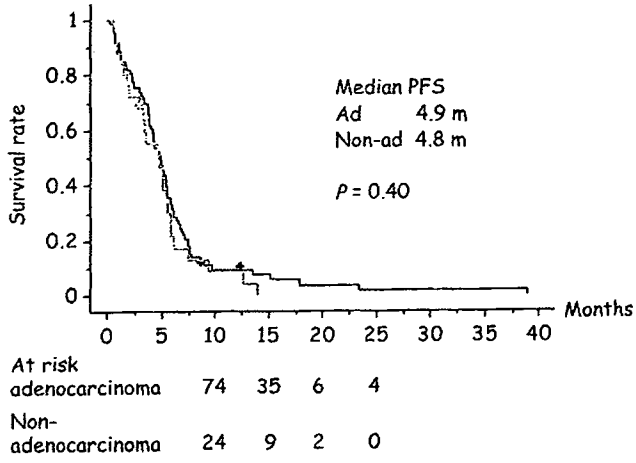

Fig. 1. Kaplan-Meier plot of progression-free survival (adenocarcinoma versus nonadenocarcinoma histology).

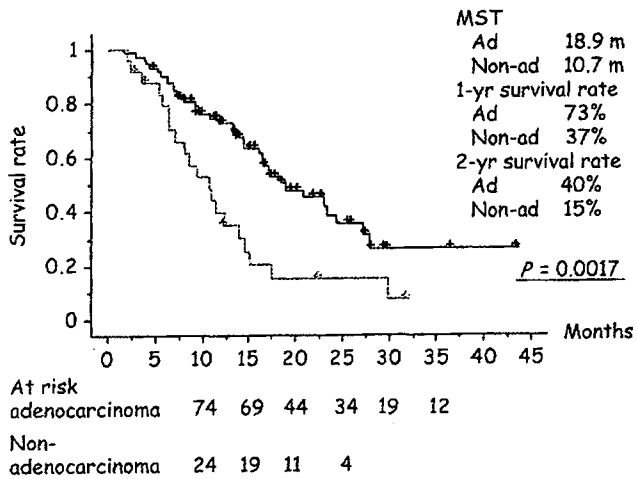

Fig. 2. Kaplan-Meier plot of progression-free survival (smoker versus non-smoker).

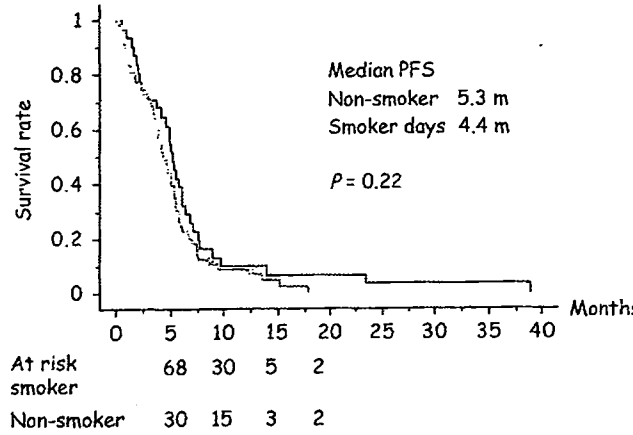

Fig. 3. Kaplan-Meier plot of overall survival (adenocarcinoma versus nonadenocarcinoma histology).

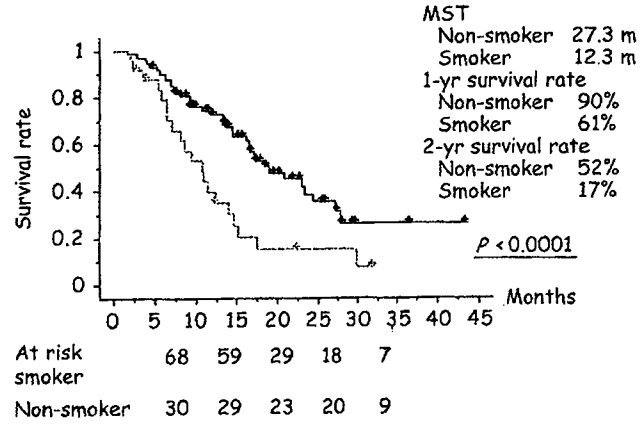

Fig. 4. Kaplan-Meier plot of overall survival (smoker versus non-smoker).

and between non-smokers versus smokers ($P < 0.0001$). Of particular note, median OS of smokers was 12.3 months, and non-smokers was 27.3 months. Two-year survival rates were 17% and 52% in smokers and non-smokers, respectively (Figs 3,4).

To identify factors influencing OS in patients who received second-line therapy ($n = 76$), multivariate analysis was performed with covariates including histology (adenocarcinoma versus other), smoking history (non-smoker versus smoker), PS (0 versus 1), docetaxel (use versus non-use) and EGFR-TKI (use versus non-use). The use of EGFR-TKI was identified as a significant prognostic factor associated with longer OS, together with non-smoking history and PS 0. The use of docetaxel was not associated with an increase in OS in this study (Table 4). When interaction terms between clinical variables and EGFR-TKI treatment were included in the model, no significant interaction was detected ($P = 0.354$ and 0.515 for smoking history × EGFR-TKI and histology × EGFR-TKI, respectively). In the exploratory Cox analysis, prognostic advantage for non-smoking history and adnocarcinoma histology was more prominent in patients who received EGFR-TKI treatment after adjustment for PS, suggesting a potential interaction between these favorable clinical variables and EGFR-TKI treatment. Compared with smokers, hazard ratio of non-smokers with or without EGFR-TKI was 0.961 (95% CI, 0.209–4.420) and 0.193 (0.083–0.449), respectively. Likewise, the hazard ratio of adenocarcinoma patients with or without EGFR-TKI was 0.429 (0.138–1.334) and 0.387 (0.187–0.800), respectively, compared with patients with other histologies.

