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Epidermal growth factor receptor mutation, but not sex and smoking, is independently associated with favorable prognosis of gefitinib-treated patients with lung adenocarcinoma

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Epidermal growth factor receptor (*EGFR*) mutations have been reported as a predictive factor for favorable prognosis of gefitinib-treated patients with lung adenocarcinoma. However, its confounding with sex and smoking makes it unclear whether the *EGFR* mutation is independently associated with prolonged patient survival. In this study, we analyzed a large-scale database to discriminate the survival impact of *EGFR* mutations against those of sex and smoking after gefitinib therapy. *EGFR* mutations in exon19 and exon21 named drug-sensitive *EGFR* mutations were examined to investigate the impact of *EGFR* mutation, sex, and smoking status on survival of 362 gefitinib-treated patients with lung adenocarcinoma. Drug-sensitive *EGFR* mutations were detected in 169 patients (46.7%). The multivariate analysis including *EGFR*, sex, and smoking status showed that drug-sensitive *EGFR* mutations were significantly related to longer overall survival (OS) ($P < 0.001$) and progression-free survival (PFS) ($P < 0.001$). In addition, we investigated the impact of sex and smoking status according to *EGFR* mutation status, and the impact of *EGFR* mutation status according to sex and smoking status on survival. Sex and smoking status were not significantly associated with longer OS and PFS according to *EGFR* mutation status. Drug-sensitive *EGFR* mutations were significantly associated with longer OS and PFS according to sex or smoking status. Our results indicated that drug-sensitive *EGFR* mutations were the only independent factor for longer survival of patients treated with gefitinib, suggesting that patient selection based on *EGFR* mutation status for gefitinib therapy will lead to a better outcome for patients with lung adenocarcinoma. (*Cancer Sci* 2008; 99: 303–308)

Epidermal growth factor receptor (*EGFR*) is a receptor tyrosine kinase that is highly expressed in cancer cells.[1] Gefitinib and erlotinib are reversible EGFR tyrosine kinase inhibitors (EGFR-TKI) used for the treatment of non-small-cell lung cancer (NSCLC) patients.[2] Previous studies have focused on identifying factors that are useful indicators when selecting candidate patients for gefitinib treatment. An adenocarcinoma histology, female sex, a never-smoking status, and an East Asian ethnicity are clinicopathological factors that are associated with sensitivity to gefitinib and these factors can be indicators for gefitinib therapy.[2,3]

Mutations in *EGFR* have been reported, particularly in NSCLC.[1,4,5] *EGFR* mutations are frequently located in exon19 and exon21 of the *EGFR* tyrosine kinase domain and play an oncogenic role in adenocarcinoma.[6] Previous studies have shown that a positive *EGFR* mutation status is significantly related to adenocarcinoma histology, female sex, a never-smoking status, and an East Asian ethnicity.[4,5,7–10] Of clinical interest, approximately 80% of patients with the *EGFR* mutation, especially exon19 deletions and the L858R mutation at exon21, are associated with sensitivity to reversible EGFR-TKIs. Positive *EGFR* mutation status is considered to predict a favorable clinical outcome for NSCLC patients treated with gefitinib, especially those from East Asia.[11–13] However, Cappuzzo *et al.* reported that *EGFR* and *HER2* gene copy numbers, but not *EGFR* mutations, were predictors for good clinical outcome of reversible EGFR-TKI therapy, especially in white populations,[14,15] but these facts have not been confirmed in patients of East Asia origin.[16–18]

Although *EGFR* mutations could be a predictor for good clinical outcome of reversible EGFR-TKI therapy, the impact of *EGFR* mutation, sex, and smoking status on patient prognosis of gefitinib treatment is still an issue of interest. Initially, female sex, never-smoking status, and adenocarcinoma histology were identified as predictive factors for having *EGFR* mutations,[4,5,7,10] therefore, in identifying predictive factors on survival after gefitinib therapy, *EGFR* mutation strongly confounds with sex, smoking status, and histology. In addition, recent reviews suggested female sex and never-smoking status as prognostic factors of NSCLCs.[19,20] Some studies have suggested that smoking status can be a useful indicator when selecting candidate patients for gefitinib treatment.[3,21] Thus, it is important to discriminate the survival impact of *EGFR* mutation against those of clinicopathological factors in patients treated with gefitinib.

To address these matters, we combined data from three institutions in Japan to establish a large-scale database and evaluated the factors that were related to clinical outcome of lung adenocarcinoma patients treated with gefitinib.

Materials and Methods

Patients and gefitinib treatment. We collected the data (clinical records and *EGFR* mutation status) of 408 NSCLC patients treated with gefitinib from the National Cancer Center Hospital (Tokyo, Japan; 207 patients), Aichi Cancer Center Hospital

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Table 1. Patient characteristics and drug-sensitive epidermal growth factor receptor (*EGFR*) mutation in 408 non-small-cell lung cancer patients treated with gefitinib

Variables	No. of patients	*EGFR* mutation (%)
Age		
<64 years	177	84 (47.5)
≥64 years	185	85 (45.9)
Sex		
Female	162	101 (62.3)
Male	200	68 (34.0)
Smoking history		
Never	170	104 (61.2)
Ever	192	65 (33.9)
0 < PY < 20	40	28 (65.0)
20 ≤ PY	152	37 (25.7)
Disease stage		
Rec	220	120 (54.5)
Adv	142	49 (34.5)
Institution		
NCC	189	83 (43.9)
ACC	91	54 (59.3)
Okayama	82	32 (39.0)

ACC, Aichi Cancer Center Hospital, Nagoya, Japan; Adv, advanced disease; *EGFR* mutation, drug-sensitive *EGFR* mutation; NCC, National Cancer Center Hospital, Tokyo, Japan; Okayama, Okayama University Hospital, Okayama, Japan; PY, pack-years; Rec, recurrent disease;

(Nagoya, Japan; 103 patients), and Okayama University Hospital (Okayama, Japan) with NHO Sanyo National Hospital (Yamaguchi, Japan; 98 patients). These cases were independently analyzed in each institution and 364 of them had been previously reported.[11,13,16,22] An additional 44 patients from Aichi Cancer Center Hospital were included in the present study. Because the majority of our patients had an adenocarcinoma histology (362 [88.7%] of 408 patients), we limited our analyses to patients with adenocarcinoma histology. Total patients consisted of: 162 (44.8%) female and 200 (55.2%) male; 170 (47.0%) never-smokers and 192 (53.0%) ever-smokers; and 220 (60.8%) recurrent disease and 142 (39.2%) advanced disease. Never-smokers were those with lifetime exposure of 100 cigarettes or less, ever-smokers were those with lifetime exposure of more than 100 cigarettes. Ever-smokers were classified into two categories based on the degree of cumulative smoking dose: 40 patients with light- to moderate-smoking status (0 < pack-years [PY] < 20), and 152 patients with heavy-smoking status (20 ≤ PY).[23] The details of the patient characteristics are shown in Table 1. All patients had initiated gefitinib treatment (250 mg/day) between November 2000 and August 2006 in each institution. In the majority of patients, gefitinib treatment was continued as long as possible until disease progression, development of unacceptable toxicity, or patients' refusal to continue treatment. Institutional review board permission and informed consent were obtained for all patients at each institution. The permission numbers of the institutional review boards related to the present study at each institution are as follows: G12-06 for National Cancer Center Hospital; No.19-12 for Aichi Cancer Center Hospital; No.1 and No.48 for Okayama University Hospital; and No.0306 for NHO Sanyo National Hospital.

Detection of *EGFR* mutations. The direct sequence with genomic DNA was used for all specimens analyzed in Okayama University Hospital and 66 specimens in National Cancer Center Hospital.[9,13] High-resolution melting analysis was used to detect exon19 deletion and mutations in exon21 in 141 specimens from National Cancer Center Hospital.[22,24] The RNA-based analysis for 59 specimens using one-step reverse transcription–polymerase chain reaction for exons 18–21 and for 44 specimens using polymerase chain reaction-based assay for exons 19 and 21 was carried out at Aichi Cancer Center Hospital.[8,25] Limited to adenocarcinoma, our 362 patients consisted of 189 patients from National Cancer Center Hospital, 91 from Aichi Cancer Center Hospital, and 82 from Okayama University Hospital. For this study, drug-sensitive *EGFR* mutations, exon19 deletion or insertion, and exon21 L858R or L861Q mutation were considered as cases with the drug-sensitive *EGFR* mutation, and others were considered as the *EGFR* wild-type.

Statistical analyses. In this study, the impact of *EGFR* mutation status on survival after gefitinib treatment was examined considering smoking and sex status as confounder or effect modifier. For this purpose, we chose overall survival (OS) and progression-free survival (PFS) as endpoints. The OS and PFS were calculated from start of gefitinib treatment until the date of death or the last follow-up for OS and until confirmed disease progression or death for PFS. The Kaplan–Meier method was applied to estimate OS and PFS. Differences in OS and PFS among groups were assessed by log–rank test. Univariable and multivariable Cox proportional hazard models combined with stratification were applied to further evaluate significance of examined factors on OS and PFS. In the multivariate model, confounders considered were age as a continuous variable, disease stage (recurrence or advanced), and institutions. Smoking status was examined in the models using cumulative exposure to smoking (PY). When necessary, χ^2-tests were applied to examine differences in categorical factors across groups. The multivariate logistic regression model was used to identify baseline factors that might independently predict for the presence of drug-sensitive *EGFR* mutations. Statistical analyses were carried out using StatView 5.0 Program for Windows (SAS Institute, Cary, NC). All statistical tests were two-sided and *P*-values less than 0.05 were defined as being statistically significant.

Results

***EGFR* mutation and clinicopathological factors.** Drug-sensitive *EGFR* mutations were present in 169 (46.7%) of 362 patients and were comprised of 95 mutations in exon19 (93 deletions and two insertions) and 74 mutations (73 L858R and one L861Q) in exon21. The relationships between the *EGFR* mutation status and clinical factors are shown in Table 1. Multivariate analysis indicated that sex ($P = 0.045$), smoking status ($P < 0.001$), and disease status ($P = 0.002$), but not institutional difference, were significantly related to drug-sensitive *EGFR* mutations.

