amphiregulin/TGF-α had been positive.

***EGFR* mutation.** *EGFR* mutations at region of exons 18 to 21 of *EGFR*, which was reported as a hotspot of mutation (refs. 9–13; from p-loop to activation loop, codon position 709-870), were screened in surgically resected tumor tissues available from the 13 NSCLC patients who were treated with gefitinib (see above). Genomic DNAs were extracted as described previously (16). Briefly, cancer tissues were immediately resuspended in 20 μL buffer containing 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.45% NP40, 0.45% Tween 20, and 0.1 mg/mL proteinase K and were incubated overnight at 55°C. The mixture was boiled for 10 minutes to inactivate the proteinase K and was used for PCR. PCR-based direct sequencing experiments were carried out with the same primers for exons 18 to 21 as described elsewhere (12).

Immunohistochemistry and tissue microarray. An independent set of 449 formalin-fixed primary tumors (285 adenocarcinomas, 121 squamous cell carcinomas, 28 large-cell carcinomas, and 15 adenosquamous cell carcinomas) and adjacent normal lung tissue samples from patients undergoing surgery at Saitama Cancer Center (Saitama, Japan) were used in this study. The histologic patterns of adenocarcinoma were divided into four distinctive subtypes: bronchioloalveolar carcinoma, acinar subtype, papillary subtype, and solid adenocarcinoma with mucin. The pathologic stage was determined according to the classification of the Union Internationale Contre le Cancer (17). Tumor tissue microarrays were constructed using these 449 formalin-fixed primary lung cancers as published previously (15).

To investigate levels of amphiregulin and TGF-α proteins in clinical samples with clinicopathologic variables, we stained the sections using Envision+ kit/horseradish peroxidase (HRP; DakoCytomation). After blocking, the following antibodies were applied in this study: a rabbit polyclonal anti-human amphiregulin antibody (Ab-1, Lab Vision Corp., Fremont, CA) used at 1:40 dilution and a mouse monoclonal anti-TGF-α antibody (Ab-2, Oncogene Science, Manhasset, NY) used at 1:80 dilution. The sections were incubated with HRP-labeled antirabbit or antimouse immunoglobulin G as the secondary antibody. Substrate chromogen was added and the specimens were counterstained with hematoxylin. Three independent investigators assessed amphiregulin and TGF-α positivity semiquantitatively without prior knowledge of the clinical follow-up data. The intensity of cytoplasmic staining was scored using the following criteria: 0 (absent), 1+ (positive), and 2+ (strongly positive). We also scored the pattern of amphiregulin staining (cytoplasmic, nuclear, or both) as reported previously (18, 19).

Statistical analysis. Statistical analyses were done using the StatView statistical program (SAS, Cary, NC) to compare patient characteristics with responses to therapy. Associations between clinicopathologic variables, including positivity for amphiregulin and/or TGF-α in serum, and the response to gefitinib were compared by Fisher's exact tests. Tumor-specific survival and 95% confidence intervals (95% CI) were evaluated with the Kaplan-Meier method, and differences between the two groups were evaluated with the log-rank test. Risk factors associated with the prognosis were evaluated using Cox proportional hazards regression model with a step-down procedure. Proportional hazards assumptions were checked and satisfied; only those variables with statistically significant results in univariate analysis were included in a multivariate analysis. The criterion for removing a variable was the likelihood ratio statistic, which was based on the maximum partial likelihood estimate (default P of 0.05 for removal from the model).

Results

Clinical characteristics. Fifty patients (Table 1) who fulfilled the selection criteria were each treated with 250 mg gefitinib daily. Median age of the patients was 62.0 years (range, 30-80 years); 36 patients were male and 14 were female. Eastern Cooperative Oncology Group (ECOG) PS was 0 for 14 patients, 1 for 26 patients, and 2 for 10 patients. Twenty-one patients had been treated with at least two lines of chemotherapy, and 34 had received platinum-based chemotherapy. At the time of study entry,

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Table 1.