Table 4. Cox regression analysis of prognostic factors for overall survival after second-line treatment: a stepwise forward procedure

Factor	Variable	*P*-value HR (95% CI)
Histology	Adenocarcinoma versus other	0.0639 –
Smoking	Non-smoker versus smoker	0.0052 0.325 (0.148–0.715)
PS	0 versus 1	0.0258 (0.258–0.917)
Docetaxel	– versus +	0.6720 –
EGFR-TKI	– versus +	0.0084 2.844 (1.306–1.823)

PS, performance status; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; HR, hazard ratio; CI, confidence interval.

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Table 5. Historical comparison of outcomes in our study and the FACS study[2]

	FACS[1] (n = 145)	This study (n = 98)
Response rate (%)	32	20
Median PFS (months)	4.5	4.8
Median OS (months)	12.3	16.5
1-year survival rate (%)	51	64
Second-line therapy, n (%)	87 (60)	76 (78)
Docetaxel	25 (17)	42 (43)
EGFR-TKI	9 (6)	29† (30)
Other	58 (40)	5 (5)

†25 patients were treated with Gefitinib. FACS, Four-Arm Cooperative Study; PFS, progression-free survival; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor.

Discussion

Since the results of a large meta-analysis revealed that platinum-based chemotherapy prolonged OS of patients with advanced NSCLC compared with best supportive care (BSC)[8] this therapy has been considered standard first-line treatment for advanced NSCLC worldwide. Median OS with carboplatin/paclitaxel – the most commonly used standard therapy outside Japan – has been reported to be 8–14 months[9–13] similar to the median OS (12.3 months) observed in the FACS trial conducted in Japan.[2,3]

Comparison of outcomes following carboplatin/paclitaxel treatment in our study with the results obtained with the same regimen in the FACS study, showed there was little difference in median PFS (4.8 versus 4.5 months), but median OS was approximately 4 months longer at our hospital (16.5 versus 12.3 months). With the recent approval of EGFR-TKIs, the use of these agents as second-line chemotherapy has increased since the FACS study was performed (30% of patients in this study versus 6% of patients in the FACS study) (Table 5). This observation suggests that the better treatment outcomes obtained in our study compared with those of FACS may be attributable to the effect of anticancer agents used in second-line and subsequent treatment, especially EFGR-TKI (gefitinib was used in most cases). In fact, the result of subgroup analysis by patient demographics in our study demonstrated a marked prolongation of OS for non-smokers and patients with adenocarcinoma, both of which are known to be factors associated with high responsiveness to EGFR-TKI. Furthermore, the multivariate analysis in patients receiving second-line treatment, revealed that EGFR-TKI use was an independent prognostic factor.

Generally, the prolongation of OS is the ultimate goal of anticancer therapy and an important clinical outcome in the evaluation of the effect of first-line treatment for NSCLC. With the emergence of potent anticancer agents in the second-line setting, therapy administered after the occurrence of progressive disease becomes a confounding factor in the interpretation of OS. To overcome this issue of confounding, there may be value in using prolongation of PFS as the primary outcome of first-line trials. Currently, the Food and Drug Administration (FDA) requires an applicant to demonstrate prolonged survival as an approval condition for new anticancer agents.[14] However, the European Agency for Evaluation of Medical Products (EMEA) has accepted PFS as the primary endpoint in some instances, and our present study result supports this view.[15]

The results of the BR21 trial showed that erlotinib significantly prolonged OS compared with placebo (6.7 versus 4.7 months, hazard ratio [HR] = 0.70). In the multivariate analysis, Asian origin (P = 0.01), adenocarcinoma histology (P = 0.004) and non-smoking status (P = 0.048) correlated with prolonged OS.[6] In the preplanned subgroup analysis in the ISEL trial, significantly longer survival was seen with gefitinib compared with placebo in patients of Asian origin (9.5 versus 5.5 months, HR = 0.66) and never-smokers (8.9 versus 6.1 months, HR = 0.67).[7] Although these two studies did not include Japanese patients, the findings might be extrapolated into Japanese populations. Since the reports of Paez *et al.*[16] and Lynch *et al.*[17] in April and May 2004, respectively, numerous studies of EGFR mutations have been conducted in a short period and studies conducted in Japan have reported a good correlation between OS and EGFR mutations in patients treated with gefitinib.[18–20] Moreover, the incidence of EGFR mutations is more frequent in women, patients with adenocarcinoma, never-smokers and Japanese patients[16,17] suggesting that there is a correlation between clinical and molecular factors and clinical benefit from EGFR-TKIs.