Impact of *EGFR*, sex, and smoking dose on survival of patient. Two hundred and sixty-three patients died, and the median follow-up period for the 99 survivors was 26.5 months (range, 1.6–58.5 months). The median survival time or the median progression-free survival time of all the patients was 15.3 and 4.0 months, respectively. The survival of patients was examined according to their *EGFR* mutation status, sex status, and smoking dose (PY = 0/0 < PY < 20/20 ≤ PY). The OS and PFS periods of patients with the drug-sensitive *EGFR* mutation and female sex status were significantly longer than those with *EGFR* wild-type (OS: $P < 0.001$; PFS: $P < 0.001$) and male sex status (OS, $P = 0.017$; PFS, $P = 0.002$) (Table 2). The OS and PFS periods of the patients were significantly related to the degree of smoking in a dose-dependent manner (OS, trend $P = 0.002$; PFS, trend $P < 0.001$) (Table 2). To further evaluate cumulative exposure to smoking, we explored two other thresholds, PY30 and PY40, similar to the PY20 analysis. As all of the analyses showed consistent trends (Supplementary Materials), we chose the commonly accepted threshold PY20 in the further analyses.[23] Multivariate analysis including *EGFR*, sex, and smoking status

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Table 2. Univariate and multivariate Cox proportional hazard models to further evaluate significance of drug-sensitive epidermal growth factor receptor (*EGFR*) mutation, sex, and smoking status on overall survival (OS) and progression-free survival (PFS) in 408 non-small-cell lung cancer patients treated with gefitinib

Co-variates	OS				PFS			
	Univariate	*P*	Multivariate†	*P*	Univariate	*P*	Multivariate†	*P*
EGFR (Mut *versus* Wt)	0.43 (0.33–0.55)	<0.001	0.48 (0.36–0.63)	<0.001	0.28 (0.22–0.35)	<0.001	0.29 (0.22–0.37)	<0.001
Sex (male *versus* female)	0.74 (0.58–0.95)	0.017	1.00 (0.71–1.40)	0.980	0.74 (0.58–0.95)	0.002	1.06 (0.78–1.45)	0.710
Smoking								
Ever PY ≥ 20	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–
Ever PY < 20	0.67 (0.47–1.00)	0.050	0.95 (0.62–1.46)	0.810	0.70 (0.49–1.02)	0.061	1.09 (0.74–1.61)	0.660
Never	0.64 (0.50–0.83)	<0.001	0.83 (0.58–1.20)	0.330	0.57 (0.45–0.72)	<0.001	0.76 (0.54–1.07)	0.120
	Trend $P = 0.002$		Trend $P = 0.61$		Trend $P < 0.001$		Trend $P = 0.15$	

†Adjusted for age, disease stage, institution, and three variables of interest (*EGFR*, sex, and smoking status). –, not applicable; Mut, drug-sensitive *EGFR* mutation; PY, pack-years; Wt, *EGFR* wild-type.

Table 3. Impact of sex and smoking according to drug-sensitive epidermal growth factor receptor (*EGFR*) mutation status on overall survival (OS) and progression-free survival (PFS) in 408 non-small-cell lung cancer patients treated with gefitinib

Co-variates	OS				PFS			
	Univariate	*P*	Multivariate†	*P*	Univariate	*P*	Multivariate†	*P*
EGFR (Mut)								
Sex (male *versus* female)	1.05 (0.70–1.56)	0.83	1.18 (0.71–1.96)	0.52	1.05 (0.70–1.56)	0.83	1.27 (0.81–1.98)	0.30
Smoking								
Ever PY ≥ 20	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–
Ever PY < 20	1.03 (0.55–1.92)	0.94	0.97 (0.49–1.89)	0.92	1.16 (0.67–2.00)	0.59	1.17 (0.65–2.10)	0.60
Never	0.94 (0.58–1.54)	0.82	0.85 (0.46–1.58)	0.61	0.84 (0.56–1.25)	0.38	0.79 (0.46–1.35)	0.39
	Trend $P = 0.94$		Trend $P = 0.84$		Trend $P = 0.34$		Trend $P = 0.32$	
EGFR (Wt)								
Sex (male *versus* female)	0.82 (0.59–1.14)	0.23	0.94 (0.60–1.47)	0.78	0.86 (0.63–1.17)	0.33	0.87 (0.56–1.35)	0.53
Smoking								
Ever PY ≥ 20	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–
Ever PY < 20	0.85 (0.46–1.59)	0.62	0.95 (0.54–1.69)	0.86	1.20 (0.69–2.10)	0.52	1.31 (0.74–2.30)	0.35
Never	0.79 (0.57–1.11)	0.17	0.93 (0.59–1.47)	0.76	0.92 (0.68–1.26)	0.61	0.98 (0.62–1.55)	0.93
	Trend $P = 0.38$		Trend $P = 0.95$		Trend $P = 0.66$		Trend $P = 0.63$	

†Adjusted for age, disease stage, institution, and two variables of interest (sex and smoking status). –, not applicable; Mut, drug-sensitive *EGFR* mutation; PY, pack-years; Wt, *EGFR* wild-type.

showed that only drug-sensitive *EGFR* mutations were significantly associated with longer OS and PFS (OS: hazard ratio = 0.48, 95% CI = 0.36–0.63, $P < 0.001$) (PFS: hazard ratio = 0.29, 95% CI = 0.22–0.37, $P < 0.001$) (Table 2).

Next, we separated the impact of *EGFR*, sex, and smoking status on survival of patients treated with gefitinib to further understanding of these factors. For this purpose, we carried out two analyses focused on the following issues: the impact of sex and smoking status, according to *EGFR* mutation status, on survival (Table 3); and the impact of *EGFR* mutation status, according to sex and smoking status, on survival (Table 4). Table 3 shows that sex and smoking status were not significantly associated with longer OS and PFS among patients with the same *EGFR* mutation status (drug-sensitive *EGFR* mutation/ *EGFR* wild-type) using univariate and multivariate analyses. Kaplan–Meier plots stratified according to sex and *EGFR* mutation status and according to smoking dose and *EGFR* mutation status are shown in Fig. 1. Regarding the impact of *EGFR* mutation status, according to sex and smoking status, on survival, drug-sensitive *EGFR* mutations were significantly related to longer OS and PFS among groups of the same sex and smoking status, as shown in Table 4.

Taken together, our results clearly indicate that drug-sensitive *EGFR* mutations are the only independent factor for favorable prognosis of patients treated with gefitinib.

Discussion

Previously, we independently reported a relationship between the positive *EGFR* mutation status and clinical benefit in Japanese patients treated with gefitinib.[11,13,16,22] Analyses of a large-scale database might not only be useful to confirm or explore factors for the favorable clinical outcome with gefitinib, but also to improve our understanding of the impact of various factors on gefitinib treatment for patients. For this purpose, we combined our data and re-analyzed the factors that were assumed to affect clinical outcomes among gefitinib-treated patients with lung adenocarcinoma. Our study indicated that drug-sensitive *EGFR* mutation status was a significant factor of longer survival among gefitinib-treated patients, although sex and smoking status were significantly associated with favorable prognosis in univariate analyses. Indeed, Han *et al.* also showed the advantage of *EGFR* mutations for gefitinib effect compared with clinical factors.[26] As a point of discussion, it has been controversial whether the *EGFR* mutation is a good prognostic factor of NSCLC or not.[27–29] One of the reasons for the discrepancy might be that *EGFR* status (wild- or mutant-type) might influence not only the natural prognosis (untreated) but also the outcome after treatment with some chemotherapeutic drugs.[30] Previous studies did not precisely analyze different kinds of chemotherapeutic drugs to estimate the prognosis of

