and placebo in advanced NSCLC with prior chemotherapy demonstrated in preliminary analysis a tendency to have improvement in overall survival but did not have a statistically significant improvement in overall survival.[11] There are many issues that need to be addressed with regard to the clinical application of gefitinib; one of the most important issues is the efficacy of gefitinib monotherapy in patients with chemotherapy-naive NSCLC,[12] and another is to establish a way to predict response to gefitinib.

Recently, it has been suggested that mutations in the EGFR tyrosine kinase domain play a critical role in determining tumor response to gefitinib in NSCLC patients.[13,14] The mutations consisted of small, in-frame deletions or substitutions clustered around the adenosine triphosphate–binding site in exons 18, 19, and 21 of the *EGFR*. After these reports, some investigators supported the belief that *EGFR* mutation is one of the strong determinants of tumor response to gefitinib.[15–17] Tumors with *EGFR* mutations tend to be more common in adenocarcinomas, female patients, nonsmokers, and those of Asian origin. In most of those studies, tumor samples that were resected by operations were used. Because it is often difficult to obtain a tumor sample from an inoperable NSCLC patient, it is necessary to establish a method for detecting mutant *EGFR* from a patient sample other than from tumor specimens.

Polymerase chain reaction (PCR) technology for the amplification of small amounts of DNA has made it possible to identify the same alterations typically observed in DNA from serum samples from NSCLC patients.[18,19] Serum DNA may provide a noninvasive and repeatable source of genotypic information that could influence treatment and prognosis, especially in advanced NSCLC patients who have received gefitinib therapy. We essentially consider that it is possible to detect the *EGFR* mutation in serum DNA. We hypothesized that serum DNA may provide useful information on *EGFR* mutations in lung cancer patients.

As described above, the usefulness of gefitinib monotherapy is controversial and that in patients without pretreatment is unclear. Because *EGFR* mutations have been shown to be strongly associated with the response of NSCLC patients to gefitinib treatment, the analysis of *EGFR* mutations is necessary to evaluate the clinical benefit of gefitinib. We therefore conducted a multicenter phase II trial for these patients. The primary objective was to evaluate the objective response rate, and secondary objectives were to estimate the disease control rate, disease-related symptom improvement rate, safety, time to progression (TTP), and overall survival (OS). In addition, as a correlative study, we planned to detect *EGFR* mutations in serum samples from NSCLC patients and evaluate the relationship between the *EGFR* mutation and clinical manifestations in NSCLC patients receiving gefitinib treatment.

PATIENTS AND METHODS

Patient Eligibility

Patients who had histologically or cytologically proven stage IIIb or IV NSCLC and no previous chemotherapy were enrolled into this trial. Radiotherapy for metastatic lesions until 3 weeks before entry was allowed on condition that these lesions were not assessed for tumor response. Patients in whom recurrence occurred after surgery were also eligible. Patient eligibility criteria included at least one measurable lesion, age of 20 years or older, Eastern Cooperative Oncology Group performance status (PS) of 0 to 2, and life expectancy of greater than or equal to12 weeks. Adequate organ and bone marrow function was necessary, defined as leukocyte counts greater than or equal to 3.0×10^6/liter, neutrophil counts greater than or equal to 1.5×10^6/liter, platelet counts greater than or equal to 100×10^9/liter, hemoglobin levels greater than or equal to 8.5 g/dl, alanine aminotransferase or aspartate aminotransferase levels less than or equal to two times the upper limit of the reference range (<100 IU/liter in the presence of liver metastases), serum bilirubin levels less than or equal to 1.5 mg/dl, serum creatinine levels less than or equal to1.5 mg/dl, and PaO_2 levels greater than or equal to 65 mmHg. Patients with any of the following were excluded: active double cancer; severe complications such as myocardial infarction within 3 months before entry or uncontrolled diabetes; symptomatic brain or bone metastasis; diarrhea more severe than grade 2 according to National Cancer Institute Common Toxicity Criteria version 2; systemic administration of steroids to treat skin diseases; pleural, pericardial, or peritoneal effusion requiring treatment; and pregnancy or lactation. All patients were required to give informed consent.

Treatment

Patients were treated with gefitinib 250 mg orally once per day. Treatment was discontinued when the disease progressed, intolerable toxicities appeared, the patients requested withdrawal, or disease-related symptoms worsened without tumor response after 8 weeks of gefitinib monotherapy. These patients received chemotherapeutic treatment after gefitinib therapy. The chemotherapy regimen consisted of platinum (cisplatin or carboplatin) plus new agents (paclitaxel, docetaxel, gemcitabine, vinorelbine, or irinotecan) in patients aged 74 years or younger and vinorelbine monotherapy in patients aged 75 years or older. If symptomatic bone or brain metastasis occurred during gefitinib monotherapy, patients received radiotherapy after gefitinib treatment.

Efficacy and Drug-Related Adverse Events

Tumor size was assessed with computed tomography or magnetic resonance imaging scans every 4 weeks from the start to cessation of protocol treatment, using Response Evaluation Criteria in Solid Tumors guidelines.[20] Disease control was judged when patients achieved the best response of complete response, partial response (PR), or stable disease (SD), which was confirmed and sustained for 4 weeks. TTP was measured as the period from the start of the treatment to an identifiable time of disease progression. OS was measured from the start of the treatment until death or the last follow-up. The Kaplan-Meier method was used to calculate these measures.

Drug adverse events were recorded and graded according to National Cancer Institute Common Toxicity Criteria

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version 2.0. Changes in physical and laboratory findings were assessed at least every 2 weeks.

Serum Sample Collection and DNA Extraction

Blood samples from patients were collected before and 14 days after the initiation of gefitinib administration. Separated serum was stocked at −80°C until use. DNA extraction from the serum samples was performed using a nonorganic method (Oncor, Gaithersburg, MD). Serum DNA was purified using Qiamp Blood Kit (Qiagen, Hilden, Germany), with the following protocol modifications. One column was used repeatedly until the whole sample had been processed. The extracted DNA was stocked at −20°C until use.

Tissue Sample Collection and DNA Extraction

Tumor specimens were obtained on protocols approved by the institutional review board. Twenty paraffin blocks of tumor material, obtained from 15 patients for diagnosis before treatment, were collected retrospectively. Eleven tumor samples were collected from primary cancer by means of transbronchial lung biopsy, one was resected by operation, and nine were from metastatic sites (four from bone, three from lymph nodes, one from the brain, and one from the colon). All specimens underwent histologic examination to confirm the diagnosis of NSCLC. DNA extraction from tumor samples was performed using the TaKaRa DEXPAT kit (TaKaRa Biomedicals, Shiga, Japan).

PCR Amplification

PCR was performed in 25-μl volumes using 15 μl of template DNA, 0.75 units of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 2.5 μl of PCR buffer, 0.8 mM dNTP, 0.5 μM of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. A set of designed primers was used to amplify exon 19 of *EGFR* (upper primer, 5′-CAGCCCCCAGCAATATCAGCCTTAGGT-3′; lower primer, 5′- CACTAGAGCTAGAAAGGGAAAGACATA-3′). Thirty cycles of amplification were performed using a thermal cycler (Perkin-Elmer, Foster City, CA) (95°C for 45 seconds, 55.5°C for 30 seconds, 72°C for 30 seconds, followed by incubation at 72°C for 10 minutes). The bands were visualized using a 2100 bioanalyzer, DNA 500 Labchip kit (Aglient Technologies, Waldbronn, Germany). If no PCR products were detected by the first PCR, an additional 20 cycles of PCR was carried out and the sample was revisualized. To confirm the deletional mutation in exon 19, and to detect the mutation in exons 18 and 21 of *EGFR*, PCR was performed again using another primer set as described previously.[13]

Sequencing

Amplification and sequencing were performed in duplicate for each sample using an ABI prism 310 (Applied Biosystems). The sequences were compared with the GenBank-archived human sequence for *EGFR* (accession no. AY588246).