Although EGFR mutations are of interest as a biomarker that can be predictive of the effect of gefitinib, especially in patients of Asian or Japanese origin, their immediate clinical application for patient selection is not always possible, due to issues including method determination, cost and convenience. Correlation between response to gefitinib and EGFR copy number determined by fluorescence *in situ* hybridization (FISH) has attracted attention in the West as an alternative potential biomarker[21,22] and this needs to be further investigated in Japan. Acknowledging the need to pay close attention to future research trends, we believe further discussion into how to select those patient populations most likely to benefit from gefitinib in routine clinical practice is required. It is important to establish whether patients could be selected on the basis of biomarker data such as EGFR mutations, or EGFR over-expression, or clinical characteristics such as histological subtype and smoking history. Nevertheless, selection of appropriate patients for EGFR-TKI therapy is undoubtedly necessary, and we hope that future research will be able to identify possible methods as soon as possible. Once identified these will require validation in large-scale prospective clinical studies.

In conclusion, this retrospective study demonstrated a marked prolongation of overall survival in patients with adenocarcinoma and non-smoking history who received carboplatin/paclitaxel as first-line treatment. Our study results suggest that the use of EGFR-TKI (especially gefitinib) after first-line treatment may be associated with an improvement in overall 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.

(*J Thorac Oncol.* 2008;3: 187–189)

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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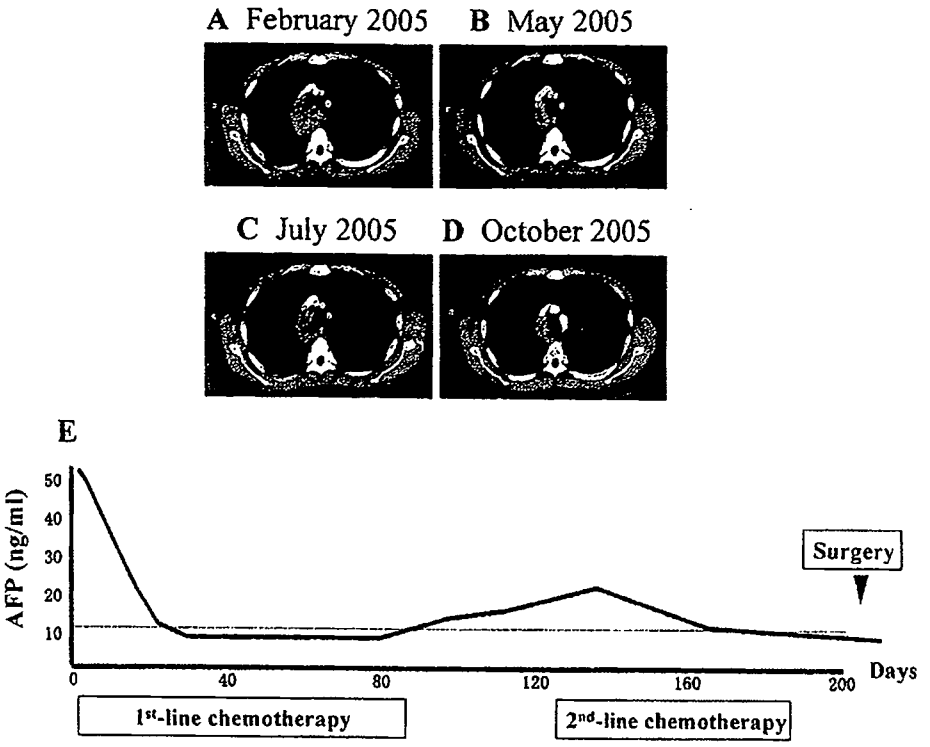

FIGURE 1. Chest computed tomography (CT) findings and serum AFP levels in the patient. *A–D*, Chest CT findings. A mass in the middle mediastinum was initially detected (*A*). The tumor had shrunk after three cycles of neoadjuvant chemotherapy (*B*), but its progression had resumed after the fourth cycle (*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.

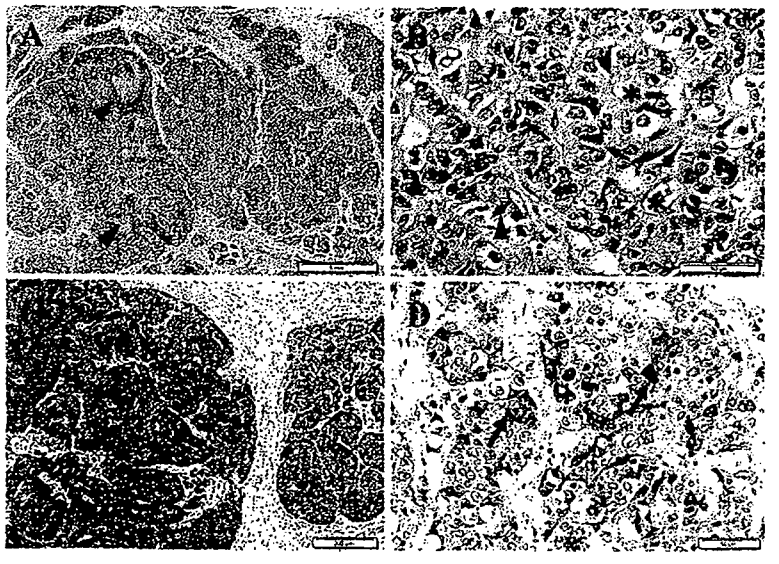

FIGURE 2. Histology and immunohistochemical analysis of the tumor specimen obtained at surgery. *A*, Hematoxylin-eosin staining revealed solid tumor nests with areas of necrosis (arrow heads). Note the homogeneous appearance of the tumor. *B*, High-power magnification of the tumor stained as in (*A*), 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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Lung cancer screening—Comparison of computed tomography and X-ray