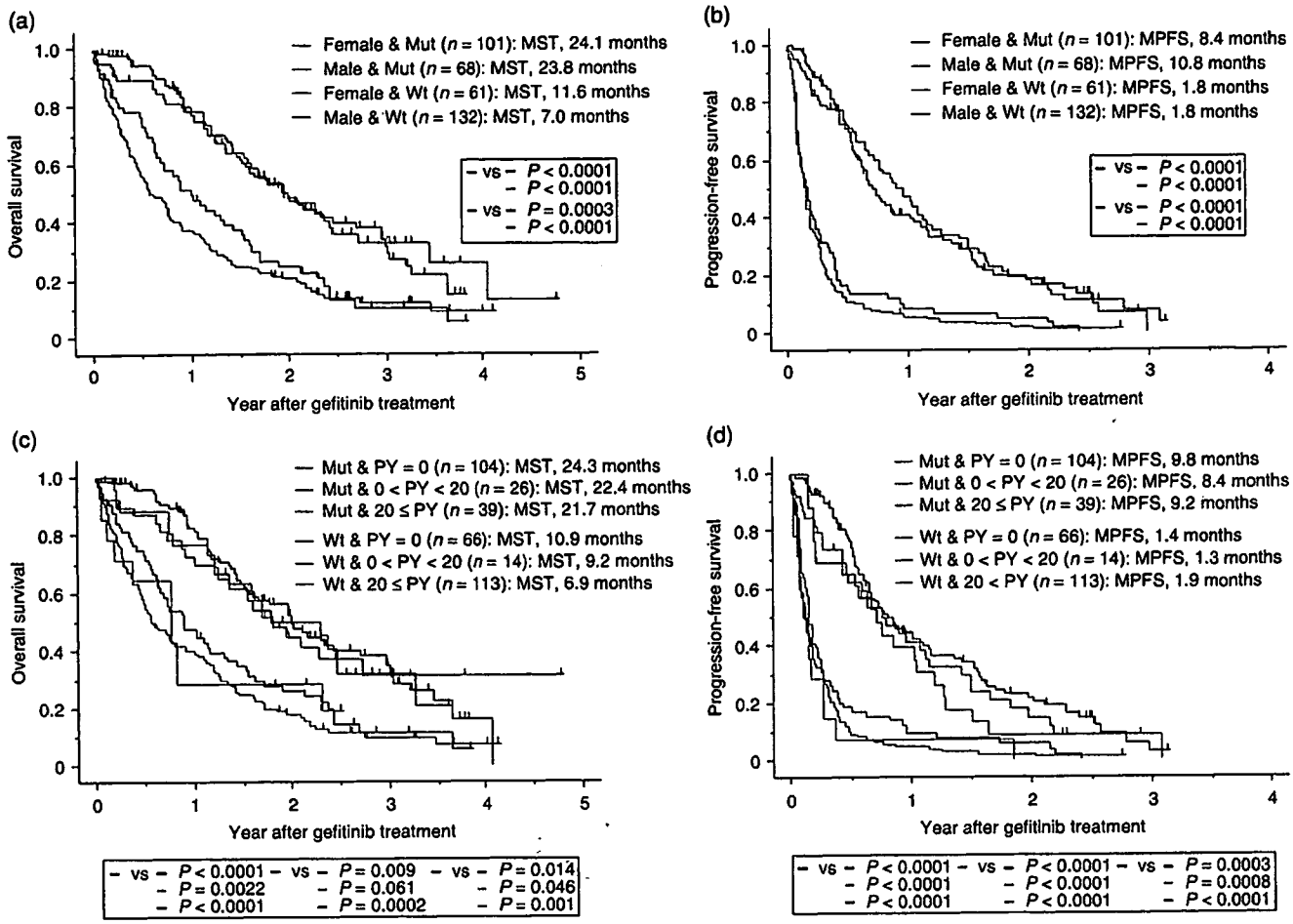

Fig. 1. Kaplan–Meier plot of survival times in 408 non-small-cell lung cancer patients treated with gefitinib. (a) Overall survival of patients classified into four groups according to epidermal growth factor receptor (*EGFR*) mutation and sex status. (b) Progression-free survival of patients classified into four groups according to *EGFR* mutation and sex status. (c) Overall survival of patients classified into six groups according to *EGFR* mutation and smoking dose status. (d) Progression-free survival of patients classified into six groups according to *EGFR* mutation and smoking dose status. Mut, drug-sensitive *EGFR* mutation; Wt, *EGFR* wild-type; PY, pack-years; MST, median survival time; MPFS, median progression-free survival time. *P*-values were calculated using the log-rank test.

Table 4. Impact of drug-sensitive epidermal growth factor receptor (*EGFR*) mutation status according to sex and smoking status on overall survival (OS) and progression-free survival (PFS) in 408 non-small-cell lung cancer patients treated with gefitinib

Co-variates	OS				PFS			
	Univariate	*P*	Multivariate†	*P*	Univariate	*P*	Multivariate†	*P*
Sex								
Male	0.43 (0.30–0.61)	<0.001	0.47 (0.32–0.69)	<0.001	0.27 (0.20–0.38)	<0.001	0.28 (0.18–0.39)	<0.001
Female	0.47 (0.32–0.68)	<0.001	0.50 (0.33–0.74)	<0.001	0.30 (0.21–0.42)	<0.001	0.30 (0.20–0.46)	<0.001
Smoking								
Ever PY ≥ 20	0.43 (0.27–0.68)	<0.001	0.44 (0.28–0.71)	<0.001	0.30(0.20–0.46)	<0.001	0.29 (0.19–0.45)	<0.001
Ever PY < 20	0.47 (0.22–1.01)	0.052	0.41 (0.15–1.01)	0.079	0.32 (0.16–0.65)	0.002	0.24 (0.09–0.61)	0.003
Never	0.45 (0.31–0.65)	<0.001	0.48 (0.32–0.71)	<0.001	0.28 (0.20–0.39)	<0.001	0.30 (0.20–0.43)	<0.001

†Adjusted for age, disease stage, and institution. PY, pack-years.

EGFR mutated/wild-type tumors. Further studies are necessary for this issue.

Our results, along with previous findings of the basic research, supported that the drug-sensitive *EGFR* mutation, but not sex or smoking status, was basically an appropriate target of gefitinib. Because of their biological features influencing the effect of gefitinib, drug-sensitive *EGFR* mutant tumors should be distinguished from *EGFR* wild-type tumors. Indeed, the IRESSA Survival Evaluation in Lung Cancer study, in which patients were not selected based on the *EGFR* mutation, did not indicate the significant survival benefit of gefitinib treatment.[31] Considering these facts, prospective studies to evaluate the

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benefit of reversible EGFR-TKIs should be designed based on *EGFR* mutation status, but not on clinical factors like smoking status. In this point, the selection criteria of the First Line IRESSA Versus Carboplatin/Paclitaxel in Asia trial, which includes smoking status but not *EGFR* mutations, is not essential to investigate the effect of gefitinib, although smoking status data are accessible. By contrast, the West Japan Thoracic Oncology Group (WJOG) has organized a randomized phase III trial of IRESSA versus cisplatin/docetaxel for patients with drug-sensitive EGFR mutations. In clinical practice, patients with *EGFR* mutations would be strongly recommended for gefitinib treatment keeping in mind that a positive *EGFR* mutation is not a perfect factor for favorable clinical outcomes.

In conclusion, our study showed that drug-sensitive *EGFR* mutation, but not sex or smoking status, is the superior factor for likely maximizing the therapeutic effect of gefitinib, indicating that drug-sensitive *EGFR* mutations, regardless of sex and smoking status, were an appropriate determinant for gefitinib treatment. Patient selection based on *EGFR* mutation status for reversible EGFR-TKI treatment will lead to better understanding of gefitinib therapy, as well as a better clinical outcome for patients with lung adenocarcinoma.

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Supplementary Materials

The following supplementary material is available for this article:

Table S1. Impact of different cumulative smoking dose on survival

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Large Cell Neuroendocrine Carcinoma of the Mediastinum with α-Fetoprotein Production

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Large cell neuroendocrine carcinoma (LCNEC) is a relatively new category of pulmonary neuroendocrine tumor. Although it was first detected in the lung, LCNEC has since been found in a variety of extrapulmonary sites. We now describe a patient who was diagnosed with LCNEC originating from the mediastinum, an extremely rare disorder. An increased serum concentration of α-fetoprotein (AFP) in the patient was reduced by chemotherapy in association with tumor shrinkage. Furthermore, the tumor was confirmed immunohistochemically to produce AFP. To our knowledge, this is the first report of a LCNEC that produces AFP.

Key Words: Large cell neuroendocrine carcinoma, α-Fetoprotein, Mediastinal tumor.

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Large cell neuroendocrine carcinoma (LCNEC) is a high-grade neuroendocrine tumor that was first detected in the lung by Travis et al.[1] The prognosis of individuals with LCNEC has been reported to be poor, with a 5-year survival rate similar to that for small cell carcinoma.[2–4] Although originally found in the lung, LCNEC has since been described in a variety of extrapulmonary locations.[5–7] Among these locations, mediastinal LCNEC is extremely rare, with only a few cases having been reported.[8,9] We now report the first case of mediastinal LCNEC with α-fetoprotein (AFP) production.

CASE REPORT

A previously healthy 35-year-old Japanese man was found to have an abnormal mass in his right mediastinum on a chest radiograph during a health checkup. The patient's general condition was fair, and symptoms such as chest pain, weight loss, or fever were not noted. He was a current smoker, having smoked 20 cigarettes a day for 15 years. Computed tomography imaging of the chest revealed a 65 × 50 mm mass in the middle mediastinum (Figure 1*A*). Serum laboratory data were within normal limits. A bronchoscopic examination revealed a compression against the outside of the trachea. No other organs appeared to be affected on extensive examination. Subsequent evaluation for serum tumor markers revealed an increased level of AFP. Other examined markers, including β-human chorionic gonadotropin, carcinoembryonic antigen, and CA19-9, were within normal limits. Thoracoscopic examination revealed that the tumor was not invading into the adjacent lung. On the basis of these findings, we considered the tumor to have originated from the middle mediastinum. A biopsy revealed poorly differentiated carcinoma with neuroendocrine features. Thymic neuroendocrine carcinoma is exclusively located in the anterior-superior mediastinum.[1] Given the tumor's location, the increase in the serum concentration of AFP, and the patient's young age, the diagnosis of embryonal carcinoma was initially favored over purely neuroendocrine neoplasm. The patient received neoadjuvant chemotherapy with bleomycin (30 mg/body) on days 2, 9, and 16, etoposide (100 mg/m^2) on days 1 to 5, and cisplatin (20 mg/m^2) on days 1 to 5. Treatment cycles were repeated every 21 days for 4 cycles. The serum AFP level had decreased to within normal limits in association with shrinkage of the tumor by the end of the third cycle of chemotherapy (Figure 1*B*, *E*). However, the AFP concentration started to increase thereafter, and progression of the tumor was confirmed after the fourth cycle of chemotherapy (Figure 1*C*, *E*). The patient then received second-line chemotherapy with cisplatin (80 mg/m^2) on day 1 and paclitaxel (200 mg/m^2) on day 1 every 21 days for three cycles before surgery. The serum AFP level again decreased in association with tumor shrinkage (Figure 1*D*, *E*). Eight months after initial detection of the tumor, the patient underwent a tumorectomy combined with right upper lobectomy and tracheoplasty, given that the tumor was found to invade the adjacent right upper lobe and trachea at the time of surgery. Histopathologic examination of the surgical specimen revealed a solid tumor nest with massive necrosis. The tumor was relatively homogeneous throughout the resection, showing sheets of cells with a high nucleus-to-cytoplasm ratio. High-power magnification of the tumor revealed that the tumor cells manifested marked neu-