A. Association between NSCLC patients' characteristics and response to gefitinib therapy (N = 50)

Variables	Total n = 50	PR + SD [disease-controlled cases; n = 20 (8 PR + 12 SD)]	PD Progressive disease case n = 30	P value
Gender				
Male	36	12	24	NS
Female	14	8	6	
Age, y				
Median		60.5	62.0	
Range		47-76	30-80	
Histologic type				
ADC	40	15	25	NS*
SCC	7	4	3	
ASC	3	1	2	
Disease stage				
IIIB	9	3	6	NS**
IV	28	9	19	
Recurrence after surgery	13	8	5	
Bone metastasis				
Yes	18	9	9	NS
No	32	11	21	
Brain metastasis				
Yes	17	6	11	NS
No	33	14	19	
ECOG PS				
0	14	7	7	0.037***, †
1	26	12	14	
2	10	1	9	
No. prior chemotherapy regimens				
1	29	13	16	NS***
2	10	2	8	
>2	11	5	6	
Prior cisplatin or carboplatin				
Yes	34	13	21	NS
No	16	7	9	
Smoking history				
Never	18	11	7	0.035*****
Former	15	4	11	
Current	17	5	12	
Serum amphiregulin				
Positive	14	2	12	0.026 †
Negative	36	18	18	
Serum TGF-α				
Positive	15	2	13	0.014 †
Negative	35	18	17	
Serum amphiregulin or TGF-α				
Positive	22	3	19	0.001 †
Negative	28	17	11	

B. Cox's proportional hazards model analysis of prognostic factors in patients with advanced NSCLCs who were treated with gefitinib

Variables	Hazard ratio (95% CI)	P value
Unvariate analysis		
AREG (+/−)	2.235 (1.050-4.761)	0.037 †
TGFA (+/−)	3.315 (1.557-7.059)	0.002 †
AREG or TGFA (+/−)	2.510 (1.197-5262)	0.018 †
Age (65≥/<65)	1.044 (0.487-2.237)	0.912
Gender (male/female)	1.555 (0.885-3.532)	0.291

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Table 1. Cont.

Variables	Hazard ratio (95% CI)	*P* value
Histological type (others/ADC †)	1.613 (0.848-4.013)	0.309
Disease Stage (IIIB/others)	1.088 (0.445-2.706)	0.8390
Perpormance status (2/0-1)	6.707 (2.544-17.682)	0.001*
Smoking history (smoker/never smoker)	1.596 (0.736-3.462)	0.237
Unvariate analysis		
AREG (+/−)	1.042 (0.384-2.825)	0.835
TGFA (+/−)	2.457 (1.072-5.625)	0.034 †
Perpormance status (2/0-1)	4.792 (1.420-18.171)	0.012 †

Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; ASC, adenosquamous cell carcinoma; NS, not significant.
*Adenocarcinoma versus other histology (squamous cell carcinoma and adenosquamous cell carcinoma).
**IIIB versus others.
***PS 0-1 versus PS 2.
****Previous chemotherapy 0-1 versus others.
*****Never versus others.
†$P < 0.05$ (Fisher's exact test).
NS: no significance † ADC adenocarcinoma
$P < 0.05$.

32 were current (17 patients) or former (15 patients) smokers. In terms of histologic diagnosis, 40 (80.0%) tumors were adenocarcinomas, 7 (14.0%) were squamous cell carcinomas, and 3 (6.0%) were adenosquamous cell carcinomas. Eight patients were classified as PR and none as CR after gefitinib therapy; 12 patients were classified as SD and 30 as PD. The tumor response rate (CR + PR / CR + PR + SD + PD) for gefitinib treatment was 16.0%, and the disease control rate (CR + PR + SD / CR + PR + SD + PD) was 40.0%. Final judgment was done in July 2005, >4 months after enrollment of the last patient. The median follow-up time had been 240 days (range, 33-1,011 days).

Serum amphiregulin/transforming growth factor-α levels, clinicopathologic features, and response to gefitinib therapy. In our recent study using a cDNA microarray to analyze gene expression in tumors from 33 patients with advanced NSCLC who had been treated with second-line to seventh-line gefitinib monotherapy, amphiregulin and TGF-α were found to be significantly overexpressed in PD cases but hardly detectable in PR cases (permutational *P*s of 9.3×10^{-12} and 0.0095, respectively; ref. 14). Hence, to establish a routine and less invasive laboratory test for prediction of drug response, we attempted to use two protein markers and attempted to establish the serologic markers. We