Trial Design and Statistical Methods

The trial was a two-stage multicenter phase II study. The primary endpoint was response rate, and secondary endpoints were disease control rate, safety, TTP, and OS. As a correlative study, *EGFR* mutations in tumor and serum samples were analyzed. The protocol and consent form were approved by the institutional review board of each participating hospital. Initially, 15 patients were recruited to the study. If one of these patients responded to treatment with gefitinib monotherapy, an additional 10 patients were recruited. If five or more of these 25 patients responded to therapy, treatment with gefitinib was concluded to be effective. According to Simon's minimax design,[21] our study, with a sample size of 25, had an 80% power to support the hypothesis that the true objective response rate was greater than 30% and a 5% significance to deny the hypothesis that the true objective response rate was less than 10%. Assuming a nonevaluability rate of less than 20%, we projected an accrual of 30 patients. In analysis of *EGFR* mutation in serum samples, the categorical variables were compared using the Fisher's exact test. A value of $p < 0.05$ was considered significant. The statistical analyses were performed using the StatView software package, version 5.0 (SAS Institute, Inc., Cary, NC).

RESULTS

Patients

From October of 2002 to August of 2003, 30 patients were enrolled into the study. Patient characteristics are summarized in Table 1. The most common histologic subtype was adenocarcinoma (25 patients [83.3%]). Three patients had undergone surgery and three had received radiotherapy to bone or brain metastases. Twenty patients were current or previous smokers. Twenty-six patients (86.7%) had good PS (0–1) and 86.7% of enrolled patients had stage IV disease. A total of 43 sites of metastatic lesions in 26 patients were diagnosed. Thirteen of the 26 patients had more than one metastatic lesion. All four patients with stage IIIb disease had pleural effusion and were ineligible for radiotherapy.

Efficacy

All patients were assessable for tumor response (Table 2). Complete response was not observed. Ten patients achieved PR, nine had SD as their best response, and 11 patients had progressive disease (PD). The objective response rate was 33.3% (95% confidence interval, 16.2–49.8%) and the disease control rate was 63.3% (95% confidence interval, 46.0–80.5%). All responders had adenocarcinoma. Of the responders, four were male patients and six were female patients. None of the prognostic factors such as gender (male versus female), PS (0–1 versus 2), smoking (never-smoker versus smoker), histology (adenocarcinoma versus nonadenocarcinoma), clinical stage (IIIb versus IV), and prior treatment (yes versus no) was significantly associated with tumor responses (Table 2). Disease control was observed in 19 patients (eight men and 11 women). A significantly higher disease control rate was observed in female patients ($p = 0.018$) and nonsmokers ($p = 0.049$). The other factors did not affect the disease control rate (Table 2).

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TABLE 1. Patient Characteristics

Characteristic	Value
No. of patients	30
Age (yr)	
Median	64
Range	44–87
Gender	
Male	18
Female	12
Histology	
Adenocarcinoma	25
Squamous-cell carcinoma	3
Large-cell carcinoma	2
Stage	
IIIB	4
IV	26
Metastatic sites	
Pulmonary	16
Bone	12
Brain	11
Others	4
ECOG performance status	
0	20
1	6
2	4
Prior treatment	
Yes	6
Operation	6
Radiation	3
No	24
Smoking	
Yes	20
Pack-years (mean ± SD)	51 ± 39
No	10

ECOG, Eastern Cooperative Oncology Group.

TABLE 2. Response to Gefitinib Monotherapy and Prognostic Factors*

	No.	PR	SD	PD	RR (%)	*p* Value	DCR (%)	*p* Value
Total	30	10	9	11	33.3		63.3	
Prognostic factors								
Gender								
Male	18	4	4	10	22.2	0.14	44.4	0.018
Female	12	6	5	1	50.0		91.7	
Smoking habit								
Smoker	20	5	5	10	25	0.231	50	0.049
Nonsmoker	10	5	4	1	50		90	
Histologic type								
Adenocarcinoma	25	10	8	7	40	0.139	72	0.327
Nonadenocarcinoma	5	0	2	3	0		40	
PS								
0–1	26	8	8	10	30.8	0.584	61.5	0.999
2	4	2	1	1	50		75	
Clinical stage								
IIIb	4	2	1	1	50	0.584	75	0.999
IV	26	8	8	19	31		62	
Prior treatment								
Yes	24	9	5	10	37.5	0.999	58.3	0.215
No	6	1	4	1	16.7		83.3	

*RR and DCR were compared between prognostic factors using Fisher's exact test.
*PR, partial response; SD, stable disease; PD, progressive disease; RR, response rate; DCR, disease control rate.

TTP and OS

At a median follow-up of 12 months, 20 patients had died and 26 patients were refractory or had become resistant to gefitinib monotherapy. Median TTP was 3.3 months (range, 0.3–19.6 months) and median OS was 10 months (range, 1.7–21.4 months) (Figure 1. Duration of response for patients with partial response was 5.8 months. OS and TTP were not affected by histologic type, smoking, PS, stage, or prior treatment. However, there was a significant difference in survival in gender (median survival time, >12 months in female patients versus 7.7 months in male patients; log-rank test, $p < 0.04$; Wilcoxon test, $p < 0.04$).

Tolerability

Table 3 shows drug-related adverse events. Twenty-six patients (86.7%) experienced drug-related adverse events, most of which were mild. Frequent adverse events included diarrhea, skin rash, and elevated transaminases. Twenty-two patients experienced skin toxicities, such as acne, pruritus, and rash. Grade 3 skin toxicities were observed in two patients, but these resolved spontaneously during treatment. Diarrhea was observed in 12 patients (40.0%) and was controlled with antidiarrheal agents such as loperamide. One patient developed grade 3 diarrhea, which required temporal interruption of therapy. Two patients developed drug-related pneumonitis; both were treated with steroid therapy, antibiotics, and oxygen inhalation and recovered within a few weeks. These patients were smokers and had not received thoracic radiotherapy. No patients experienced hematologic toxicities.

Postgefitinib Treatment

Twenty-five patients became resistant or were refractory to gefitinib monotherapy. Eight of these patients received neither chemotherapy nor radiotherapy because of deterioration of PS in four patients and withdrawal of informed consent to chemotherapy in three patients. One patient underwent palliative surgery and two received radiotherapy for symptomatic brain metastases. Fifteen patients received chemotherapy as postgefitinib treatment (platinum-based chemotherapy in 14 patients and vinorelbine monotherapy in one patient). Five patients achieved PR and four showed SD by the second-line chemotherapy.

EGFR Mutations in Tumor Samples

Twenty tumor samples were obtained from 15 patients retrospectively. Sequencing of exons 18, 19, and 21 in *EGFR* was performed in 12 of 20 samples under the same PCR conditions. *EGFR* mutations were detected in four tumor

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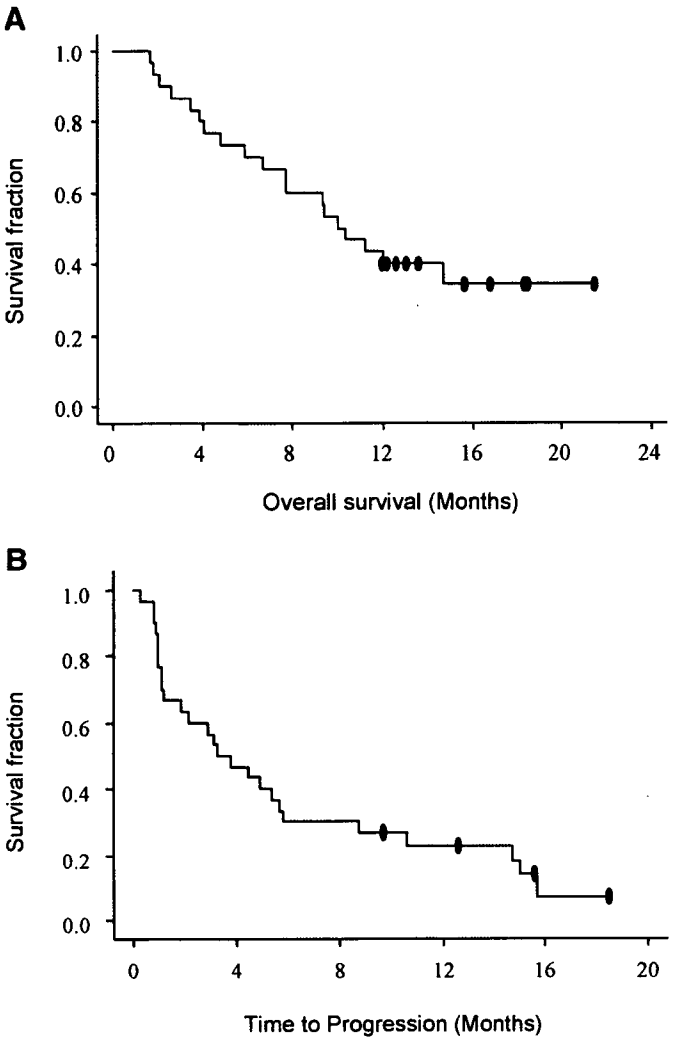