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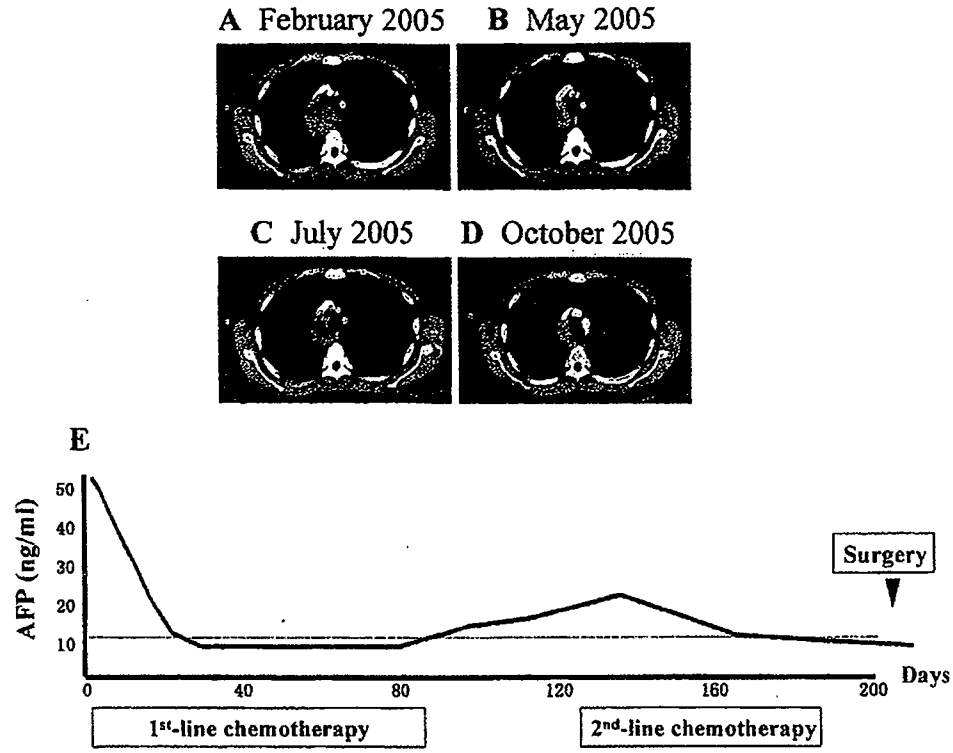

FIGURE 1. Chest computed tomography (CT) findings and serum AFP levels in the patient. *A–D*, Chest CT findings. A mass in the middle mediastinum was initially detected (*A*). The tumor had shrunk after three cycles of neoadjuvant chemotherapy (*B*), but its progression had resumed after the fourth cycle (*C*). The tumor shrank again in response to second-line chemotherapy (*D*). *E*, Time course of the serum concentration of AFP. The AFP level was initially increased, it decreased to within normal limits (dotted line) in association with tumor shrinkage during first-line chemotherapy, but it started to increase again after the third cycle. The serum AFP level again decreased in association with tumor shrinkage during second-line chemotherapy.

roendocrine features, such as frequent rosette structures and trabecular arrangements, nuclear moldings, and prominent mitoses (Figure 2*A*, *B*). The tumor cells also had abundant nucleoli. Immunohistochemical analysis showed the tumor cells to be diffusely positive for CK7 and neuroendocrine markers including CD56, chromogranin A (Figure 2*C*), and synaptophysin as well as negative for CD5, CD30, human chorionic gonadotropin, placental alkaline phosphatase, hepatocyte antigen, and thyroid transcription factor-1. No regions of the specimen showed features of a germ cell tumor or hepatoid carcinoma. On the basis of the morphology and staining characteristics of the tumor, a pathologic diagnosis of LCNEC was made. A small number of tumor cells showed subtle but unequivocal positive staining for AFP (Figure 2*D*). Thoracic radiotherapy was not able to be given because the patient suffered from thoracic empyema after surgery. Despite intensive chemotherapy, he died of extensive recurrence of carcinoma 4 months after the surgery.

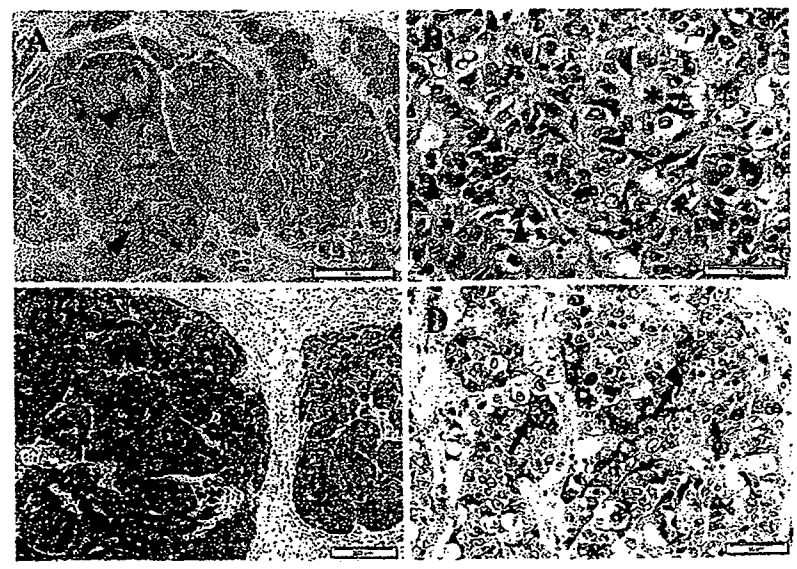

FIGURE 2. Histology and immunohistochemical analysis of the tumor specimen obtained at surgery. *A*, Hematoxylin-eosin staining revealed solid tumor nests with areas of necrosis (arrow heads). Note the homogeneous appearance of the tumor. *B*, High-power magnification of the tumor stained as in (*A*), showing numerous rosettes (asterisk), abundant cytoplasm, chromatin clearing with occasionally prominent nucleoli, nuclear molding (arrows), and frequent mitosis (arrow heads). *C*, Immunohistochemical staining for chromogranin A revealed diffuse and intense cytoplasmic staining. *D*, Immunohistochemical staining for AFP, showing a focus of tumor cells positive for AFP (arrows). Scale bars: 1 mm, 50 μ.

DISCUSSION

LCNEC is a relatively new category of pulmonary neuroendocrine tumor, with affected individuals reported to have a prognosis intermediate between those with atypical carcinoid lung cancer and those with small cell lung cancer.[10] Recent clinical studies indicate a 5-year survival rate of 27 to 67% even if patients are at pathologic stage I.[2–4] Since its original detection in the lung, LCNEC has been found in a variety of extrapulmonary locations including gastrointestinal sites and the uterine cervix.[5–7] The present case was identified as LCNEC originating in the mediastinum. Given the age of the patient and the tumor location, a diagnosis of embryonal carcinoma was initially considered, but no morphologic or immunohistochemical features indicative of embryonal carcinoma were found on extensive pathologic analysis of the surgical specimen. Primary mediastinal LCNEC is an extremely rare disorder and has been described in only a few case reports to date.[8–9]

In the present case, the increased serum AFP level decreased in association with tumor shrinkage in response to chemotherapy, and the tumor was confirmed immunohistochemically to produce AFP. AFP is the main component of fetal serum in mammals. It is synthesized by visceral endoderm of the yolk sac and fetal liver, but expression of the *AFP* gene is greatly reduced at the time of birth. AFP-producing carcinoma has been recognized for decades and reported in various locations including the lung and mediastinum.[11] In contrast to the present case, however, most cancers that produce AFP show morphologic features similar to hepatocellular carcinoma. With regard to neuroendocrine tumors, some case reports indicate that small cell carcinoma can also produce AFP.[12,13] As far as we are aware, however, the present case is the first reported example of LCNEC producing AFP. Given that the concept of LCNEC is relatively new, this may not be that surprising, and previous reports of small cell carcinoma may actually have been diagnosed as LCNEC today. Our case raises the possibility that the origin of mediastinal neuroendocrine tumors including LCNEC may be mediastinal primordial germ cells. Examination of germ cell tumor markers in neuroendocrine tumors may shed light on this matter.

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Matuzumab and cetuximab activate the epidermal growth factor receptor but fail to trigger downstream signaling by Akt or Erk

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Molecular inhibition of the epidermal growth factor receptor (EGFR) is a promising anticancer strategy, and monoclonal antibodies (mAbs) to EGFR are undergoing extensive evaluation in preclinical and clinical trials. However, the effects of anti-EGFR mAbs on EGFR signaling have remained unclear. We have now examined the effects of 2 anti-EGFR mAbs, matuzumab (EMD72000) and cetuximab (Erbitux), both of which are currently under assessment for treatment of various cancers, on EGFR signal transduction and cell survival in nonsmall cell lung cancer cell lines. Similar to EGF, matuzumab and cetuximab each induced phosphorylation of EGFR at several tyrosine phosphorylation sites as a result of receptor dimerization and activation of the receptor tyrosine kinase. In contrast to the effects of EGF, however, EGFR activation induced by these antibodies was not accompanied by receptor turnover or by activation of downstream signaling pathways that are mediated by Akt and Erk and are important for regulation of cell proliferation and survival. In addition, clonogenic survival assays revealed that matuzumab and cetuximab reduced the survival rate of H292 cells, in which they also inhibited the EGF-induced activation of Akt and Erk. Although we have examined only a few cell lines, our results indicate that the antitumor effects of matuzumab and cetuximab depend on inhibition of EGFR downstream signaling mediated by Akt or Erk rather than on inhibition of EGFR itself.

Key words: EGF receptor; signal transduction; matuzumab; cetuximab; nonsmall cell lung cancer

The epidermal growth factor receptor (EGFR, also known as ErbB1), a member of the ErbB family of receptor tyrosine kinases, is a 170-kDa plasma membrane glycoprotein composed of an extracellular ligand binding domain, a transmembrane region and an intracellular tyrosine kinase domain with a regulatory COOH-terminal segment.[1] Binding of ligand to EGFR induces receptor dimerization, activation of the receptor kinase and autophosphorylation of specific tyrosine residues within the COOH-terminal region of the protein.[1] These events trigger intracellular signaling pathways that promote cell proliferation and survival.[2,3]

EGFR is frequently overexpressed in many types of human malignancy, with the extent of overexpression being negatively correlated with prognosis.[4,5] Recognition of the role of EGFR in carcinogenesis has prompted the development of EGFR-targeted therapies that include both small-molecule tyrosine kinase inhibitors (TKIs) that target the intracellular tyrosine kinase domain and monoclonal antibodies (mAbs) that target the extracellular domain.[6–8] Among EGFR-TKIs, gefitinib and erlotinib have been extensively evaluated in nonsmall cell lung cancer (NSCLC), and sensitivity to these drugs has been correlated with the presence of somatic mutations in the EGFR kinase domain or with EGFR gene (*EGFR*) amplification.[9–16] Among anti-EGFR mAbs, cetuximab (Erbitux), a chimeric mouse-human antibody of the immunoglobulin (Ig) G1 subclass, has proved efficacious in the treatment of irinotecan-refractory colon cancer[17] and was recently approved by the U.S. Food and Drug Administration for the treatment of patients with head and neck squamous cell carcinoma.[18] Several clinical studies of anti-EGFR mAbs such as matuzumab (EMD72000, humanized IgG1) and cetuximab are ongoing for other types of cancer including NSCLC.[19–24] Anti-EGFR mAbs bind to the extracellular ligand binding domain of the receptor and are thereby thought to block ligand binding.[18,25] The antitumor effects of these mAbs are thus thought to be attributable to inhibition of EGFR signaling as well as to other mechanisms such as antibody-dependent cellular cytotoxicity.[18,26] However, the detailed effects of anti-EGFR mAbs on EGFR signaling have remained unclear.[27–30]

We have now examined in detail the effects on EGFR signal transduction of 2 anti-EGFR mAbs, matuzumab and cetuximab, both of which are used clinically, to provide insight into the mechanisms of their antitumor effects.