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KEYWORDS
Lung cancer;
Computed tomography;
Mass screening;
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Morbidity;
Mortality

Summary Recent studies on lung cancer screening with CT disclosed a discrepancy between its efficiency in detecting early lung cancer and a lack of proof for decreasing mortality from lung cancer. The present study, in a city in Japan where an X-ray screening program is provided, bi-annual CT screening was performed for X-ray screening negative subjects for 4 years. Ten patients with lung cancer were detected among 22,720 person-year subjects (0.044%) through the X-ray screening. Among the X-ray screening-negative subjects, 3305 subjects participated in a CT screening program resulting in the detection of 15 patients with lung cancer (0.454%). All 15 cases detected by CT screening and 5 of the 10 cases detected by X-ray screening were at stage IA. In respect of gender, histological type and CT findings, patients detected by CT screening had a better prognostic profile than those detected by X-ray screening. Survival was significantly better in the former than the latter, both in its entirety comparison and in a comparison limited to patients who underwent surgery. In conclusion, CT screening might have the potential to detect lung cancer with good prognostic factors not limited to early detection. Sufficiently long follow-up time, therefore, would be required to evaluate the efficacy for decreasing lung cancer mortality with CT screening.

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1. Introduction

Lung cancer is the leading cause of cancer death in many countries worldwide. Hope of decreasing death from lung cancer by early detection has encouraged studies for lung cancer screening by chest X-ray [1–4], sputum cytology and low-dose spiral computed tomography (CT) [5–13].

Recently, a large scale study on lung cancer screening by CT (International Early Lung Cancer Action Program, or I-ELCAP) resulted in a diagnosis of lung cancer in 484 participants out of 31,567 asymptomatic persons at risk for lung cancer, a high ratio of clinical stage I of 85% in the diagnosed patients, and a high estimated 10-year survival rate of 88% in the subgroup with clinical stage I lung cancer, confirming the previous reports on CT screening [10]. On the other hand, another international study failed to show a decline in advanced lung cancer diagnoses and lung cancer deaths by CT screening when compared with estimated numbers by means of 2 prediction models, although it again disclosed significant efficacy in the early detection of lung cancer [14], revealing a discrepancy between the studies. Clarifying the characteristics of lung cancer detected by CT screening may help to explain this discrepancy. The present study, performed in a single region, compared the results of lung cancer screenings by low-dose CT with those by conventional chest X-ray in terms of efficacy and the characteristics of the detected lung cancers.

2. Materials and methods

2.1. Study region and subject recruitment

The study was conducted in an anonymous city located in a suburb in Chiba prefecture next to Tokyo, Japan. The municipal office has, for decades, provided its residents older than 40 years with an annual health-screening program including chest X-ray. All subjects participating in this program, on the day of the chest X-ray screening, were informed of the free-of-charge and research-based low-dose CT screening program to take place at a later date, together with its potential benefits and risks. Those who gave their written informed consent for the study became candidates for enrolling in the CT screening for lung cancer. Subjects who had abnormalities detected on the basis of the X-ray screening, and were judged to require further examinations, were excluded from enrollment in the CT screening program. New subjects were recruited every year for each screening. With encouragement, repeat of the screening at the next opportunity depended on the individual's will.

2.2. Lung cancer screening by chest X-ray and CT

For screening with X-ray, images in 10×10 cm miniature radiograms were obtained with mobile X-ray equipment (Model MXO-15B, Toshiba Medical Systems Co., Otawara, Japan) on X-ray film rolls (X-ray film HX, Konica Minolta Holdings, Inc., Tokyo, Japan) with an X-ray mirror-camera (CM5-100, Canon Inc., Tokyo, Japan). The technical parameters consisted of tube voltage of 130 kV with adjustment of mAs by photo-timer, and a distance of 120 cm from the tube to film with a 2.0-mm aluminum filter. The film rolls were reviewed on dedicated illuminant miniature X-ray film viewers equipped with magnifying glasses. For screening with CT, images were obtained by mobile low-dose spiral CT equipment (W950SR, Hitachi Medical Co, Tokyo, tube voltage of 120 kV, electric current of 50 mA, rotation of $0.5\,s^{-1}$, collimation of 10 mm, interval of 10 mm). The images were reviewed on CRT with personal computer-based viewing and reporting system. Each image of X-ray and CT was reviewed by 2 independent expert pulmonologists, and any pulmonary or endobronchial nodule suggesting a lesion requiring further examinations was compared with a previous study when available. Then, the final judgment was given by several reviewers' consensual decision and the results were classified into 4 categories; no nodule (category I), nodules requiring no further examinations (category II), nodules suggesting non-malignant lesion requiring further examinations (including lesions suggesting active tuberculosis, category III), and nodules suggesting malignant lesions (category IV). There was no communication between X-ray and CT reviews. Further examinations consisted of conventional-dose CT with thin-section scanning, ranging from 0.5 to 2 mm thickness according to the requirement, in all patients with categories III and IV, follow-up studies by CT, and invasive diagnostic procedures including bronchoscopy, CT-guided biopsy and video-assisted thoracotomy when required.