FIGURE 1. Kaplan-Meier curve showing (*A*) overall survival and (*B*) time to progression in all patients.

samples (33.3%). Three of them had a 15–base pair deletion (E746_A750del) in exon 19. Another of them had L858R in exon 21. The histologic types in patients with *EGFR* mutations were adenocarcinoma in three and large-cell carcinoma in one. All patients with E746_A750del in tumor samples had adenocarcinoma. The responses to gefitinib in these four patients were PR in two, SD in one, and PD in one. There were no responders among nine patients without an *EGFR* gene mutation.

EGFR Mutations in Serum Samples

The serum DNA in serum samples from 27 NSCLC patients was examined. Serum DNA was detected in all 54 samples at concentrations of up to 1720 ng/ml.

Exon 19 of *EGFR* in pretreatment serum samples obtained from 21 of 27 patients (77%) was detected (Figure 2 *A*). The lower band was also detected in 10 of 27 (37%) pretreatment serum samples. Sequencing of the PCR products confirmed that the upper and lower bands corresponded to wild-type and E746_A750del, respectively (Figure 2 *B*). No

TABLE 3. Drug-Related Adverse Events

	NCI-CTC Grade	No. of Patients	%
Diarrhea	1	8	26.7
	2	3	10.0
	3	1	3.3
Nausea	1	8	26.7
	2	2	6.7
	3	0	0.0
Vomiting	1	2	6.7
	2	0	0.0
	3	0	0.0
Skin toxicity	1	15	50.0
	2	5	16.7
	3	2	6.7
Elevation of transaminases	1	4	13.3
	2	1	3.3
	3	2	6.7
Pneumonitis	1	0	0.0
	2	0	0.0
	3	2	6.7

NCI-CTC, National Cancer Institute Common Toxicity Criteria.

point mutation in exon 18, 19, or 21 was detected in the PCR products from serum samples. Wild-type *EGFR* was detected in all 10 of the deletion-positive cases. The pattern of bands was reproducible when using another primer set.[13]

When compared according to histologic type, E746_A750del was detected in eight of 25 (32%) cases of adenocarcinoma, in zero of three cases of squamous carcinoma, and in two of two cases of large-cell carcinoma (Table 4). In contrast, the serum *EGFR* status was not correlated statistically with either the clinical response, the gender, or the recorded adverse effects (Table 5).

In serum samples obtained after the initiation of gefitinib treatment, 19 of 27 (70%) cases were wild-type–positive and 14 of 27 (52%) cases were deletion-positive (Figure 2 *C*). In the posttreatment serum samples, E746_A750del was more frequently observed. Furthermore, the deletional mutant of *EGFR* was significantly more frequently observed in samples from patients who showed a PR or SD (12 of 16 cases [75%]) than in samples from patients with PD (two of 11 cases [18%]) ($p = 0.0063$, Fisher's exact test) (Table 6). The deletional mutant *EGFR* was more frequently detected in female patients (six of nine cases [67%]) than in male patients (eight of 18 cases [44%]), but this difference was not significant (Table 6). No correlations were seen statistically between the presence of mutation and the adverse effects.

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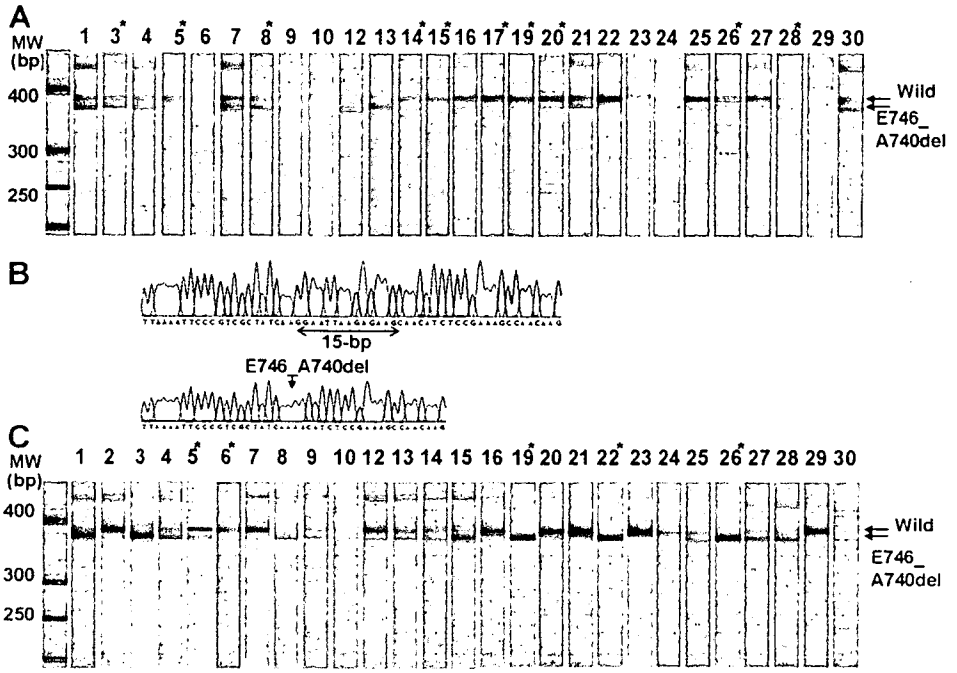

FIGURE 2. (*A*) Detection of genomic *EGFR* in the serum of pretreatment patients. (*B*) The sequences of the PCR products from patient 19 (days 0 and 14) are shown. (*C*) PCR of the serum samples obtained on day 14. Serum-derived genomic DNA PCR was performed. Exon 19 of *EGFR* in serum obtained from the patients was amplified by PCR, and the products were detected using a Bioanalyzer. A second round of PCR (20 cycles) was performed when no band was detected in the first round of PCR (30 cycles). Row numbers indicate the patient number. *Band detected in the first round of PCR.

TABLE 4. Frequency of Serum *EGFR* in Lung Cancer Patients According to Histology and Response to Gefitinib*

	Pre		Post	
	Wild	Deletion	Wild	Deletion
Adenocarcinoma	18/23	8/23	15/22	13/22
Squamous-cell carcinoma	1/2	0/2	3/3	0/3
Large-cell carcinoma	2/2	2/2	1/2	1/2

*A total of 27 samples were obtained from 28 patients both before and after treatment. A pretreatment sample of patient 2 and a posttreatment sample of patient 17 were lacking.

TABLE 5. Frequency of Serum *EGFR* in Lung Cancer Patients According Response to Gefitinib and Gender: Detection of Deletion-Type Mutation on Day 0*

	+	−	*p* Value
Response			
PR/SD	8	9	
PD	2	8	0.2305
Gender			
Male	5	12	
Female	5	5	0.4153

*A total of 27 samples were obtained from 28 patients both before and after treatment. A pretreatment sample of patient 2 and a posttreatment sample of patient 17 were lacking. SD, stable disease; PD, progressive disease; PR, partial response; +, deletion-positive; −, wild-type.