Material and methods

Cell culture and reagents

The human NSCLC cell lines NCI-H292 (H292), NCI-H460 (H460) and Ma-1 were obtained as previously described[31] and were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Matuzumab and cetuximab were kindly provided by Merck KGaA (Darmstadt, Germany) and Bristol Myers (New York, NY), respectively; gefitinib was obtained from AstraZeneca (Macclesfield, UK); and trastuzumab (Herceptin; Genentech, South San Francisco, CA) was obtained from Chugai (Tokyo, Japan). Neutralizing antibodies to EGFR (clone LA1) were obtained from Upstate Biotechnology (Lake Placid, NY).

Immunoblot analysis

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites, the membrane was incubated consecutively with primary and secondary antibodies, and immune complexes were detected with the use of enhanced chemiluminescence reagents, as described previously.[31] Primary antibodies to the specific intracellular phosphorylation sites of EGFR (pY845, pY1068 or pY1173), to Erk, to phospho-Akt and to Akt were obtained from Cell Signaling Technology (Beverly, MA); those to the extracellular domain of EGFR (clone 31G7) were from Zymed (South San Francisco, CA); those to the intracellular domain of EGFR (EGFR 1005) and to phospho-Erk were from Santa Cruz Biotechnology (Santa Cruz, CA); and those to β-actin (loading control) were from Sigma. Horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG were obtained from Amersham Biosciences (Little Chalfont, UK).

Chemical cross-linking assay

Cells were incubated first with 1 mM bis(sulfosuccinimidyl) suberate (BS^3; Pierce, Rockford, IL) for 20 min at 4°C and then with

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; mAb, monoclonal antibody; NSCLC, nonsmall cell lung cancer; Ig, immunoglobulin; BS^3, bis(sulfosuccinimidyl) suberate; PE, R-phycoerythrin; PI3K, phosphoinositide 3-kinase.

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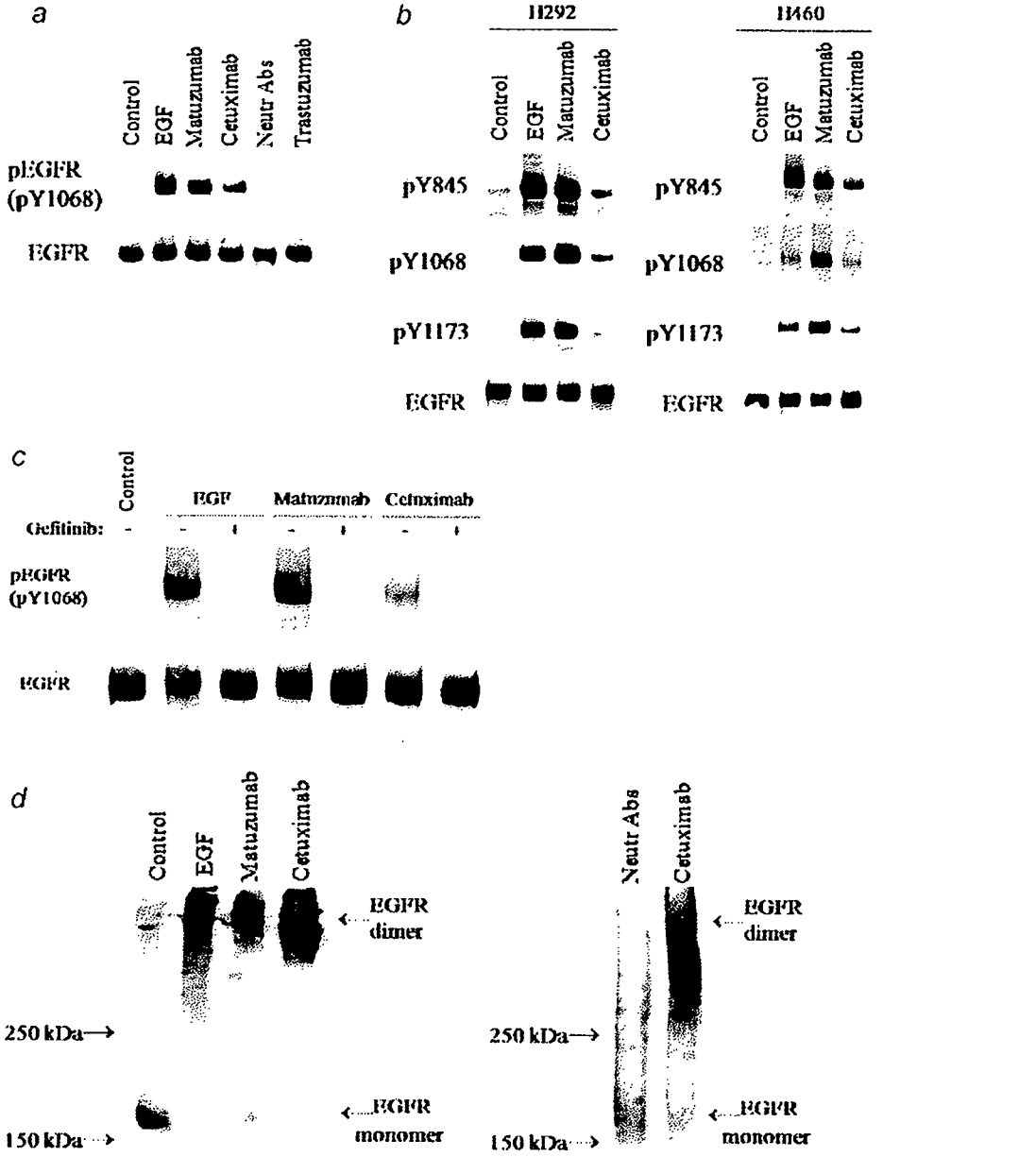

FIGURE 1 – EGFR phosphorylation induced by matuzumab or cetuximab as a result of receptor dimerization and activation of the receptor tyrosine kinase. (*a*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence (Control) or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM), trastuzumab (50 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR (pY1068) and to total EGFR (the extracellular domain). (*b*) H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y845-, Y1068- or Y1173-phosphorylated forms of EGFR and to total EGFR (the extracellular domain). (*c*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), EGF (100 ng/ml) or gefitinib (10 μM), as indicated. Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR and to total EGFR (the extracellular domain). (*d*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM) or EGF (100 ng/ml). The cells were then washed and exposed to the chemical cross-linker BS^3 after which cell lysates were subjected to immunoblot analysis with antibodies to EGFR (the intracellular domain). The positions of EGFR monomers and dimers as well as of molecular size standards are indicated.

250 mM glycine for 5 min at 4°C to terminate the cross-linking reaction, as described previously.[31] Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis on a 4% gel and subjected to immunoblot analysis with rabbit polyclonal antibodies to the intracellular domain of EGFR (EGFR 1005).

Immunofluorescence analysis

Cells were grown to 50% confluence in 2-well Lab-Tec Chamber Slides (Nunc, Naperville, IL), deprived of serum overnight, and then incubated with 200 nM matuzumab or EGF (100 ng/ml) for 4 hr at 37°C. They were fixed with 4% paraformaldehyde for

30 min at 4°C, permeabilized with 0.1% Triton X-100 for 10 min, and exposed to 5% nonfat dried milk for 1 hr at room temperature. The cells were stained with rabbit polyclonal antibodies to the intracellular domain of EGFR (EGFR 1005) for 1 hr at room temperature and then incubated for an additional 45 min with Alexa 488-labeled goat antibodies to rabbit IgG (Molecular Probes, Eugene, OR). Cell nuclei were counterstained for 5 min at room temperature with 4′,6-diamidino-2-phenylindole (Sigma) at 2 μg/ml. The chamber slides were mounted in fluorescence mounting medium (DakoCytomation, Hamburg, Germany), and fluorescence signals were visualized with a fluorescence microscope (Eclipse E800; Nikon, Kawasaki, Japan). Negative controls (secondary antibodies alone) did not yield any substantial background staining.

Flow cytometry

Cells were deprived of serum overnight and then incubated with 200 nM matuzumab or EGF (100 ng/ml) for 4 hr at 37°C. They were isolated by exposure to trypsin, and aliquots of ~1.0×10^6 cells were incubated for 2 hr at 4°C either with an R-phycoerythrin (PE)-conjugated mouse mAb to EGFR (clone EGFR.1; Becton Dickinson, San Jose, CA), which does not interfere with the binding of EGF to EGFR,[32] or with a PE-conjugated isotype-matched control mAb (Becton Dickinson). The cells were then examined by flow cytometry (FACScalibur, Becton Dickinson) to detect the intensity of EGFR staining at the cell surface.

Clonogenic assay

Cells were plated in triplicate at a density of 200 per 25-cm^2 flask containing 10 ml of medium and were cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated in medium alone for 7 days at 37°C, fixed with methanol:acetic acid (10:1, v/v), and stained with crystal violet. Colonies containing >50 cells were counted for calculation of the surviving fraction as follows: (mean number of colonies)/(number of inoculated cells × plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for untreated controls.

Results

Matuzumab and cetuximab induce EGFR phosphorylation in a manner dependent on the receptor tyrosine kinase activity

With the use of immunoblot analysis, we first examined the effects of the anti-EGFR mAbs matuzumab and cetuximab on EGFR phosphorylation in human NSCLC H292 cells, which express wild-type EGFR. Incubation of the serum-deprived cells for 15 min with EGF, matuzumab or cetuximab-induced phosphorylation of EGFR on tyrosine-1068 (Y1068), whereas treatment of the cells with neutralizing antibodies to EGFR or with trastuzumab, a mAb specific for HER2 (ErbB2), had no such effect (Fig. 1*a*). Furthermore, like EGF, matuzumab and cetuximab each induced phosphorylation of EGFR on Y845, Y1068 and Y1173 in H292 and H460 cells (Fig. 1*b*), the latter of which are also human NSCLC cells that express wild-type EGFR.