The X-ray screening was repeated every year. Because of research resource limitation, the city was geographically divided into 2 areas, and the CT screening was performed alternatively in only one area each year, resulting in screening in the same area every 2 years. Inter-screening tracking of the subjects without categories III and IV was not allowed because of local regulations. Both screenings were performed from 2001 to 2004 in each fiscal year, with follow-up periods until September 2007. The entire study was approved by the Ethics Committee of the Chiba Foundation for Health Promotion & Disease Prevention.

2.3. Image analysis of detected lung cancer

Thin-section images of detected nodules definitively diagnosed as primary lung cancer were retrospectively reviewed and classified into 3 categories; pure ground glass attenuation (GGA), part solid (GGA with a central solid part) and solid nodule [15,16].

2.4. Statistics

Comparisons of frequency were performed by Student's t-test, and survival curves were drawn by Kaplan–Meier's method followed by comparison with log rank test. Differences with p values of less than 0.05 (two tailed) were judged as statistically significant.

3. Results

The total numbers of person-years for X-ray and CT screening in the 4-year period were 22,720 and 3305, with actual subject numbers of 8246 and 2550, respectively. Characteristics of the subjects are summarized in Table 1. In this

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Table 1 Characteristics of subjects

Year	Total no. of subjects	Age (years)[a]	Sex		No. of baseline study	No. of repeat study
			No. of male (median age; range)	No. of female (median age, range)		
X-ray screening						
2001	5,309	59 (40–93)	1776 (63; 40–85)	3,533 (57; 40–93)	101	5,208
2002	5,417	58 (40–89)	1828 (62; 40–86)	3,589 (56; 40–89)	927	4,490
2003	5,782	59 (40–92)	2018 (63; 40–88)	3,764 (57; 40–92)	848	4,934
2004	6,212	60 (40–94)	2167 (63; 40–91)	4,045 (58; 40–94)	794	5,418
Total	22,720*	59 (40–94)	7789 (63; 40–91)	14,931 (57; 40–94)	2670	20,050*
CT screening						
2001 (area A)	729	65 (50–87)	326 (65; 50–85)	403 (66; 50–87)	729	NA
2002 (area B)	762	65 (50–84)	314 (66; 50–84)	448 (64; 50–81)	762	NA
2003 (area A)	838	65 (50–85)	361 (65; 50–83)	477 (64; 50–85)	519	319
2004 (area B)	976	65 (50–83)	419 (65; 50–83)	557 (64; 50–80)	540	436
Total	3,305*	65 (50–87)	1420 (65; 50–85)	1,885 (65; 50–87)	2550	755

[a] Median (range).
* Numbers in terms of person-years.

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table, the actual number for CT screening is equal to the total number of subjects who participated in the baseline study because the screening was started at 2001. However, in the X-ray screening, the number of subjects participated in the repeat study at 2001 ($n=5208$) plus total number of subjects participated in the baseline study ($n=2670$) dose not result in the actual total number ($n=8246$) in 4 years, because the screening had been started before 2001; there were some subjects who had participated in the screening before 2001 and did not at 2001. In addition, the subject number of repeat study is greatly exceeds the number of baseline study in every year, because many subjects had already participated in the screening before 2001. Smoking status of the subjects is summarized in Fig. 1 according to gender and screening method. The subjects of CT screening consisted of a significantly higher proportion of smokers than those of X-ray screening ($p<0.001$ for males, and $p=0.0014$ for females, χ^2 test). Total accrual numbers of further examinations (categories III and IV) were 313 (78 category III and 235 category IV) of 22,720 (1.4%) in X-ray screening, and 337 (67 category III and 270 category IV) of 3305 (10.2%) in CT screening.

All lung cancers were found exclusively from category IV in both screenings. Lung cancers were found in 10 patients (0.044% of the 22,720 screened) through the X-ray screening, 4 patients by baseline (0.1498%, or 4 out of 2670) and 6 patients by repeat screening (0.030%, 6 out of 20,050). Among them, 5 (50%) patients had stage IA lung cancer. With CT screening, 15 patients (0.454% of the 3305 screened) with primary lung cancer were found. They were exclusively found in baseline screening, and all 15 lung cancers were stage IA. Patient characteristics are summarized in Table 2. With X-ray screening, lung cancer was detected in 0.040% (6/14,931) of female participants, and in 0.051% (4/7789) of male participants, with a female-to-male ratio of the detection rate of 0.78. In contrast, with CT screening, it was detected in 0.58% (11/1885) of female, and in 0.28% (4/1420) of male participants, with a female-to-male ratio of 2.07. The proportion of adenocarcinoma was 86.7% (13/15) in patients detected through CT screening, significantly higher than that (50%, or 5/10) in patients detected through X-ray screening ($p=0.0455$, χ^2 test). The constitution of the image type of lesions, that is, pure GGA, part solid and solid types, was significantly different between these 2 groups ($p=0.0001$, χ^2 test), and those detected by CT screening were more likely to be pure GGA or part solid than those detected by X-ray screening. Standard lobectomy with hilar and mediastinal lymph node dissection was performed in 6 of the 10 lung cancer patients detected by X-ray and in 14 of the 15 patients detected by CT screening (Table 2). Retrospective re-review of X-rays of patients with CT screening-detected lung cancer disclosed that the corresponding lesion was visible on X-ray in only one case, #CT-12.