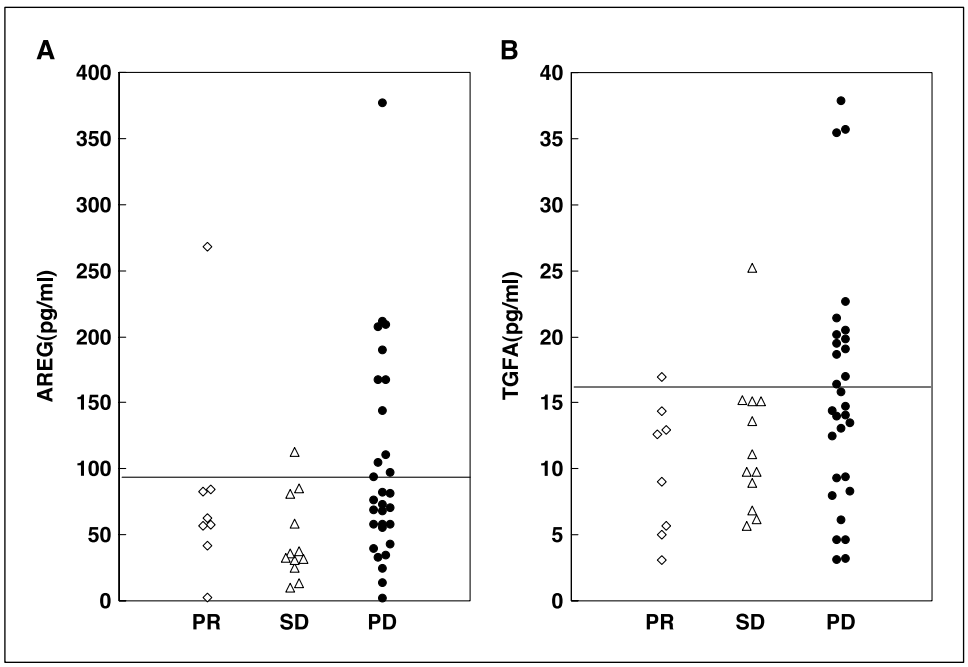

Figure 1. ELISA assays for concentrations of amphiregulin (*AREG*; *A*) and TGF-α (*TGFA*; *B*) in serum from 50 NSCLC patients treated with gefitinib therapy. Eight showed PR, 12 showed SD, and showed 30 PD. *Horizontal lines,* cutoff concentrations for positivity above or negativity below the lines.

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applied ELISA assays for both proteins using serum samples from an independent set of 50 other NSCLC patients treated with gefitinib. Judgments of "positive" or "negative" were based on measured concentrations of each protein that fell above or below cutoff values (93.8 pg/mL for amphiregulin and 15.6 pg/mL for TGF-α), which had been set by drawing receiver-operated characteristic curves according to optimal diagnostic accuracy and likelihood ratios discriminating PD cases from disease-controlled cases (PR + SD). Fourteen (28.0%) of the 50 serum samples were judged as positive for amphiregulin and 15 (30.0%) patients were positive for TGF-α according to these cutoff values (Fig. 1).

As shown in Table 1, we analyzed associations between serum amphiregulin and TGF-α positivity and response to gefitinib therapy in this group of patients. Twelve (40.0%) serum samples from the 30 PD patients were positive for amphiregulin, whereas 18 of 20 (90.0%) samples from disease-controlled (PR or SD) patients were negative (P = 0.026 by Fisher's exact test). Thirteen of 30 (43.3%) samples from PD patients were positive for TGF-α, whereas 18 of 20 (90.0%) samples from PR or SD patients were negative (P = 0.014 by Fisher's exact test). At least one of the two proteins was positive in 19 of 30 (63.3%) serum samples from PD patients, whereas 17 of 20 (85.0%) samples from PR + SD patients were negative (P = 0.001 by Fisher's exact test), indicating that a combined assay using both amphiregulin and TGF-α should be a good predictor for poor response in 63% of PD cases. The false-positive rate for indicating PD response was only 15.0%.

Table 1 also shows associations between clinicopathologic factors and responses to gefitinib therapy among the 50 patients. In this study, both performance status (0/1 versus 2; P = 0.037 by Fisher's exact test) and smoking history (current and former smoker versus never-smoker; P = 0.035 by Fisher's exact test) as well as amphiregulin and/or TGF-α positivity were significantly associated with the response to gefitinib. As mentioned above, 12 of 14 patients with amphiregulin-positive values as well as 13 of 15 patients with TGF-α-positive scores revealed poor response to gefitinib therapy. Of 22 patients with positive values for either marker, 19 were judged as PD.