To determine whether the antibody-induced phosphorylation of EGFR requires the kinase activity of the receptor, we examined the effect of gefitinib, a specific EGFR-TKI. H292 cells were deprived of serum and then exposed to matuzumab, cetuximab or EGF for 15 min in the absence or presence of gefitinib. EGFR phosphorylation on Y1068 induced by EGF, matuzumab or cetuximab was completely blocked by gefitinib (Fig. 1*c*). These findings thus indicated that, like EGF, matuzumab and cetuximab each induce EGFR phosphorylation by activating the tyrosine kinase of the receptor.

Matuzumab and cetuximab induce EGFR dimerization

Ligand-dependent EGFR dimerization is responsible for activation of the receptor tyrosine kinase.[33,34] To examine whether matuzumab or cetuximab induces EGFR dimerization, we incubated serum-deprived H292 cells with the mAbs for 15 min and then exposed the cells to the chemical cross-linker BS^3. Immunoblot analysis of cell lysates with antibodies to the intracellular domain of EGFR revealed that matuzumab and cetuximab each induced EGFR dimerization to an extent similar to that observed with EGF, whereas only the monomeric form of the receptor was detected in control cells or in cells treated with neutralizing antibodies to EGFR (Fig. 1*d*). These data thus suggested that matuzumab and cetuximab activate EGFR through induction of receptor dimerization.

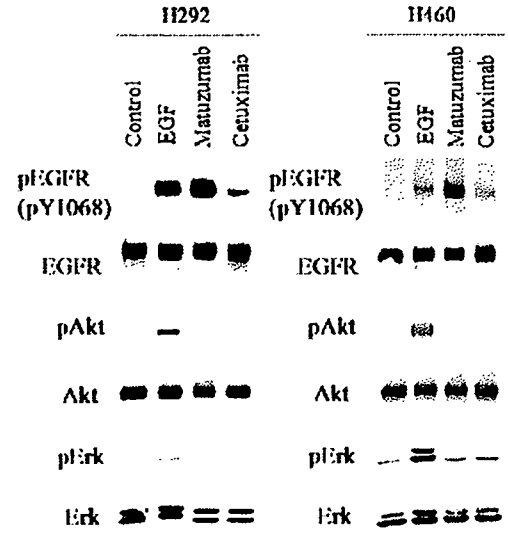

FIGURE 2 – Failure of matuzumab or cetuximab to activate Akt or Erk. H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR, to phosphorylated Akt and to phosphorylated Erk as well as with antibodies to total EGFR (the extracellular domain), Akt or Erk.

Matuzumab and cetuximab fail to induce signaling downstream of EGFR

EGFR signaling is transduced by 2 main pathways mediated by phosphoinositide 3-kinase (PI3K) and Akt and by Ras, Raf and Erk.[35,36] To determine whether EGFR phosphorylation induced by matuzumab or cetuximab is accompanied by activation of these pathways, we examined the levels of phosphorylated (activated) Akt and Erk in H292 and H460 cells treated with these antibodies for 15 min after serum deprivation. In contrast to the effects of EGF, neither matuzumab nor cetuximab induced the phosphorylation of Akt or Erk in H292 or H460 cells (Fig. 2). These results thus indicated that matuzumab and cetuximab induce EGFR activation but fail to activate the downstream Akt and Erk signaling pathways.

Matuzumab and cetuximab do not induce EGFR downregulation

Endocytic trafficking of EGFR is important for full activation of Erk and PI3K.[37] To examine further the defect in signaling downstream of EGFR activation by matuzumab or cetuximab, we determined the effects of these mAbs on receptor turnover. H292 or H460 cells were deprived of serum and then cultured with EGF, matuzumab or cetuximab for various times up to 24 hr, after which the levels of phosphorylated and total EGFR, Akt and Erk were measured. In both H292 and H460 cells treated with EGF, the amount of total EGFR decreased in a time-dependent manner

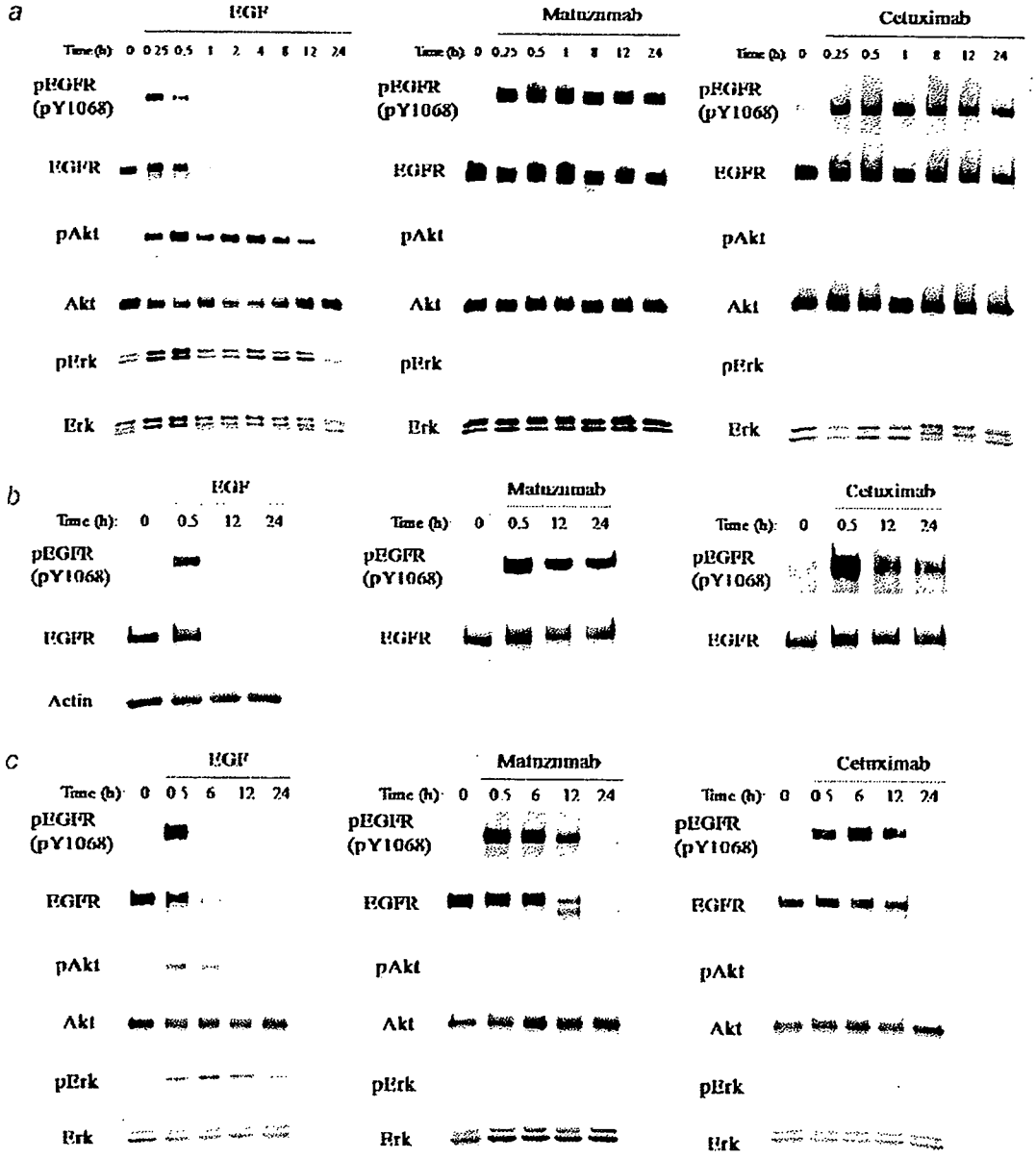

FIGURE 3 – Lack of EGFR turnover in cells treated with matuzumab or cetuximab. (*a*) H292 cells were deprived of serum overnight and then incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), respectively. Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the extracellular domain), Akt or Erk. (*b*) H292 cells deprived of serum overnight were incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR, to total EGFR (the intracellular domain) or to β-actin (loading control). (*c*) H460 cells deprived of serum overnight were incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), after which cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the intracellular domain), Akt or Erk. (*d*) H292 cells plated on chamber slides were deprived of serum overnight and then incubated for 4 hr in the absence or presence of matuzumab (200 nM) or EGF (100 ng/ml). The cells were fixed, permeabilized, and stained with antibodies to EGFR and Alexa 488-labeled secondary antibodies (green). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Fluorescence signals were visualized with a fluorescence microscope, and the merged images are shown. Scale bar, 20 μm. (*e*) H292 cells were deprived of serum overnight and then incubated for 4 hr in the absence or presence of matuzumab (200 nM) or EGF (100 ng/ml). The cells were stained with either a PE-conjugated mAb to EGFR (right peaks) or a PE-labeled isotype-matched mAb (left peaks) and analyzed by flow cytometry. Representative histograms of relative cell number versus PE fluorescence are shown.