Survival curves of the patients with detected lung cancer are shown in Fig. 2. Survival of the patients detected by CT screening was better than that of the patients detected by X-ray screening with statistical significance (Fig. 2A). Survival of the patients undergoing surgery was also compared between the patient groups detected by CT and X-ray screenings, again disclosing better survival in the CT screened patients than in the X-ray-detected patients with

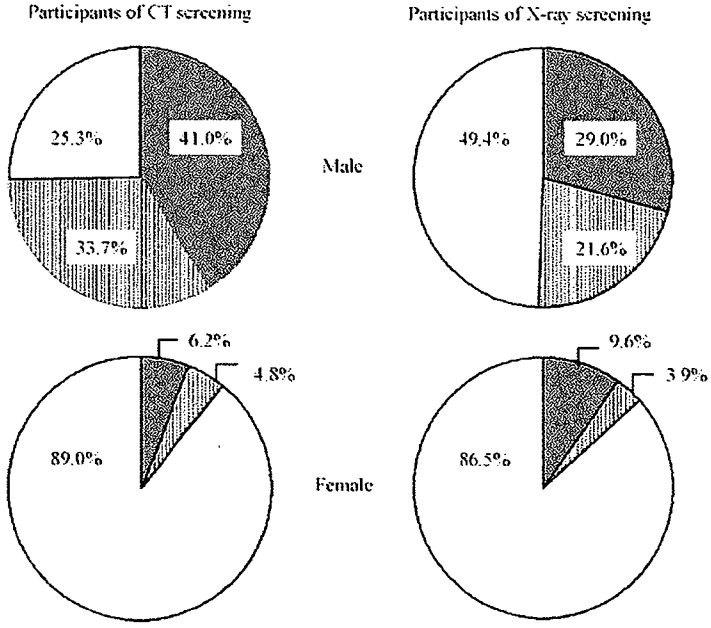

Fig. 1 Smoking status of the participants in the 2 screening programs. Closed, shaded and open areas represent current, ex- and never smokers, respectively. Smoking rates of the participants in the X-ray screening were similar to the general statistics in Japan, in both male and female populations, whereas those of the participants, especially in males, in the CT screening were significantly higher than in participants of the X-ray screening ($p<0.001$ for males, and $p=0.0014$ for females, χ^2 test), very possibly because of smokers' motivation to participate in CT screening.

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Table 2 Characteristics of detected lung cancer

Case no.	Age	Sex	Size (mm)	Histology	Stage	Image type	Treatment	Visible on X-ray
With X-ray screening								
X-1	75	F	30[a]	Ad	p-IA	Solid	S[b]	NA
X-2	75	M	28	Sm	p-IB[c]	Solid	S and C	NA
X-3	70	F	20	Ad	p-IA	Solid	S	NA
X-4	77	M	50	Sq	c-IIIA	Solid	R	NA
X-5	74	M	20	Sq	c-IIIA	Solid	R	NA
X-6	78	F	8	Sm	c-IIIA	Solid	C and R	NA
X-7	51	F	25	Ad	p-IA	Solid	S	NA
X-8	47	M	27	Ad	c-IIIA	Solid	C and R	NA
X-9	69	F	10	Carcinoid	p-IA	Solid	S	NA
X-10	62	F	27	Ad	p-IA	Solid	S	NA
With CT screening								
CT-1	64	M	10	Ad	p-IA	Pure GGA	S	No
CT-2	72	F	11	Ad	p-IA	Pure GGA	S	No
CT-3	64	M	20	Ad	p-IA	Pure GGA	S	No
CT-4	63	F	15	Ad	p-IA	Part solid	S	No
CT-5	71	F	15	Ad	p-IA	Part solid	S	No
CT-6	79	M	14	Sq	p-IA	Solid	S	No
CT-7	66	F	7	Ad	p-IA	Pure GGA	S	No
CT-8	60	F	8	Ad	p-IA	Part solid	S	No
CT-9	67	F	15	Ad	p-IA	Part solid	S	No
CT-10	58	F	9	Ad	p-IA	Pure GGA	S	No
CT-11	63	F	10	Ad	p-IA	Pure GGA	S	No
CT-12[d]	59	M	23	Non-small	c-IA	Solid	BSC	Yes
CT-13	70	F	10	Ad	p-IA	Part solid	S	No
CT-14	62	F	10	Ad	p-IA	Part solid	S	No
CT-15	61	F	30	Ad	p-IA	Pure GGA	S	No

[a] Maximum diameter.
[b] S, R, C and BSC represent surgery, radiotherapy, chemotherapy and best supportive care, respectively.
[c] Postoperative evaluation of the tumor size determined the stage of IB.
[d] This patient was diagnosed as having clinical stage IA non-small cell lung cancer not further specified together with concomitant advanced esophageal cancer by staging procedures.

statistical significance (Fig. 2B). Two patients, detected by X-ray screening were dead after surgery, both from lung cancer recurrence (case #X-2 and 10).

4. Discussion

The present lung cancer screenings recruited subjects not limited to a high-risk group. First, the X-ray screening program was provided for general residents in a certain city in Japan, and the next screening program with CT was offered to the participants of the X-ray program, while excluding subjects who were judged to require further examinations by the X-ray screening. Therefore, the present CT screening program was eventually a screening for roentgen-negative lung cancer. The present study was preliminary and had several shortcomings: (1) sample size was relatively small, (2) there were some deviations in characteristics of the subjects; the subjects of X-ray screening consisted of less smokers and younger population especially in female than the subjects of CT screening, (3) examination was repeated every 2 years in the CT screening program, and (4) no inter-screening follow-up for counting lung cancer occurrence and death was performed, resulting in a lack of estimation of the true frequency of lung cancer occurrence in the subjects during the study period. In particular, the primary issues being the small sample size and the deviations in subject characteristics ostensibly limit this study's ability to make definitive conclusion. This kind of study solely enables us to evaluate screening efficacy by comparing CT screening with X-ray screening in terms of the characteristics of the detected lung cancer.