The median survival time of amphiregulin-negative patients treated with gefitinib was significantly longer than that of

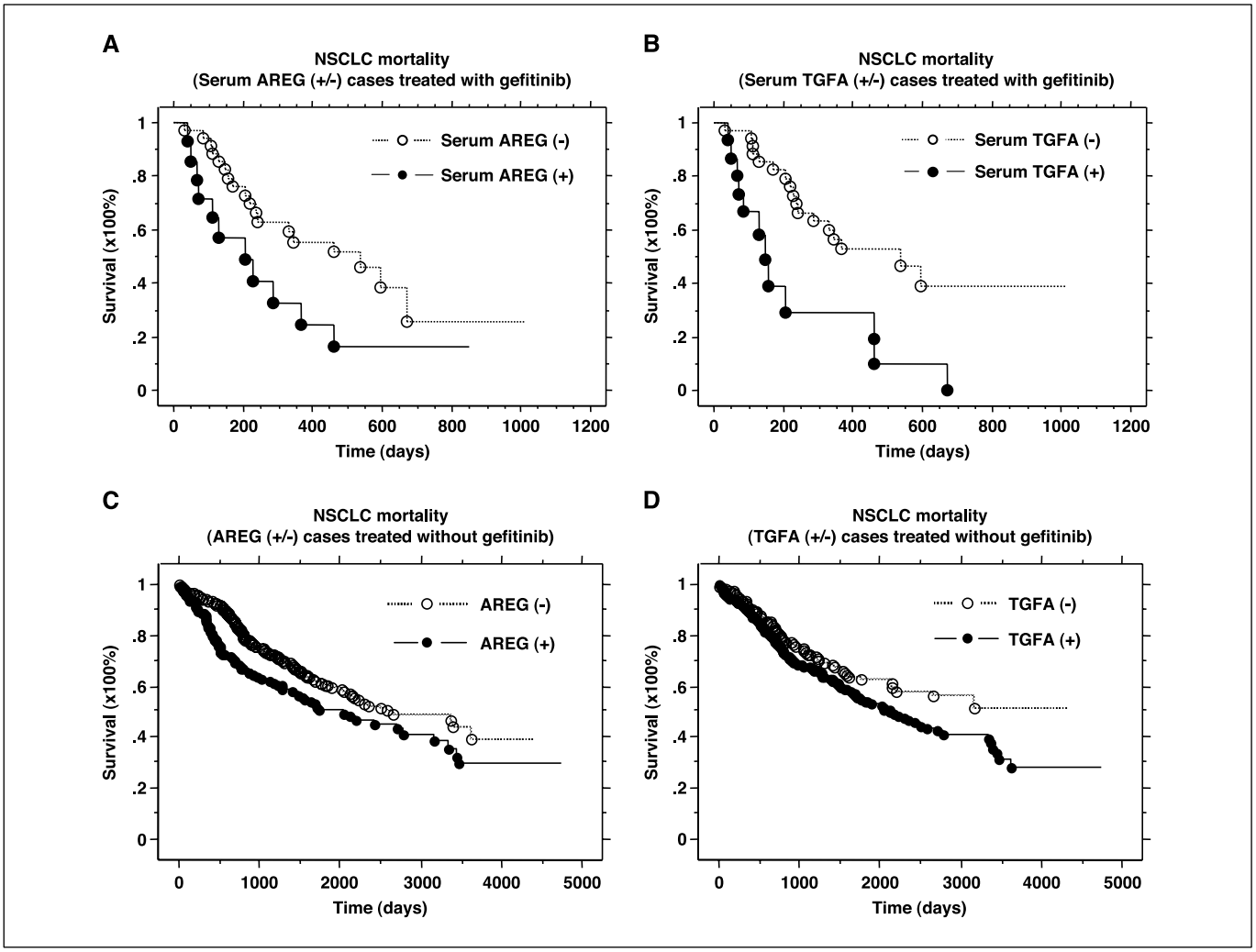

Figure 2. Kaplan-Meier analysis of tumor-specific survival of 50 gefitinib-treated patients according to positive or negative concentrations of amphiregulin (*A*) or TGF-α (*B*) in serum and that of 449 gefitinib-untreated NSCLC patients according to amphiregulin (*C*) or TGF-α (*D*) expression on tissue microarrays. Differences between groups were evaluated with the log-rank test.