(Figs. 3*a*–3*c*), an effect that has been shown to be the result of receptor internalization and degradation.[30,38] In parallel with this EGFR downregulation, the extent of EGF-induced tyrosine phosphorylation of EGFR also decreased and was virtually undetectable by 4–6 hr (Figs. 3*a*–3*c*). The phosphorylation of Akt and Erk induced by EGF persisted for at least 12 hr but had declined by 24 hr in both cell lines (Figs. 3*a* and 3*c*). In contrast, the levels of phosphorylated and total EGFR in H292 cells treated with

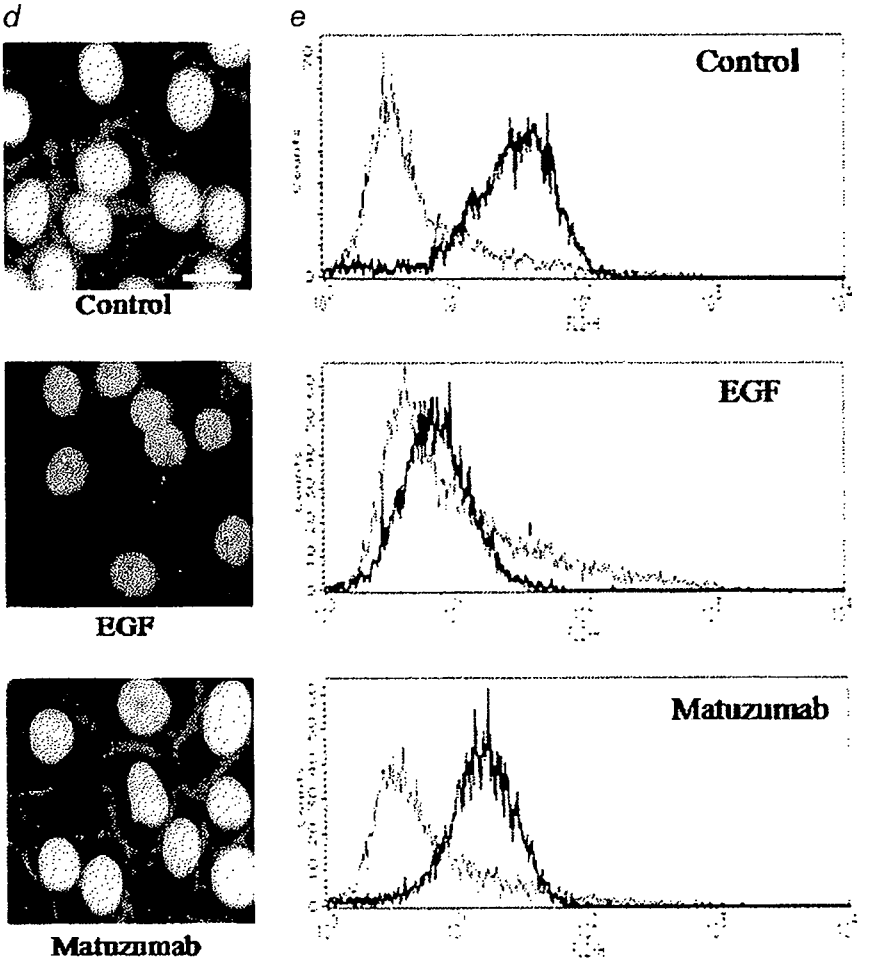

FIGURE 3 – CONTINUED

matuzumab or cetuximab for 24 hr were similar to those apparent after exposure to the antibodies for only 15 or 30 min (Figs. 3*a* and 3*b*). A marked delay in EGFR turnover was also apparent in H460 cells treated with matuzumab or cetuximab (Fig. 3*c*), although EGFR dephosphorylation and downregulation had occurred by 24 hr. Neither matuzumab nor cetuximab induced the activation of Akt or Erk or affected the total amounts of these proteins over a period of 24 hr in either cell line (Figs. 3*a* and 3*c*). We eliminated the possibility that the antibodies to the extracellular domain of EGFR used for the immunoblot analysis shown in Figure 3*a* bind only to the unoccupied form of EGFR (as a result of competition with EGF, matuzumab or cetuximab) by performing the immunoblot analysis shown in Figures 3*b* and 3*c* with antibodies to the intracellular domain of EGFR. These results thus suggested that downregulation of EGFR is impaired in cells treated with matuzumab or cetuximab, likely explaining the failure of these antibodies to activate downstream signaling by Akt and Erk.

To confirm that the inability of the anti-EGFR mAbs to induce EGFR downregulation is attributable to a failure to induce internalization-dependent receptor degradation, we treated serum-deprived H292 cells with matuzumab or EGF for 4 hr and then examined the expression of EGFR by immunofluorescence analysis (Fig. 3*d*) or flow cytometry (Fig. 3*e*). Whereas EGFR was localized at the cell surface in control cells, treatment with EGF resulted in internalization and a decrease in the fluorescence intensity of EGFR. In contrast, EGFR remained at the surface of cells treated with matuzumab. These data suggested that, in contrast to EGF-EGFR complexes, antibody-EGFR complexes remain at the cell surface and do not undergo internalization and degradation.

TABLE I – CHARACTERISTICS OF NSCLC CELL LINES

Cell line	*EGFR* mutation	*EGFR* copy number
H292	Wild type	Polysomy
H460	Wild type	Monosomy
Ma-1	del E746-A750	Gene amplification

Effects of matuzumab and cetuximab on EGF-induced signaling and cell survival

We next determined whether matuzumab or cetuximab inhibits ligand-dependent EGFR signal transduction. To examine also whether the effects of these antibodies are dependent on *EGFR* status, we studied 3 human NSCLC cell lines: 2 cell lines (H292, H460) that possess wild-type *EGFR* alleles and 1 (Ma-1) with an *EGFR* mutation in exon 19 that results in deletion of the residues E746–A750. Our recent fluorescence in situ hybridization analysis[31] revealed that *EGFR* copy number is increased (polysomy) in H292 cells and that H460 cells exhibit monosomy for *EGFR*. Ma-1 cells were also found to manifest *EGFR* amplification (Table I).[31] We treated serum-deprived cells of the 3 NSCLC lines with

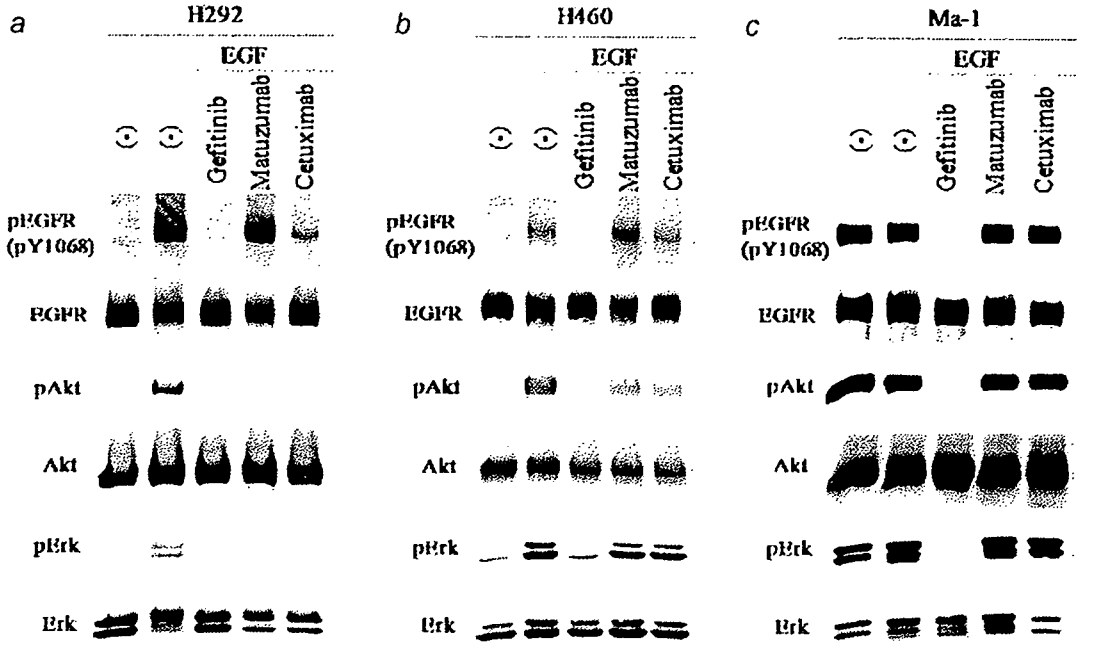

FIGURE 4 – Effects of matuzumab and cetuximab on EGF-induced EGFR signaling. H292 (*a*), H460 (*b*) and Ma-1 (*c*) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or gefitinib (10 μM) and then for an additional 15 min in the additional absence or presence of EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the extracellular domain), Akt or Erk.

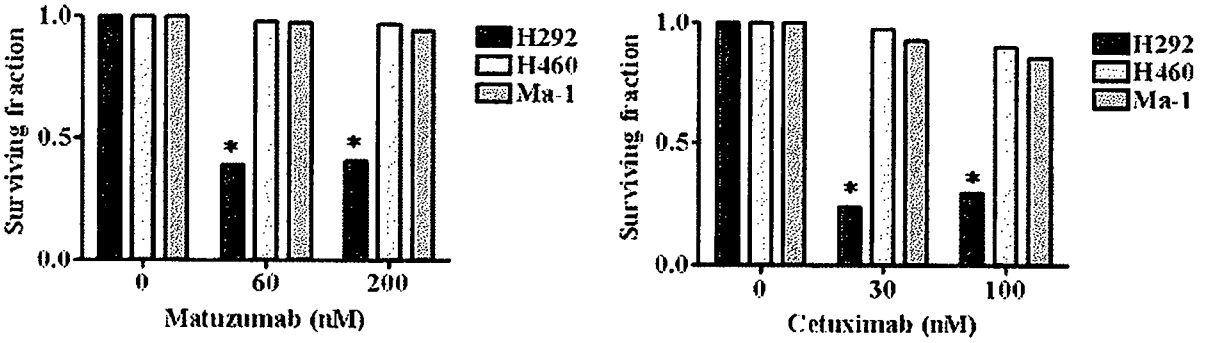

FIGURE 5 – Effects of matuzumab and cetuximab on cell survival. H292, H460 or Ma-1 cells were plated at a density of 200 cells per 25-cm^2 flask in triplicate and cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated with medium alone for 7 days before determination of the number of colonies containing >50 cells for calculation of the surviving fraction. Data are means of triplicates from a representative experiment. *$p < 0.001$ *versus* the corresponding value for cells not exposed to mAb (Student's *t*-test).

matuzumab, cetuximab or gefitinib for 15 min and then stimulated them with EGF for 15 min. Gefitinib prevented the phosphorylation of EGFR, Akt, and Erk induced by EGF in H292 (Fig. 4*a*) and H460 (Fig. 4*b*) cells. The level of EGFR phosphorylation in EGF-treated H292 or H460 cells was not substantially affected by matuzumab or cetuximab, likely because these antibodies also induce EGFR phosphorylation. However, whereas matuzumab and cetuximab did not substantially affect EGF-dependent phosphorylation of Akt or Erk in H460 cells, they markedly inhibited these effects of EGF in H292 cells. As we showed previously,[31] EGFR, Akt, and Erk are constitutively activated in the *EGFR* mutant cell line Ma-1 cell (Fig. 4*c*). Furthermore, whereas gefitinib blocked the phosphorylation of each of these 3 proteins in Ma-1 cells, matuzumab and cetuximab did not.