Comparison of *EGFR* Mutation Status between Tumor Samples and Serum Samples

Pairs of tumor samples and serum samples were obtained from 12 patients retrospectively (Table 7). The *EGFR* mutation status in the tumors was consistent with those in serum of seven of 12 of the paired samples. Among the other five patients, *EGFR* mutation was negative in the tumor and positive in the serum in four patients, and in the other patient it was positive in the tumor and negative in the serum, from whose tumor sample L858R was detected.

TABLE 6. Frequency of Serum *EGFR* in Lung Cancer Patients According to Response to Gefitinib and Gender: Detection of Deletion-Type Mutation on Day 14*

	+	−	*p* Value
Response			
PR/SD	12	4	
PD	2	9	0.0063
Gender			
Male	8	10	
Female	6	3	0.4197

*A total of 27 samples were obtained from 28 patients both before and after treatment. A pretreatment sample of patient 2 and a posttreatment sample of patient 17 were lacking. SD, stable disease; PD, progressive disease; PR, partial response; +, deletion-positive; −, wild-type.

DISCUSSION

The overall response of 33.3% in this phase II study was comparable not only to that achieved in Japanese population enrolled in the IDEAL-1 trial (27.5%)[7] but also to a retrospective analysis conducted of patients in Japan.[22] Gefitinib monotherapy appeared to be equally effective in patients with chemotherapy-naive NSCLC and in patients with pretreated NSCLC.

Drug-related adverse events were generally mild compared with cytotoxic chemotherapy. Grade 3 pulmonary toxicities were observed in two patients. In this study, the

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TABLE 7. *EGFR* Mutation Status in Tumor Samples and Serum Samples*

					EGFR Mutation Status			
					Serum Samples			
					Pre		Post	
No.	Gender	Histology	Response	Tumor Sample	Wild	Mutation	Wild	Mutation
43	M	Large	SD	Wild	+	+	–	+
45	M	SCC	PD	Wild	ND	ND	+	–
52	F	SCC	PD	Wild	+	–	+	–
53	M	Adeno	PD	Wild	–	–	+	–
55	M	Adeno	PR	L858R	+	+	–	–
57	F	Adeno	SD	Wild	–	–	+	+
61	M	Large	PD	E746-A750 del	+	+	+	–
64	M	Adeno	PD	Wild	+	–	+	–
70	M	Adeno	PD	Wild	+	+	+	–
72	M	Adeno	SD	E746-A750 del	+	–	–	+
75	F	Adeno	PR	E746-A750 del	+	+	+	+
77	M	Adeno	PD	Wild	+	–	+	+

*Pairs of both tumor samples and serum samples were obtained from 12 patients. M, male; F, female; SD, stable disease; PD, progressive disease; PR, partial response; SCC, squamous-cell carcinoma; Adeno, adenocarcinoma; Large, large-cell carcinoma; ND, not determined.

incidence of drug-related pneumonitis was 6.7% and was comparable to results of other studies.[23,24] Therefore, gefitinib monotherapy as a first-line treatment appears to be equally tolerable as a second-line treatment.

Thirteen of 22 patients who became resistant or were refractory to gefitinib monotherapy received salvage chemotherapy. The objective response rate was 30.8%, comparable to that of first-line chemotherapy. These results suggest that cancer cell populations that are sensitive to gefitinib might not be identical to those sensitive to chemotherapeutic drugs such as platinum agents or taxanes.

Somatic mutations in the tyrosine kinase domain of the *EGFR* gene were reported, and these mutations induced increased activity of *EGFR* and sensitivity to gefitinib in vitro and the predictive factor of response to gefitinib.[13,14] We evaluated *EGFR* gene status in 13 tumor samples and detected *EGFR* gene mutation in four tumors. Objective responses were achieved in two patients, but one patient showed PD whose tumor had a 15–base pair deletion mutation in exon 19. This suggested that response to gefitinib may not be determined by *EGFR* mutation in exon 19 or 21, and other mechanisms may relate to gefitinib resistance.

The detection of EGFR mutation from serum samples was carried out as a correlative study. These results provided us two major findings: (1) E746_A750del was detectable in serum sample obtained from NSCLC patients; and (2) E746_A750del was frequently observed in posttreatment serum samples obtained from the PR and SD patients.

It may be explained that DNA derived from destructive tumor cells that have responded to gefitinib may be more frequently observed in the circulating blood. Previous reports regarding detection of mutations in serum did not elucidate the changes in mutation status during treatment. We would like to do this in the next experiments to confirm our speculation. Our hypothesis is that serum detection of *EGFR* mutation will be a convenient means of predicting the sensitivity to gefitinib, although we could only demonstrate the feasibility of the *EGFR* mutation in serum in this report. We need to develop a highly sensitive methodology to improve the predictability of this assay.

In comparison of the mutation status of *EGFR* in actual tumors with serum DNA obtained from the same patients before treatment, 70% of patients who had sequence data obtained from both serum and tumor samples were conforming. Esteller et al. reported detection of aberrant promoter hypermethylation of tumor suppressor genes (*p16*, *DAP*, *GSTP1*, and *MGMT*) in serum DNA obtained from NSCLC patients and demonstrated that 73% of serum samples showed abnormal methylated DNA in the patients with the methylated primary tumors.[19] Another report investigating a point mutation of the *p53* gene and hypermethylation of *p16* in plasma DNA from breast cancer patients demonstrated that 66% of the patients with at least one molecular event in tumor DNA had some alteration in plasma DNA.[25] We believe that the sensitivity of our assay is equivalently sensitive to those of these previous reports.

CONCLUSION

In conclusion, 250 mg of oral gefitinib monotherapy as a first-line treatment produces obvious antitumor activity, with acceptable toxicities. Oral gefitinib monotherapy as a first-line treatment merits investigation in further clinical trials. Using serum samples from NSCLC patients, the *EGFR* mutation was detected. The detection of E746_A750del in the serum of untreated patients was not a predictor of gefitinib response in this study. However, further prospective studies using serum samples may be necessary to confirm this con-

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clusion. The presence of *EGFR* mutation in serum may be a useful biomarker for monitoring gefitinib response.

ACKNOWLEDGMENTS

We thank the patients who enrolled into the trial and the investigators who enrolled them.

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The FASEB Journal express article 10.1096/fj.05-4034fje. Published online December 22, 2005.

Dimerization and the signal transduction pathway of a small in-frame deletion in the epidermal growth factor receptor

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ABSTRACT

A short, in-frame deletional mutant (E746-A750del) is one of the major mutant forms of epidermal growth factor receptor (EGFR) and has been reported to be a determinant of response to EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib. However, the biological and pharmacological functions of mutational EGFR remain unclear. To clarify these biological functions of deletional EGFR, we examined the cellular response to EGF ligand stimulation. Dimerization and phosphorylation of EGFR were observed without any ligand stimulation in the 293(D) cells transfected with deletional EGFR as compared with those transfected with wild-type EGFR (293(W) cells). When the 293(D) cells were exposed to gefitinib, an immunoblotting analysis revealed remarkable inhibition of AKT phosphorylation but not phospho-p44/42 MAPK. To examine the cellular response in a lung cancer cell line intrinsically expressing deletional EGFR, phospho-EGFR, and downstream reactions were monitored under EGF stimulation with a beads-based mulitiplex assay. EGFR and its downstream proteins were constitutively phosphorylated in the PC-9 cells without any ligand stimulation as compared with A549 lung cancer cells expressing wild-type EGFR. In conclusion, deletional EGFR is constitutively active and phosphorylates p44/42 MAPK and AKT in the cells, although the fact that the EGFR phosphorylation in the PC-9 cells is still modulated by EGF stimulation cannot be ignored. Gefitinib-inhibited phospho-AKT predominantly in deletional EGFR expressing cells.