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Table 2. Cox proportional hazards model analysis of prognostic factors in patients with advanced NSCLCs who were treated with gefitinib

Variables	Hazard ratio (95% CI)	*P*
Univariate analysis		
Amphiregulin (+/−)	2.235 (1.050-4.761)	0.037*
TGF-α (+/−)	3.315 (1.557-7.059)	0.002*
Amphiregulin or TGF-α (+/−)	2.510 (1.197-5.262)	0.016*
Age (≧65/<65 y)	1.044 (0.487-2.237)	0.912
Gender (male/female)	1.555 (0.685-3.532)	0.291
Histologic type (others/ADC)	1.613 (0.648-4.013)	0.309
Disease stage (IIIB/others)	1.098 (0.445-2.706)	0.8390
PS (2/0-1)	6.707 (2.544-17.682)	<0.001*
Smoking history (smoker/never smoker)	1.596 (0.736-3.462)	0.237
Multivariate analysis		
Amphiregulin (+/−)	1.042 (0.384-2.825)	0.935
TGF-α (+/−)	2.457 (1.072-5.625)	0.034*
PS (2/0-1)	4.792 (1.420-16.171)	0.012*

*$P < 0.05$.

amphiregulin-positive patients ($P = 0.032$ by log-rank test; Fig. 2*A*). The same was true of TGF-α-negative patients ($P = 0.001$ by log-rank test; Fig. 2*B*). We also used univariate analysis to evaluate associations between patient prognosis and other factors, including age, gender (male versus female), PS (0/1 versus 2), disease stage (IIIB versus other stages), smoking history (current and former smoker versus never smoker), histologic classification (adenocarcinoma versus other histologic types), serum amphiregulin status (positive versus negative), and serum TGF-α status (positive versus negative; Table 2). Among these variables, serum amphiregulin positivity [odds ratio (OR), 2.235; 95% CI, 1.050-4.761; $P = 0.037$], serum TGF-α positivity (OR, 3.315; 95% CI, 1.557-7.059; $P = 0.002$), and PS (OR, 6.707; 95% CI, 2.544-17.682; $P < 0.001$) were significantly associated with poor prognosis. However, multivariate analysis revealed that serum TGF-α status (OR, 2.457; 95% CI, 1.072-5.625; $P = 0.034$) and PS (OR, 4.792; 95% CI, 1.420-16.171; $P = 0.012$) were significant independent prognostic factors for advanced NSCLC patients who received gefitinib therapy.

To determine whether there is a mechanistic relationship between *EGFR* mutations and levels of serum amphiregulin and TGF-α, we carried out PCR-based direct sequencing of the EGFR tyrosine kinase domain (exons 18-21) using tumors from 13 patients whose sera were also analyzed by ELISA. As shown in Table 3, *EGFR* mutations were detected in 4 of 8 (50%) patients showing PR or SD condition after gefitinib treatment and in 2 of 5 (40%) PD cases, indicating that there was no significant association between *EGFR* mutations and the levels of serum amphiregulin and TGF-α.

Expression status of amphiregulin and transforming growth factor-α in unselected lung cancer tissues. To investigate the clinical significance of amphiregulin and TGF-α overexpression, we also examined the expression of amphiregulin and TGF-α proteins by means of tissue microarrays containing NSCLC tissues from unselected 449 patients who underwent surgical resection. Amphiregulin was mainly detected at cytoplasm and/or nucleus of tumor cells as reported elsewhere (18, 19). TGF-α was mainly stained at the cytoplasm of tumor cells. As shown in Table 4, gender (higher in male; $P < 0.001$ by Fisher's exact test), histologic type (higher in nonadenocarcinoma; $P < 0.001$ by Fisher's exact test), and smoking history (higher in current and former smokers; $P < 0.001$ by Fisher's exact test) were significantly associated with the amphiregulin positivity. A similar tendency was observed in the TGF-α analysis (higher in male, $P = 0.001$; higher in nonadenocarcinoma, $P < 0.001$; and higher in current and former smokers, $P = 0.005$ by Fisher's exact test). The amphiregulin positivity was significantly higher in nonbronchioloalveolar ($P = 0.004$) and nonpapillary ($P = 0.011$) subtypes of adenocarcinoma, whereas no significant difference in the TGF-α positivity was observed.