CT screening detected lung cancer at a frequency of approximately 10 times that of X-ray screening, even though CT screening was provided for X-ray screening-negative subjects. In addition, all lung cancers detected by CT were at stage IA, whereas only 6 of 10 lung cancers detected by X-ray screening were at stage IA, consequently resulting in better survival in the former compared to the latter. Characteristics of the lung cancer detected through CT screening were significantly different from those through X-ray screening. First of all, all 15 lung cancers detected by CT were adenocarcinomas except for one with non-small cell lung cancer not further specified, whereas only 5 of 10 lung cancers detected by X-ray screening were adenocarcinomas, the latter ratio being similar to that of the general statistics in Japan. Secondly, the female-to-male ratio of the detection rate with CT screening (2.07) was substantially higher than that with

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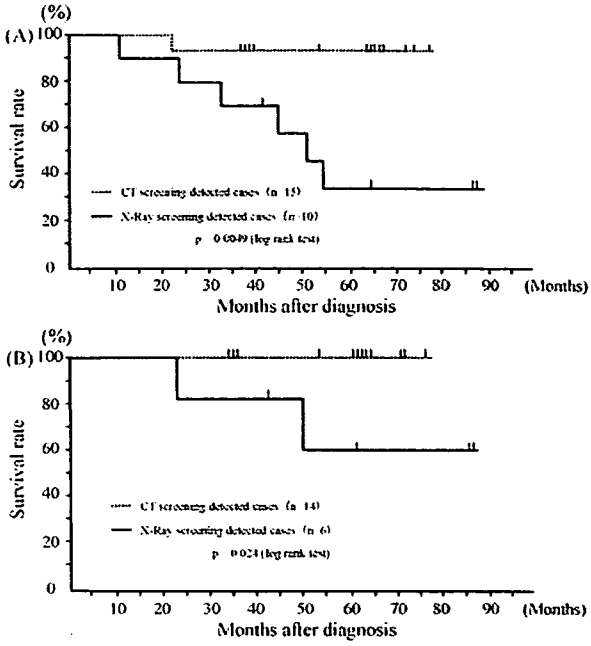

Fig. 2 Survival curves of patients with lung cancer according to screening method. Patients detected by CT screening ($n = 15$) survived significantly longer than patients detected by X-ray screening ($n = 10$, A). Comparison between the 2 groups limited to the subpopulations undergoing surgery showed a similar result (B). The curves were drawn by Kaplan–Meier's method, and compared with the log rank test.

X-ray screening (0.78). Considering that the female-to-male ratio of patients with lung cancer in the general statistics of Japan was 0.41 [17], the ratio with CT screening seemed extraordinarily high, and may actually be biased. As a matter of fact, the ratio with X-ray screening of 0.78 also seemed high, suggesting strong bias with screening. Thirdly, when assessed with thin-section CT, the image type of the lung cancer detected by CT screening contained a significantly larger portion of pure GGA or part solid type than that by X-ray screening. This is quite reasonable because nodules of GGA type are notoriously invisible on X-ray. The existence of GGA either as pure GGA or in part solid nodules in thin-section CT represents air-spaces in lung adenocarcinoma tissue, and very likely corresponds to either type A, B, or C of peripheral small adenocarcinoma [15,16,18–21] according to Noguchi's classification [22]. Such lung adenocarcinomas, in most cases, are characterized by a slow-growing nature and good prognosis with lung resection [15,16,18–22].

Effective cancer screening requires several conditions including the followings: (1) the screening is capable of detecting corresponding cancer at a high frequency, (2) prognosis of patients with screening-detected cancer is significantly better than that of patients found by symptoms, (3) less patients with advanced cancer and deaths from the cancer are shown by the screening, and (4) the screening is affordable in respect to human resource and cost. Many previous studies [5,11,13,23–27] and two recent studies [10,14] on lung cancer screening by CT provided evidence for the first 2 conditions. The present study also supports these previous study results. Bach et al., however, cast doubt on lower number of patients with advanced disease and lung cancer deaths by CT screening [14]. The discrepancy between the high frequency of early detection resulting in good prognosis of the detected patients and a lack of decrease in advanced disease and death may be partly explained by overdiagnosis through screening. That is to say, in spite of a definitive histological diagnosis, many early lung cancers detected through screening would not progress rapidly to the point of being clinically overt in the individual's lifetime. In fact, lung cancers detected via the present CT screening seemed to possess less malignant propensity, because the majority (13 out of 15 patients) were classified into either pure GGA or part solid type adenocarcinomas by thin-section CT findings, and because they were found predominantly in female non-smokers. In particular, detection and diagnosis in one patient (#CT-12) was apparently overdiagnosed because he died from concomitant advanced esophageal cancer while his lung cancer was at clinical stage IA. Nevertheless, the rest of the lung cancers detected by the present CT screening would have very possibly progressed to clinically overt and fatal cancer if left untreated, making it needless to refer in particular to the I-ELCAP study [10], in which 8 patients with clinical stage I cancer detected by CT screening did not receive treatment, with all of them dying within 5 years. In addition, any individual with pulmonary nodules judged to require further examinations through X-ray screening was excluded from enrollment to the CT screening. Most lung cancers detected by X-ray screening would have been detected by CT screening if no X-ray screening had been provided. Therefore, the present CT screening has the potential to reduce advanced lung cancer or death from lung cancer in the future, but not within a few years. Although Bach et al. failed to demonstrate a decrease in advanced disease and death from lung cancer [14], the reason for the negative result may be related to a relatively short median follow-up period of 3.9 years. Needless to say, large-scale randomized controlled studies that eliminate biases would have advantages for drawing definitive conclusions. Hence, results from randomized controlled studies such as the National Lung Screening Trial in the United States and the NELSON Trial in Europe are awaited [14,28,29]. It is important, however, to understand that a substantially long follow-up period, although difficult to be estimated from this study, would be required even in the case of well-sophisticated randomized controlled studies. Considerations on potential harm and cost would also be an important issue.