Key words: mutation • gefitinib • tyrosine kinase

Epidermal growth factor receptor (EGFR) belongs to the ErbB family (1) and contains an extracellular ligand-binding domain, a transmembrane domain, and a tyrosine kinase domain. Wild-type EGFR is unphosphorylated and exists as a monomer in the unstimulated conditions. Binding of ligands such as EGF and TGF-α leads to the dimerization of EGFR, phosphorylation of tyrosine residues (2), and stimulation of the phosphorylation pathway downstream. This signaling pathway is considered to be closely related to cellular growth, differentiation, and the development of malignant phenotypes of cancer cells (1, 2). Increased

expression of EGFR and gene amplification of EGFR are often observed in several types of tumors such as lung and breast cancers.

Recently, many small anticancer molecules have been developed that target EGFR. Gefitinib (Iressa) is an orally available EGFR tyrosine kinase inhibitor. Previous clinical studies have demonstrated that EGFR expression levels in tumors did not correlate with the clinical response to gefitinib (3). On the other hand, EGFR mutation in adenocarcinoma of the lung was reported to be a determinant of sensitivity for EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib (4, 5). To date, over 30 types of EGFR mutation have been reported in lung cancer. The in-frame, 15 base deletional mutation (delE746-A750 type deletion) is one of the most common of these EGFR mutations. We previously demonstrated that overexpression of deletional EGFR increased the cellular sensitivity to tyrosine kinase inhibitors targeting EGFR in human HEK293 cells in vitro (6). However, it remains unclear how deletional EGFR alters dimerization and the downstream signaling pathways from these heterodimers. Recently, Tracy et al. demonstrated that another major mutant EGFR (L858R) altered signal transduction downstream (7). In addition, it has been suggested that the L858R mutation is a hyper-response to ligand stimulation (7). We hypothesized that the deletional EGFR is constitutively active. The aim of this study was to clarify the downstream of the signaling and its function of the deletional EGFR.

Technically, a beads-based multiplex assay (Bio-Plex phosphoprotein assay) (8) allowed us to analyze numbers of phosphoproteins simultaneously after ligand stimulation.

MATERIALS AND METHODS

Reagents

Gefitinib (Iressa, ZD1839) was provided by AstraZeneca (Cheshire, UK).

Cell culture

The human embryonic kidney HEK293 cell line and human nonsmall-cell lung cancer (NSCLC) cell line A549 and cervix epitheloid cancer cell line HeLa were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 medium supplemented with 10% FBS. The human NSCLC cell line PC-9 was established at the Tokyo Medical University (9, 10), and was maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Rockville, MD).

Plasmid construction and transfection

The construction of the mock expression plasmid vector (empty vector) and of the wild-type of EGFR and the 15-bp deletional EGFR (delE746-A750 type deletion) vectors that posses the same deletion site observed in PC-9 cells has been described in another paper in detail (6, 11, 12). The plasmids were transfected into the HEK293 cells, and the transfectants were selected by Zeosin (Sigma). The stable transfectants (pooled cultures) of the empty vector, wild type of EGFR, and its deletion mutant were designated as 293(M), 293(W), and 293 (D) cells, respectively.

Reverse-transcription PCR

Five micrograms of total RNA from each cultured cell line was converted to cDNA with a GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA). The primers used for the PCR were EGFR (forward), 5′-AAGTTAAAATTCCCGTCGCTATCA-3′ and (reverse) 5′-GAGCCAATATTGTCTTTGTGTTCC-3′. PCR amplification consisted of 28 cycles (95°C for 45 s, 57.5°C for 30 s, and 72°C for 45 s) followed by incubation at 72°C for 7 min. The RT-PCR products were analyzed with a 2100 Bioanalyzer and DNA 500 kit (Agilent Technologies, Waldbronn, Germany).

Chemical cross-linking

After treatment with or without EGF (Sigma), monolayer cells were washed twice with ice-cold phosphate buffered saline containing 0.33 mM $MgCl_2$ and 0.9 mM $CaCl_2$ (PBS(+)) and chemically cross-linked for 20 min at room temperature with freshly prepared 1.5 mM bis(sulfosuccinimidyl) suberate (BS^3, Pierce, Rockford, IL). To terminate the reaction, a final concentration of 20 mM glycine was added for an additional 5 min. For immunoblot analysis, the cells were washed twice with ice-cold PBS(+) and lysed with M-PER mammalian protein extraction reagent (Pierce). The lysate was centrifuged at 20,000 *g* for 10 min, and the protein concentration of the supernatant was measured with a BCA (bicinchoninic acid) protein assay (Pierce).

Ligand stimulation

After reaching 70–80% confluence, cultured cells were stimulated with EGF, TGF-α, and HB-EGF for 10 min under nonstarved conditions or serum-starved conditions. The cells were washed twice with ice-cold PBS(+), and lysed for immunoblotting.

Drug treatment

After reaching 70–80% confluence, cultured cells, were exposed to various concentrations of gefitinib and stimulated or not stimulated with EGF (100 ng/ml) for 10 min under nonstarved conditions or serum-starved conditions. The cells were washed twice with ice-cold PBS(+) and lysed for immunoblotting.

Immunoblot analysis

Immunoblot analysis was performed as described previously (12). Equivalent amounts of protein were separated by electrophoresis on a SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was probed with a mouse monoclonal antibody against EGFR (Transduction Lab, San Diego, CA), a phospho-EGFR antibody (specific for Tyr1068), p44/42 mitogen-activated protein kinase (p44/42 MAPK), phospho-p44/42 MAPK, AKT, phospho-AKT, nuclear factor-κB (NF-κB) inhibitor α (IκB-α), and phospho-IκB-α antibody (Cell Signaling Technology, Beverly, MA) as the first antibody, followed by a horseradish peroxidase-conjugated secondary antibody. The bands were visualized with electrochemiluminescence (ECL, Amersham, Piscataway, NJ), and images of blotted patterns were analyzed with NIH image software (National Institutes of Health, Bethesda, MD).

Phosphoprotein assay

A panel of phosphoproteins was measured in duplicate using a bead-based mulitiplex assay (Bio-Plex phosphoprotein assay, Bio-Rad, Hercules, CA), according to the manufacturer's instructions (8, 13). The EGFR-transfected 293 cells and NSCLC cells cultured in the serum-free medium for 24 h were stimulated by the addition of EGF at a final concentration of 100 ng/ml for the indicated time intervals. After incubation, the cells were rinsed with ice-cold Cell Wash Buffer and collected. The lysate was centrifuged at 1,700 *g* for 20 min. The protein concentration was calculated with a DC (detergent compatible) protein assay (Bio-Rad). The Bio-Plex assay was customized to detect and quantify phosphoproteins of EGFR, p44/42 MAPK, activating transcription factor 2 (ATF-2), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), IκB-α, and signal transducer and activator of transcription 3 (STAT-3). The prepared first antibody with coupled beads was captured under 96-well plates, and samples (17.5 μg each) were incubated overnight at room temperature. Samples were incubated with biotin-labeled detection antibodies followed by further incubation with the fluorescence-labeled avidin reporter. The level of phosphoproteins bound to the beads was indicated by the intensity of the reporter signal. The signal was measured with Bio-Plex Manager software (Bio-Rad) interfaced with a Bio-Plex Reader (Bio-Rad). In this assay, we used the lysates of untreated HeLa cells as the background control for all phosphoprotein assays provided by the Bio-Plex phosphoprotein assay (14). This experiment was repeated in duplicate.