Table 3. Clinicopathologic characteristics and *EGFR* mutation status of 13 NSCLC patients

Case	Age, y	Gender	Smoking status	Histologic type	ECOG PS	Serum amphiregulin	Serum TGF-α	*EGFR* mutation	Response to gefitinib
1	69	Female	Never	ADC	1	Negative	Negative	del E746-A750	PR
2	66	Female	Never	ADC	1	Negative	Negative	del E746-A750	PR
3	57	Male	Never	ADC	1	Negative	Negative	L858R	PR
4	64	Male	Former	SCC	1	Negative	Positive	ND	PR
5	64	Female	Never	SCC	0	Negative	Negative	L858R	SD
6	53	Male	Current	ASC	1	Negative	Negative	ND	SD
7	47	Male	Former	ADC	0	Negative	Negative	ND	SD
8	48	Female	Never	ADC	1	Negative	Negative	ND	SD
9	46	Male	Current	ADC	0	Negative	Negative	L858R	PD
10	53	Male	Former	ADC	1	Negative	Positive	L858R	PD
11	59	Male	Former	ADC	2	Positive	Negative	ND	PD
12	80	Male	Never	SCC	2	Positive	Positive	ND	PD
13	69	Female	Never	ADC	0	Negative	Negative	ND	PD

Abbreviation: ND, no mutation was detected.

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Table 4. Association between amphiregulin/TGF-α positivity in NSCLC tissues and patients' characteristics (N = 449)

Variables	Total (N = 449)	Amphiregulin status			TGF-α status		
		Amphiregulin positive (n = 163)	Amphiregulin negative (n = 286)	P	TGF-α positive (n = 301)	TGF-α negative (n = 148)	P
Gender							
Male	310	134	176	<0.001*	223	87	0.001*
Female	139	29	110		78	61	
Age, y							
<65	215	77	148	NS	144	71	NS
≥65	234	86	138		157	77	
Histologic type							
ADC	285	78	207	<0.001 †,*	170	115	<0.001 †,*
SCC	121	61	60		94	27	
LCC	28	19	9		26	2	
ASC	15	5	10		11	4	
Dominant histologic subtype (n = 273)							
Papillary	218	52	166	0.011*	127	91	NS
Nonpapillary	55	23	32		32	23	
BAC	138	27	111	0.004 †	78	60	NS
Non-BAC	135	48	87		81	54	
Disease stage							
I + II + IIIA	359	130	229	NS	248	111	NS
IIIB	90	33	57		53	37	
pT							
$T_1 + T_2$	320	108	212	NS	217	103	NS
$T_3 + T_4$	129	55	74		84	45	
pN							
$N_0 + N_1$	336	114	222	NS	221	115	NS
$N_2 + N_3$	113	49	64		80	33	
Smoking history							
Never smoker	139	25	114	<0.001 †	80	59	0.005 †
Smoker	310	138	172		221	89	

Abbreviations: LCC, large-cell carcinoma; BAC, bronchioloalveolar carcinoma.
*P < 0.05 (Fisher's exact test).
†Adenocarcinoma versus other histology.

The median survival time of amphiregulin-negative patients was significantly longer than that of amphiregulin-positive patients (P = 0.013 by log-rank test; Fig. 2C). Similarly, the median survival time of the TGF-α-negative patients was longer than that of the negative patients (P = 0.029 by log-rank test; Fig. 2D). We also applied univariate analysis to evaluate associations between patient prognosis and several factors, including age, gender, pT stage ($T_1 + T_2$ versus $T_3 + T_4$), pN stage ($N_0 + N_1$ versus $N_2 + N_3$), smoking history, histologic classification, amphiregulin status (positive versus negative), and TGF-α status. Among these variables, amphiregulin positivity, TGF-α positivity, elderly, male, nonadenocarcinoma histology, pT stage, and pN stage were significantly associated with poor prognosis (Table 5). However, multivariate analysis revealed that neither amphiregulin nor TGF-α status was an independent prognostic factor for surgically treated NSCLC patients who were not treated with gefitinib (Table 5).