Finally, we performed a clonogenic assay to determine whether cell survival is affected by the differences in EGF-dependent signaling among H292, H460 and Ma-1 cells after treatment with matuzumab or cetuximab (Fig. 5). Matuzumab and cetuximab each induced a marked reduction in the survival rate of H292 cells, consistent with the inhibition of EGF-dependent EGFR downstream signaling by these antibodies in these cells. In contrast, neither mAb affected the survival of H460 or Ma-1 cells, consistent with the lack of inhibition of EGF-dependent or constitutive EGFR downstream signaling by matuzumab or cetuximab in these cell lines. These results suggested that the effects of matuzumab and cetuximab on EGF-dependent or constitutive EGFR downstream signaling are correlated with their effects on cell survival in NSCLC cell lines.

Discussion

The effectiveness of treatment with anti-EGFR mAbs has been thought to be based on prevention of ligand binding to EGFR and consequent inhibition of EGFR activation.[18,25,26] Matuzumab and cetuximab have recently been developed as EGFR-inhibitory mAbs for clinical use.[17–22,25] A structural study revealed that cetuximab binds to the extracellular ligand binding domain (domain III) of EGFR,[25] and matuzumab is also thought to bind to domain III on the basis of its observed competition with EGFR ligands.[18] We have now shown that matuzumab and cetuximab induced phosphorylation of EGFR at several sites, including Y845, Y1068 and Y1173. These findings are consistent with previous observations that mAb 225, the mouse mAb equivalent to cetuximab, is able to induce EGFR dimerization and activation.[38,39] Cetuximab was also recently shown to induce phosphorylation of EGFR in head and neck squamous cell carcinoma cell lines[29] as well as in NSCLC cell lines including H292.[40] These in vitro results appear to contradict observations that matuzumab and cetuximab inhibit EGFR phosphorylation in vivo.[28,41,42] This apparent discrepancy may be due to the more complex cellular environment in vivo, including the presence of stromal cells that interact with tumor cells. We have also now shown that gefitinib, a specific EGFR-TKI, completely blocked EGFR phosphorylation induced by matuzumab or cetuximab, confirming that this effect of the antibodies is dependent on the intrinsic tyrosine kinase activity of EGFR. Furthermore, our cross-linking analysis showed that matuzumab as well as cetuximab activated EGFR through induction of receptor dimerization. Although recent structural analysis has revealed that cetuximab restricts the range of the extended conformation of EGFR that is required for ligand-induced receptor dimerization,[25] matuzumab and cetuximab likely induce EGFR dimerization in a manner dependent on their immunologically bivalent binding capacities, as was previously shown for mAb 225.[39] We found that neutralizing antibodies to EGFR did not activate EGFR, even though they also recognize the external domain of EGFR and compete with EGFR ligands for receptor binding.[43] The neutralizing antibodies did not induce EGFR dimerization, however, likely accounting for their inability to activate EGFR. This difference in the ability to induce EGFR dimerization between matuzumab and cetuximab on the one hand and the neutralizing antibodies on the other might be due to differences in the corresponding binding sites on EGFR.

To examine the mechanism by which matuzumab and cetuximab exert antitumor effects despite their induction of EGFR activation, we investigated the effects of antibody-induced EGFR activation on EGFR downstream signal transduction. We found that EGFR activation induced by matuzumab or cetuximab was not accompanied by activation of downstream signaling pathways mediated by Akt and Erk, both of which play an important role in regulation of cell proliferation and survival.[35,36] Moreover, we found that the antibody-EGFR complexes were not removed from the plasma membrane, in contrast to the rapid receptor turnover induced by EGF. In response to ligand binding, the ligand-EGFR complex is rapidly internalized and then either recycled back to the cell surface or proteolytically degraded.[44–46] The internalized EGFR interacts with various signaling proteins that are important for sustained activation of the major signaling pathways mediated by PI3K-Akt and Erk.[44,47] The activity of the PI3K-Akt and Erk pathways is thus greatly reduced in cells that are defective in internalization of ligand-EGFR complexes as a result of their expression of a mutant form of dynamin.[37] Furthermore, expression in glioblastoma cells of an EGFR chimeric protein that does not undergo internalization resulted both in a reduction in the extent of EGFR-dependent activation of Akt and Erk as well as in inhibition of tumor growth.[48] These observations thus suggest that inhibition of EGFR turnover by matuzumab or cetuximab is likely responsible for the failure of these mAbs to activate Akt and Erk.

We examined the effects of matuzumab and cetuximab on EGF-dependent EGFR signaling and on cell survival in 3 NSCLC cell lines of differing *EGFR* status. The inhibition of EGF-dependent activation of Akt and Erk by these antibodies appeared related to the inhibition of clonogenic cell survival in the 3 cell lines. With regard to NSCLC cell lines harboring wild-type *EGFR* alleles, matuzumab and cetuximab markedly inhibited EGF-dependent phosphorylation of Akt and Erk in H292 cells but not in H460 cells. Both antibodies inhibited cell survival in H292 cells but not in H460 cells. These results suggest that the antitumor effects of matuzumab and cetuximab depend on inhibition of EGFR downstream signaling such as that mediated by Akt and Erk rather than on inhibition of EGFR itself. Our present data are consistent with previous observations that cetuximab did not inhibit EGFR phosphorylation completely even in cells sensitive to this antibody.[27,30] It is possible that the difference in sensitivity to matuzumab and cetuximab between the 2 cell lines expressing wild-type EGFR in the present study is due to the difference in gene copy number, given that we found an increase in *EGFR* copy number in H292 cells compared with that in H460 cells.[31] A previous clinical study showed that *EGFR* copy number correlated with the response to cetuximab treatment in individuals with colorectal cancer.[49] *EGFR* copy number was not determined by fluorescence in situ hybridization in previous clinical studies of NSCLC patients treated with matuzumab or cetuximab.[19,22–24] Several clinical studies of the therapeutic efficacy of anti-EGFR antibodies in NSCLC patients are underway, and investigation of the potential of molecular markers including *EGFR* copy number to predict clinical response is warranted. Matuzumab and cetuximab failed to inhibit both activation of Akt and Erk and clonogenic cell survival in Ma-1 cells, which express a mutant form of EGFR that shows an increased sensitivity to EGFR-TKIs such as gefitinib and erlotinib.[9–16] We recently showed that cells expressing EGFR mutants exhibit constitutive, ligand-independent receptor dimerization and activation,[31] likely explaining the lack of effect of matuzumab or cetuximab on EGFR signaling or cell survival in such cells. However, previous studies showed that cetuximab exerted an antitumor effect in a cell line with an *EGFR* mutation, whereas several other cell lines with *EGFR* mutations were resistant to cetuximab.[27,30] Our results are consistent with clinical observations showing that the presence of an *EGFR* mutation is not a major determinant of a positive response to cetuximab in individuals with NSCLC or colorectal cancer.[22,50,51]

In conclusion, we have shown that EGFR turnover is impaired in cells treated with the anti-EGFR mAbs matuzumab or cetuximab, resulting in inhibition of EGFR downstream signaling. Although our study is limited by the small number of cell lines analyzed, our findings provide important insight into the mechanisms by which anti-EGFR mAbs exert their antitumor effects, and they suggest that it may be possible to predict the therapeutic efficacy of such mAbs by assessment of EGFR signal transduction.

Acknowledgements

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Multicentre prospective phase II trial of gefitinib for advanced non-small cell lung cancer with epidermal growth factor receptor mutations: results of the West Japan Thoracic Oncology Group trial (WJTOG0403)

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The purpose of this study was to evaluate the efficacy of gefitinib and the feasibility of screening for epidermal growth factor receptor (*EGFR*) mutations among select patients with advanced non-small cell lung cancer (NSCLC). Stage IIIB/IV NSCLC, chemotherapy-naive patients or patients with recurrences after up to two prior chemotherapy regimens were eligible. Direct sequencing using DNA from tumour specimens was performed by a central laboratory to detect *EGFR* mutations. Patients harbouring *EGFR* mutations received gefitinib. The primary study objective was response; the secondary objectives were toxicity, overall survival (OS), progression-free survival (PFS), 1-year survival (1Y-S) and the disease control rate (DCR). Between March 2005 and January 2006, 118 patients were recruited from 15 institutions and were screened for *EGFR* mutations, which were detected in 32 patients – 28 of whom were enrolled in the present study. The overall response rate was 75%, the DCR was 96% and the median PFS was 11.5 months. The median OS has not yet been reached, and the 1Y-S was 79%. Thus, gefitinib chemotherapy in patients with advanced NSCLC harbouring *EGFR* mutations was highly effective. This trial documents the feasibility of performing a multicentre phase II study using a central typing laboratory, demonstrating the benefit to patients of selecting gefitinib treatment based on their *EGFR* mutation status.
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Gefitinib, a tyrosine kinase inhibitor (TKI), is an orally active small molecule that functions as a selective epidermal growth factor receptor (*EGFR*) inhibitor (Ranson *et al*, 2002). Two phase II trials (Fukuoka *et al*, 2003; Kris *et al*, 2003) for previously treated non-small cell lung cancer (NSCLC) (IDEAL-1 and -2, respectively) have documented favourable objective responses in 14–18% of patients. However, in a phase III trial (Thatcher *et al*, 2005), no survival benefit of gefitinib was observed when compared with best-supportive care (BSC) for previously treated NSCLC. In contrast, we have seen a significant survival benefit of erlotinib compared with BSC as a salvage therapy (BR21); erlotinib is also an *EGFR*-TKI and its chemical structure, which is based on quinazoline, is quite similar to that of gefitinib (Shepherd *et al*, 2005). Although we do not know whether differences between gefitinib and erlotinib were responsible for these different outcomes, appropriate patient selection to identify good responders is likely crucial for revealing the clinical benefits of the *EGFR*-TKI family.

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