RESULTS

Dimerization and phosphorylation of wild-type EGFR and deletional EGFR

The EGFR expression level in the 293 cells transfected with the empty vector (293(M)), wild-type EGFR (293(W)), and deletional EGFR (293(D)), in the A549 and PC-9 NSCLC cells, and in the HeLa cervix epitheloid cancer cells were determined by RT-PCR and immunoblot analysis (Fig. 1). The PCR products were separated into wild-type EGFR (upper band) and deletion mutant EGFR (lower band) by the different lengths of the sequences. Overexpression of EGFR was detected in the 293(W) and 293(D) cells. Only a small amount of intrinsically EGFR was detected in 293(M) cells by RT-PCR. We sequenced exon 19 and 20 of EGFR in HEK293 cells, but no mutations were detected (data not shown). A high level of EGFR expression was detected in the PC-9 cells, and a moderate level was detected in the A549 and HeLa cells. The EGFR protein levels closely matched the mRNA expression levels in all cells.

Dimerization of EGFR, the first step in the EGFR signaling pathway, was examined by chemical cross-linking. The 293(M), 293(W), and 293(D) cells were treated with EGF (10 ng/ml) for 10 min under nonstarved conditions (Fig. 2*A*). Cells were incubated with the cross-linking reagent BS^3. Dimerization and phosphorylation of EGFR were determined by immunoblot with anti-EGFR and anti-phospho EGFR antibodies. Dimerization and expression of EGFR were not detected in the 293(M) cells. Dimerized EGFR with a molecular weight of ~400 kDa was detected in the 293(W) cells. Increased phosphorylation and dimerization of the deletional EGFR were detected without EGF stimulation in the 293(D) cells by the chemical cross-linking and immunoblot assay, respectively. When the cells were stimulated with the EGF ligand (10 ng/ml for 10 min), increased phospho-EGFR dimers were observed in the 293(W) cells, whereas no response of EGFR to EGF was observed in the 293(D) cells. We quantified the levels of monomeric and dimerized EGFR in the 293(W) and 293(D) cells densitometrically under nonstarved conditions (Fig. 2*A*, right). In the

293(W) cells, the dimer/monomer ratio was increased ~40% by the addition of EGF. Addition of EGF slightly increased the dimer/monomer ratio (~20%) in the 293(D) cells.

We investigated the dimerization and phosphorylation status of EGFR in the EGFR-transfected cell lines under serum-starved conditions (Fig. 2*B*). The transfected cells were exposed by EGF (10 ng/ml for 10 min) after serum starvation for 24 h. No expression of EGFR dimer or monomer was detected in the 293(M) cells. Addition of EGF resulted in an increase in dimerized and phosphorylated EGFR in the 293(W) cells. Dimerization and phosphorylation of the deletional EGFR were detected in the 293(D) cells in the absence of EGF after serum starvation. Expression of dimerized EGFR in the 293(D) cells was unchanged by EGF stimulation. These findings demonstrated that the deletional mutant EGFR was constitutively dimerized and phosphorylated without any ligand stimulation even under starved conditions and are consistent with the results under nonstarved conditions (Fig. 2*A*). The ratio of dimerized to monomeric EGFR in 293(W) and 293(D) cells was analyzed densitometrically under serum-starved conditions (Fig. 2*B*, right). The dimer/monomer ratio in the 293(W) cells was markedly increased (~3 fold) by addition of EGF. Under unstimulated conditions, the dimer/monomer ratio of the 293(D) cells was higher than that of the 293(W) cells, and the ratio was unchanged by addition of EGF.

These results suggest that the cells expressing the wild-type of EGFR responded to EGF for their dimerization and phosphorylation and that the deletion mutant of EGFR was dimerized and phosphorylated constitutively without any ligand stimulation.

Phosphorylation of EGFR, p44/42 MAPK, and AKT in the EGFR-transfected 293 cells

The p44/42 MAPK and AKT are major downstream targets of EGFR. We examined the phosphorylation status of p44/42 MAPK and AKT with EGF addition in the transfectants. The transfected cells were treated with EGF (10 ng/ml) for 10 min under nonstarved conditions (Fig. 3*A*). The phosphorylation levels of EGFR in the 293(D) cells were higher without any ligand stimulation than that of the 293(W) cells. Phospho-EGFR in the 293(W) cells was increased after EGF stimulation. These findings are consistent with Fig. 2. Even under unstimulated conditions, increased phosphorylation of p44/42 MAPK and AKT was observed in the 293(D) cells. In the 293(W) cells, increased phosphorylation of p44/42 MAPK and AKT was observed with the addition of EGF especially p44/42 MAPK was remarkably phosphorylated. On the other hand, in the 293(D) cells, phosphorylation of p44/42 MAPK and AKT was not increased with the addition of EGF. We quantified the phosphorylation levels of p44/42 MAPK and AKT densitometrically in the transfectants in response to EGF. The addition of EGF increased phosphorylation of p44/42 MAPK in the 293(M) cells (~3.4 fold) and in the 293(W) cells (~2.5 fold) (Fig. 3*B*), suggesting no difference in response in regard to p44/42 MAPK to EGF. Increased phosphorylation of AKT in the 293(M) cells (~2.1 fold) and in the 293(W) cells (~1.3 fold) was observed. By contrast, EGF decreased the phosphorylation of p44/42 MAPK and AKT in the 293(D) cells ~30% and ~20% (Fig. 3*C*). These findings suggest that the p44/42 MAPK and AKT pathways are activated in cells expressing the deletional EGFR without ligand stimulation.

Next, we examined the dose-dependent response of EGFR status in the transfected cells to the other ligands, TGF-α, and HB-EGF, after serum starvation (Fig. 4). The hyperphosphorylated EGFR in the 293(D) cells was unchanged by stimulation with these ligands either. By contrast, a dose-dependent increase in EGFR phosphorylation was observed in the 293(W) cells in response to stimulation by TGF-α and HB-EGF. Both HB-EGF and EGF strongly increased EGFR

phosphorylation in the 293(W) cells compared with TGF-α. We also examined the downstream in response to these ligands. In the 293(W) cells, increased phosphorylation of p44/42 MAPK and AKT was observed in response to addition of the ligands (EGF, TGF-α, and HB-EGF). p44/42 MAPK was markedly phosphorylated by the ligands. In the 293(D) cells, addition of ligands further increased p44/42 MAPK phosphorylation. The AKT phosphorylation in the 293(D) cells was unchanged by stimulation with these ligands. These findings are consistent with the data in Fig. 3*A*, and suggest that the p44/42 MAPK and AKT pathways are both activated in cells constitutively expressing the deletional EGFR.

Effect of gefitinib on phosphorylation of EGFR, p44/42 MAPK, and AKT in the EGFR-transfected 293 cells

Previously, we demonstrated that 293(D) cells were hypersensitive to EGFR-targeted tyrosine kinase inhibitors such as gefitinib and ZD6474, as compared with 293(W) cells (6). To examine the specific action of these tyrosine kinase inhibitors on deletional EGFR signal transduction, we exposed the 293 transfectants to gefitinib, and its cellular response was examined under nonstarved conditions with an immunoblot analysis (Fig. 5*A*). In the 293(W) cells, phosphorylation of p44/42 MAPK was not inhibited by exposure to a low dose of gefitinib (0.01 μM) but phosphorylation of AKT was inhibited by exposure to gefitinib (0.01 μM). In contrast, exposure to gefitinib decreased phospho-EGFR in the 293(D) cells that are hypersensitive to gefitinib. Phosphorylation of AKT was completely inhibited by 0.01 μM gefitinib exposure, while the inhibition of p44/42 MAPK phosphorylation was not remarkable in the 293(D) cells. The effect of 0.01 μM gefitinib on phosphorylated p44/42 MAPK and AKT in the transfectents measured densitometrically. p44/42 MAPK phosphorylation in the 293(M) and 293(W) cells was unaltered by gefitinib exposure for 3 h, but it decreased in the 293(D) cells (~20%) (Fig. 5*B*). Gefitinib increased phosphorylation of AKT ~1.3 fold in the 293(M) cells. Gefitinib inhibited AKT phosphorylation was ~70% in the 293(W) cells, and decreased it ~99% in the 293(D) cells (Fig. 5*C*).