Discussion

Gefitinib was developed as a "selective" inhibitor of EGFR tyrosine kinase. However, no clear association between EGFR levels in cancer cells and response to gefitinib has been found *in vitro* or *in vivo* (4, 14). Multivariate analysis of patients in previous studies suggested that the response rate in females might be higher than in males and higher in patients with adenocarcinomas than in patients with squamous cell carcinomas (5, 6). Recent clinical studies have suggested that individuals in whom gefitinib is efficacious are more likely to have adenocarcinomas of the bronchioloalveolar or dominant papillary subtype and have no history of smoking (6, 20). Recently, studies by Lynch et al. (9) and Paez et al. (10) showed that genetic mutations in the tyrosine kinase domain of EGFR were associated with sensitivity of NSCLCs to gefitinib. Clinicopathologic determinants of gefitinib sensitivity, including *EGFR* mutations, are predictive to a certain extent; however, previous reports and our observations have suggested that no known factors can perfectly predict the response of NSCLC to gefitinib treatment (9–14). Moreover, the clinical benefit of treatment of gefitinib is not restricted to objective response, such as PR, and should be extended to SD patients (14). In previous clinical trials (5, 6), one group of patients showed marked improvement of symptoms and prolonged stabilization of disease without any measurable reduction in tumor size. These findings

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agree with our study; the survival time of our PR + SD group of patients was significantly prolonged compared with that of PD patients ($P = 0.011$ by log-rank test).

Analyses of mutations or expression profiles are time-consuming procedures and require biopsy samples containing a sufficient number of cancer cells. Because patients with advanced NSCLC are rarely candidates for surgical resection of their tumors and invasive biopsy for pathologic diagnosis is not essential for selection of their treatment protocols, the above approaches are not appropriate for routine diagnosis of drug response. In fact, the incidence of major complications related to transbronchial biopsy is reportedly as high as 0.5% to 6.8% (21). Hence, we considered it urgent to establish a safe and less invasive system for predicting chemosensitivity of individual patients, such as a serologic test, which would be readily available at any hospital.

We found recently that amphiregulin and TGF-α, both encoding ligands for EGFR and other ErbBs, were significantly overexpressed in tumor cells of nonresponders but hardly detectable in those of responders to gefitinib (14). Examination of the serum levels of amphiregulin and TGF-α in a subset of patients with advanced NSCLC revealed that amphiregulin and TGF-α proteins in the serum of such patients might have a high diagnostic value for predicting poor response to gefitinib. The positivity of these proteins in serum of nonresponders was 12 of 30 (40.0%) for amphiregulin and 13 of 30 (43.3%) for TGF-α. Furthermore, a combination of the two markers improved the overall sensitivity for detection of nonresponders to 63.3%. One of the important advantages of a prediction system using these two markers is its ability to specifically discriminate PD cases from PR or SD patients compared with analysis of *EGFR* mutation that mainly selects patients with PR to gefitinib but has no significant power to indicate patients with SD (9–13). In fact, statistical analysis supports the hypothesis that the levels of serum amphiregulin and TGF-α were biologically independent of *EGFR* mutation status (Table 3).

Table 5. Cox proportional hazards model analysis of prognostic factors in patients with advanced NSCLCs who were not treated with gefitinib

Variables	Hazard ratio (95% CI)	*P*
Univariate analysis		
Amphiregulin (+/−)	1.420 (1.075-1.880)	0.013*
TGF-α (+/−)	1.403 (1.034-1.908)	0.029*
Age (≧65/<65)	1.474 (1.115-1.949)	0.006*
Gender (male/female)	1.600 (1.164-2.193)	0.004*
Histologic type (others/ADC)	1.401 (1.063-1.848)	0.017*
T factor ($T_3 + T_4$ / $T_1 + T_2$)	1.931 (1.451-2.571)	<0.001*
N factor ($N_2 + N_3$ / $N_0 + N_1$)	2.882 (2.174-2.882)	<0.001*
Multivariate analysis		
Amphiregulin (+/−)	1.078 (0.797-1.456)	0.627
TGF-α (+/−)	1.280 (0.929-1.764)	0.130
Age (≧65/<65)	1.943 (1.456-2.593)	<0.001*
Gender (male/female)	1.587 (1.120-2.252)	0.010*
Histologic type (others/ADC)	1.133 (0.831-1.543)	0.431
T factor ($T_3 + T_4$ / $T_1 + T_2$)	1.695 (1.261-2.278)	0.063
N factor ($N_2 + N_3$ / $N_0 + N_1$)	3.174 (2.353-4.274)	<0.001*

*$P < 0.05$.