We examined the dose-dependent effect of gefitinib (0.02, 0.2, 2 μM) on EGFR and its downstream signaling in all of the transfectants under serum-starved conditions. Phosphorylation of EGFR was not detected in the 293(W) cells under the 24 h serum-starved conditions, and gefitinib had no effect on it (Fig. 6*A*). Hyperphosphorylation of EGFR was dose dependently inhibited by gefitinib in the 293(D) cells. p44/42 MAPK and AKT were slightly phosphorylated in the 293(W) cells, and their degree of phosphorylation was unaltered by exposure to gefitinib. By contrast, gefitinib dose-dependently decreased phosphorylation of p44/42 MAPK, and AKT in the 293(D) cells. Under EGF stimulation (Fig. 6*B*), gefitinib dose-dependently decreased phosphorylation of EGFR, p44/42 MAPK, and AKT in the 293(W) cells. Phosphorylation of EGFR in the 293(D) cells was completely inhibited by a low concentration of gefitinib (0.02 μM), and phosphorylation of p44/42 MAPK and AKT was dose-dependently inhibited by gefitinib. A low concentration of gefitinib inhibited EGFR phosphorylation and its signal in the 293(D) cells under serum-starved conditions. The phosphorylation of EGFR (and its signal) induced by EGF-addition in the 293(W) cells was inhibited by gefitinib dose dependently.

These data suggest that gefitinib inhibited the AKT signaling pathway more strongly than the p44/42 MAPK signaling pathway in the cells expressing the deletional mutant EGFR.

Response to EGF stimulation in the EGFR-transfected 293 cells

We performed the quantitative phosphoprotein analysis in the 293(W) and 293(D) cells by a beads-based multiplex assay. Downstream from the reaction, we monitored the phosphorylation status of p44/42 MAPK, JNK, and p38 MAPK. We also examined the phosphorylation status of ATF-2 that is located downstream of the MAPK pathway, IκB-α that is a member of the AKT pathway, and STAT-3 that is found downstream of the other signaling pathway. Even under unstimulated conditions, EGFR was hyperphosphorylated in the 293(D) cells but not in the 293(W) cells (Fig. 7*A*). Increased phosphorylation of EGFR was observed in the 293(W) cells (~40 fold), but the phosphorylation of EGFR in the 293(W) cells was much lower than that of the deletional EGFR in the 293(D) cells. IκB-α phosphorylation in the transfected cells was as low as in the HeLa cells, which was used as a negative control (Fig. 7*B*). Under unstimulated conditions, phosphorylation of p44/42 MAPK was greater in the 293(D) cells than in the 293(W) cells (~3 fold) (Fig. 7*C*). A large increase in phosphorylation of p44/42 MAPK in response to the addition of EGF was observed in the 293(W) cells (~15 fold), and a smaller increase was observed in the 293(D) cells (~3.5 fold). These differences in phosphorylated p44/42 MAPK in the 293(W) and 293(D) cells detected in the beads-based multiplex assay are consistent with the result of immunoblotting (Fig. 4). ATF-2 was phosphorylated in the 293(W) cells (~1.5 fold) and 293(D) cells (~1.7 fold) by addition of EGF (Fig. 7*D*). By contrast, the JNK in the 293(W) cells was phosphorylated by addition of EGF (~2 fold), but not phosphorylated in the 293(D) cells (Fig. 7*E*). Phosphorylation of p38 MAPK and STAT-3 was not detected in either type of cell (data not shown). These findings suggest that the p44/42 MAPK and AKT pathways are both phosphorylated without any ligand stimulation in the cells expressing the deletional mutant EGFR.

Response to EGF stimulation in PC-9 cells intrinsically expressing deletional EGFR

The hyperphosphorylation and increased dimerization of deletional EGFR has been demonstrated by ectopic expression of deletional EGFR. To examine whether this phenomenon is also observed in the lung cancer cells intrinsically expressing deletional EGFR, we monitored the phosphorylation of EGFR and its related molecules in the PC-9 cells as compared with the A549 cells. The PC-9 cells and A549 cells express the deletional and wild-type EGFR, respectively. PC-9 cells also express a small amount of wild-type EGFR, and so these cell lines mimic the 293(D) cells. We examined the phosphorylation of EGFR and its downstream signaling molecules in these cells by immunoblotting (Fig. 8*A*). Increased phosphorylation of EGFR was observed in the PC-9 cells even under unstimulated conditions, but not in the A549 cells. The addition of EGF markedly increased the EGFR phosphorylation in the A549 cells compared with the PC-9 cells. Under unstimulated conditions, p44/42 MAPK, and AKT were more phosphorylated in the PC-9 cells than in the A549 cells. Increased phosphorylation of p44/42 MAPK and AKT was observed in the A549 cells after addition of EGF, and p44/42 MAPK was markedly phosphorylated. A small increase in phosphorylation of p44/42 MAPK and AKT was observed in the PC-9 cells in response to the addition of EGF. The increased phosphorylation of IκB-α in the PC-9 cells was observed even under unstimulated conditions. The addition of EGF increased the phosphorylation of IκB-α in the A549 cells but did not alter it in the PC-9 cells. These findings suggest a difference in reactivity to the EGF stimulation between the A549 and PC-9 cells.

Next, examination was performed by a beads-based mulitiplex assay. Increased phosphorylation of EGFR was observed in the PC-9 cells, even under unstimulated conditions, but not in the A549 cells (Fig. 8*B*). These findings are consistent with the results of immunoblotting. EGFR

phosphorylation was markedly increased in the A549 cells (~100-fold), and to a lesser extent increased in the PC-9 cells (~1.4-fold). IκB-α was phosphorylated in the PC-9 cells in the absence of EGF stimulation (Fig. 8*C*), suggesting that the AKT pathway, including IκB-α, was activated in the PC-9 cells expressing deletional EGFR, compared with A549 cells. p44/42 MAPK and ATF-2 were phosphorylated without EGF stimulation in the PC-9 cells compared with the A549 cells (Fig. 8*D* and *E*). A large increase in phosphorylation of p44/42 MAPK and ATF-2 was observed in the A549 cells (~13 fold and ~4.3 fold), and a smaller increase was observed in the PC-9 cells (~3.5-fold and ~1.7-fold). JNK was not phosphorylated in either the A549 or the PC-9 cells. Phosphorylation of JNK in response to EGF stimulation was detected in the A549 cells (~2.6-fold), but not in the PC-9 cells (Fig. 8*F*). No phosphorylation of p38 MAPK and STAT-3 was detected in either cell line (data not shown). These differences in phosphoproteins in the A549 and PC-9 cells are consistent with the results of the beads-based multiplex assay in the EGFR-transfected cells (293(W) and 293(D)) (Fig. 7).

To determine whether there was a significant difference between unstimulated and EGF-stimulated conditions, a statistical analysis was performed using the *t* test. There were significant differences between increased phospho-EGFR, phospho-p44/42 MAPK, phospho-ATF-2, and phospho-JNK ($P>0.01$) in the A549 cells under unstimulated and EGF-stimulated conditions. There were also significant differences in the increased phospho-EGFR, phospho-p44/42 MAPK, phospho-ATF-2, and phospho-IκB-α ($P>0.01$) in the PC-9 cells. The fact that phosphorylation of EGFR, p44/42 MAPK, and ATF-2 in the PC-9 cells is still modulated by EGF stimulation cannot be ignored. This pathway is considered to be preferentially regulated by wild-type EGFR.

DISCUSSION

Previous studies have demonstrated that mutational EGFR is a major factor against determining gefitinib sensitivity. We analyzed the characteristics of deletional EGFR with cells expressing deletional EGFR.

In Fig. 3, phosphorylation of p44/42 MAPK and AKT was increased by EGF in the 293(M) cells, although the EGFR was not overexpressed in these cells. There is a rich cross-talk among the ErbB family that regulates the cellular effects mediated by these receptors. However, ErbB-2 (HER2), ErbB-3 (HER3), and ErbB-4 (HER4) are not expressed, and intrinsic EGFR was weakly expressed in the 293 cells (15). Activation of p44/42 MAPK and AKT in response to EGF might be mediated by the intrinsic EGFR. On the other hand, constitutive activation of EGFR and its downstream pathway is due to the mutant EGFR in the 293(D) cells, suggesting that the mutant EGFR shows a dominant phenotype.

In Fig. 3, p44/42 MAPK and AKT were activated under unstimulated conditions in the 293(D) cells. In these transfectants, EGF did not enhance the phosphorylation of p44/42 MAPK and AKT in contrast to those in the 293(W) cells. The phosphorylation of AKT in these cells seems to be decreased by the addition of EGF (Fig. 3*A*). It can be speculated that EGF negatively regulated the activation of mutant EGFR as a feedback mechanism. It is uncertain whether EGF itself negatively regulates the mutant EGFR. Although the mechanism of this phenomenon remains uncertain, a feedback mechanism might be postulated as a possible explanation. It was reported that leucine-rich repeats and immunoglobulin-like domains 1 (LIRG1) is a negative regulator of EGFR (16) and its transcription was up-regulated by EGF stimulation and caused consequently

degradation of EGFR. Thus the feedback mechanisms, including that mediated by LIRG1, should be clarified in the future study.

The 293(D) cells transfected with deletional EGFR were hypersensitive to the growth-inhibitory effect of EGFR tyrosine kinase inhibitor, including gefitinib (6). AKT phosphorylation was completely suppressed by 0.01 μM gefitinib in the 293(D) cells (Fig. 5). It is suggested that deletional EGFR signaling inclines toward the AKT pathways, and this is correlated with cellular sensitivity to gefitinib. The response to gefitinib in the 293(M) cells cannot be ignored. We speculate that the difference in cell response to gefitinib may be attributable to cell dependency on EGFR in the 293(M), 293(W), and 293(D) cells. Since the 293(W) and 293(D) cells seem to be largely dependent on EGFR, therefore, gefitinib effectively inhibited the AKT phosphorylation in these cells. Cell growth in 293(M) cells, on the other hand, is regulated by other signaling pathways. Then the MAPK and AKT pathways did not respond to gefitinib. In addition, the PC-9 cells intrinsically expressing deletional EGFR were also hypersensitive to gefitinib (11, 17), and the AKT pathway was more sensitively inhibited by gefitinib as compared with p44/42 MAPK pathway (18). These findings are consistent with the evidence seen in the 293(D) cells. The altered downstream pathway in the cells expressing other types of mutant EGFR was previously reported. The L858R and delL747-P753insS were basically unphosphorylated and these mutants were markedly phosphorylated compared with wild-type EGFR by ligand stimulation (4, 5). Activated AKT signaling pathways, but not MAPK pathway was observed in the transfectants of L858R and delL747-P753insS. Taken together, preferential activation of AKT pathway was commonly observed between these EGFR mutant cells. On the other hand, there were some differences between delE746-A750, L858R, and delL747-P753insS; constitutive active in delE746-A750 vs. hyperresponse to ligand stimulation in L858R and delL747-P753insS. These EGFR mutations except for T790M are considered to be "gain of function," although detailed differential function will be clarified in future studies.

We examined the phosphorylation status of EGFR and its downstream events in PC-9 cells intrinsically expressing deletional mutant EGFR in addition to the ectopic expression system. However, the PC-9 cells also express low levels of wild-type of EGFR. It can thus be considered that these cells mimic the 293(D) cells. In the PC-9 cells, IκB-α is activated. IκB-α binds to NF-κB and suppresses this function (19). Kapoor reported that NF-κB activating signal from EGFR is mediated by the PI3-kinase/AKT pathway (20). We used immunoblotting to investigate the phosphorylation of AKT in the PC-9 cells after EGF stimulation. Increased phosphorylation of AKT was induced even under unstimulated conditions and considered that the activation (or phosphorylation) of IκB-α occurred via the AKT pathway machinery. Therefore, the phosphorylation of IκB-α is due to the activation of the AKT pathway in the PC-9 cells.

In the PC-9 cells, increased phosphorylation of p44/42 MAPK and ATF-2 (21, 22) was detected with the addition of EGF. In addition, phosphorylation of IκB-α was also increased by the ligand stimulation in the PC-9 cells, suggesting that this signaling pathway might be mediated by wild-type of EGFR.

We demonstrated that deletional EGFR was hyperphosphorylated and dimerized in a steady state in the 293(D) cells. This mutant is considered to be constitutively active. The activation mutation in EGFR is consistent with that of the c-KIT mutation in GIST that is a target for gleevec (23). The c-KIT mutations in GIST have also been reported as gain-of-function mutations (24). The role of

mutant EGFR and its function in transformation activity and carcinogenesis requires clarification in future studies.

Downstream of the signaling pathway, p44/42 MAPK and AKT pathways are activated in the 293(D) cells, but the AKT pathway was more strongly suppressed by gefitinib. Therefore, the AKT pathway must interact with cellular hypersensitivity to the EGFR targeted tyrosine kinase inhibitor in cells expressing deletional mutant EGFR.

In this study, we focused on the short, in-frame deletional mutant (E746_A750del). Now more than 30 types of mutation have been reported in clinical lung cancer specimens. In the next step, we will examine the biological function of other types of mutants of EGFR differentially, with the aim of selecting clinically meaningful mutations.

ACKNOWLEDGMENTS

This work was supported by funds for the Third Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (12217165).

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Received April 23, 2005; accepted October 25, 2005

Fig. 1

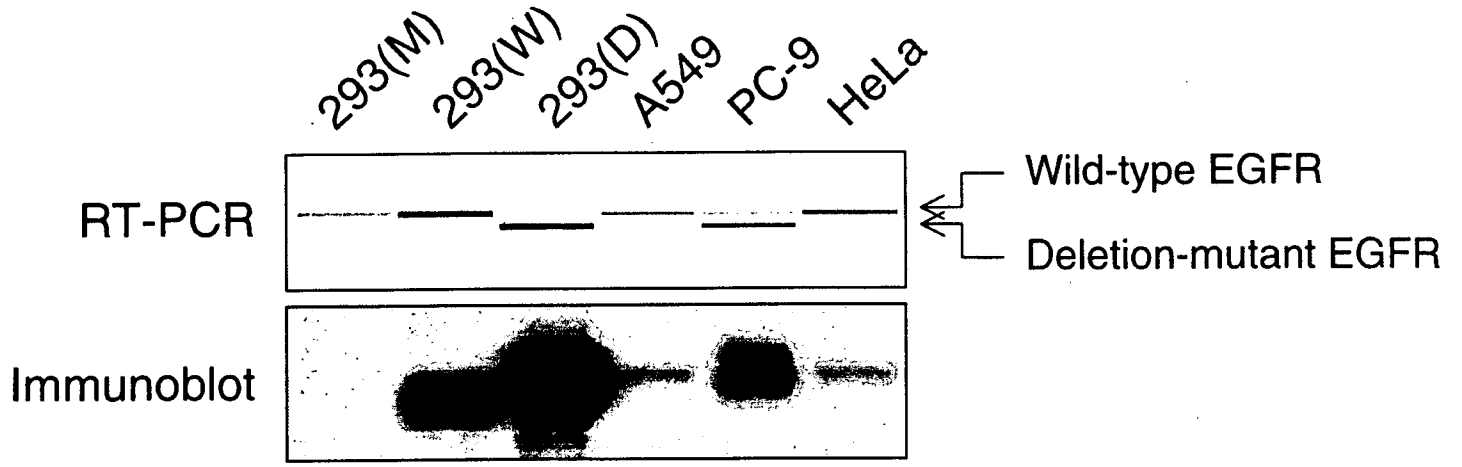

Figure 1. Expression status of epidermal growth factor receptor (EGFR) The EGFR expression level was determined by RT-PCR and immunoblot analysis. The RT-PCR products were analyzed with a 2100 Bioanalyzer. The level of EGFR protein expression was measured by immunoblot with anti-EGFR.