Although further evaluation of these serologic markers in clinical settings will be necessary, eventually they should, in combination with a limited number of other possible markers that we found to be significantly positive in PD patients in our microarray analysis (14), enable laboratories to more precisely select in advance the patients who will actually benefit from gefitinib treatment. The prospective trial to evaluate the reliability of the serum marker–based prediction system is in progress in our institute.

The present study also showed that the survival time of patients with positive values for serum amphiregulin or TGF-α was significantly shorter than for patients with negative values. In particular, diagnosis of these markers in serum seemed to be independently related to poor prognosis. An initial analysis of the primary end point of a phase III trial (Iressa Survival Evaluation in Lung Cancer with 1,692 patients; http://www.astrazeneca.com/pressrelease/4245.aspx) revealed that gefitinib failed to significantly prolong survival in comparison with placebo in the overall population (hazard ratio, 0.89; $P = 0.11$; median, 5.6 versus 5.1 months) or in patients with adenocarcinoma (hazard ratio, 0.83; $P = 0.07$; median, 6.3 versus 5.4 months), although there was a statistically significant improvement in objective response rate in these populations, and there were survival benefits in a prospective subgroup of patients of Oriental origin or who had never smoked. Our own results suggest that serum concentrations of amphiregulin and/or TGF-α could be useful for selection of patients who are likely to respond poorly to gefitinib and receive no clinical benefit from it while extending the survival benefit of gefitinib to a larger population of patients who should in fact receive this treatment. At present, detecting the serum amphiregulin and TGF-α is the only routine and significant predictor for poor response of advanced NSCLCs to gefitinib treatment. In this sense, serum amphiregulin and TGF-α positivities could be useful for decision of the gefitinib treatment option, providing that a standardized diagnostic kit available at every hospital is developed and its validation in a larger clinical setting is completed.

The significance of the EGFR ligand autocrine loop in growth and survival of lung cancer cells is indisputable (22, 23). However, the role of amphiregulin and TGF-α in the development and progression of lung cancer is not well understood, although several lines of evidence suggest that overexpression of amphiregulin is associated with shortened survival of patients with NSCLC (22). Our data also revealed that the expression of amphiregulin and TGF-α proteins in NSCLC tissues is likely to correlate with a poor prognosis of surgically treated patients to a certain extent. In addition, male, nonadenocarcinoma, and smoking history were associated with amphiregulin and TGF-α positivity in NSCLC tissues, and some histologic subtypes of adenocarcinoma (nonpapillary and nonbronchioloalveolar subtypes) were associated with amphiregulin positivity. Recently, other investigators reported that gefitinib was particularly effective in adenocarcinoma, especially the dominant papillary and/or bronchioloalveolar subtype (6, 20); their data independently suggested the possible relationship between amphiregulin/TGF-α expression and histologic types that are resistant to gefitinib. On the other hand, antiapoptotic activity of amphiregulin in human lung adenocarcinoma cells was reported recently (23). To investigate whether the antiapoptotic activity of amphiregulin leads to resistance of NSCLC cells to gefitinib therapy, we previously did a biological assay using

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a gefitinib-sensitive but amphiregulin-nonexpressing NSCLC cell line, PC-9, and found that the antitumor activity of gefitinib on PC-9 cells was decreased dramatically by autocrine secretion of amphiregulin (14). That evidence strongly suggests that although growth factor signaling by the EGFR is extremely complicated at every step because of the multiplicity of ligands, dimerization partners, effectors, and downstream pathways (22, 23), amphiregulin might be a principal activator of the ligand-receptor autocrine pathway that leads to the resistance of cancer cells to gefitinib.

In conclusion, the present study showed that the status of amphiregulin and TGF-α in serum could be an important predictor of the response of NSCLCs to gefitinib. Measurement of serum amphiregulin and TGF-α levels is obviously a routinely feasible, relatively noninvasive, and inexpensive method of predicting response to this drug.

Acknowledgments

Received 5/5/2005; revised 7/21/2005; accepted 8/17/2005.

Grant support: "Research for the Future" Program Grant 00L01402 of the Japan Society for the Promotion of Science (Y. Nakamura).

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Increases of Amphiregulin and Transforming Growth Factor-α in Serum as Predictors of Poor Response to Gefitinib among Patients with Advanced Non–Small Cell Lung Cancers

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Cancer Res 2005;65:9176-9184.

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