In conclusion, the present study confirmed the capability of CT screening in detecting early stage lung cancer at a high frequency, and suggested that CT screening-detected lung cancer might have less malignant propensity than X-ray screening-detected or symptom-detected lung cancer. In CT screening for lung cancer, a considerably extended follow-up period would be essential for evaluating its effectiveness in decreasing lung cancer mortality.

Conflict of interest

There exists no potential conflict of interest with regard to the manuscript in every author.

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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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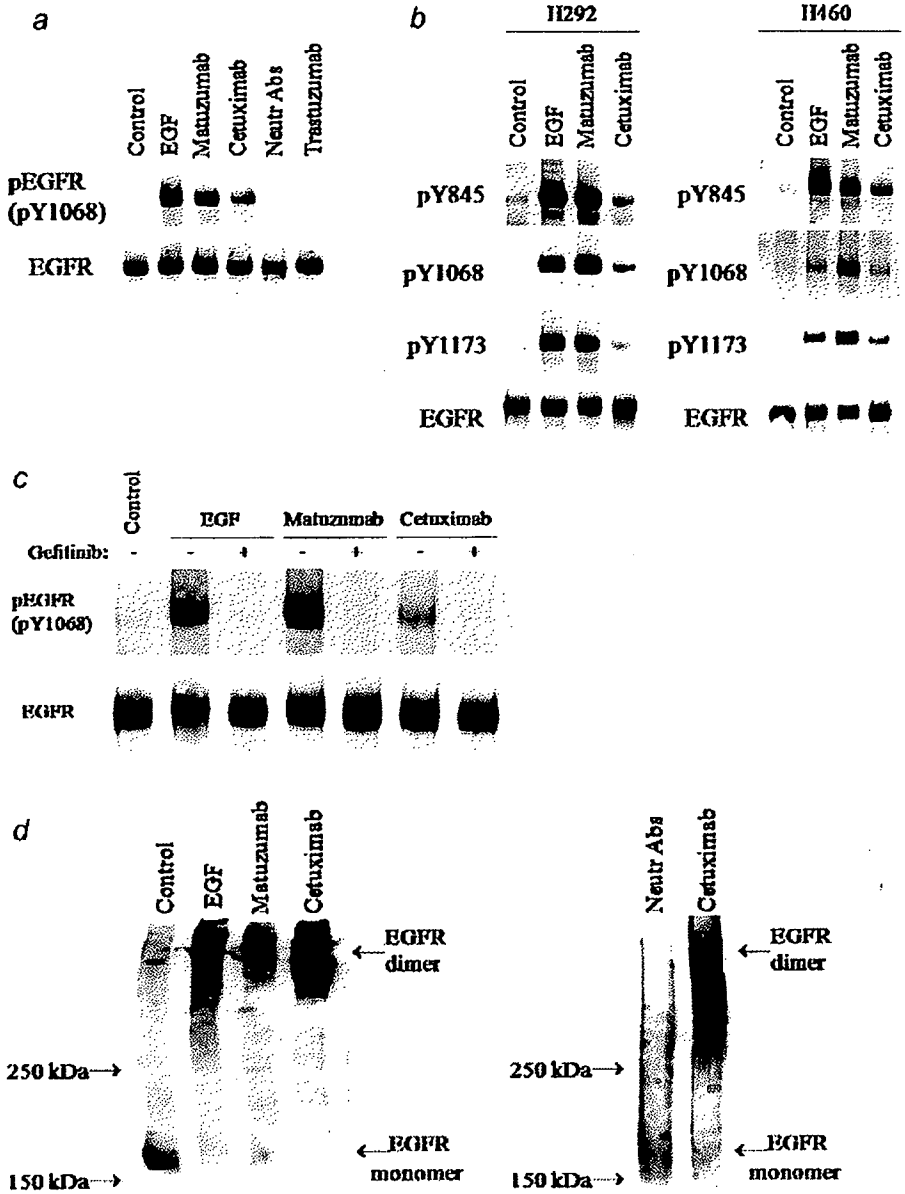

FIGURE 1 – EGFR phosphorylation induced by matuzumab or cetuximab as a result of receptor dimerization and activation of the receptor tyrosine kinase. (*a*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence (Control) or presence of matuzumab (200 nM), cetuximab (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.

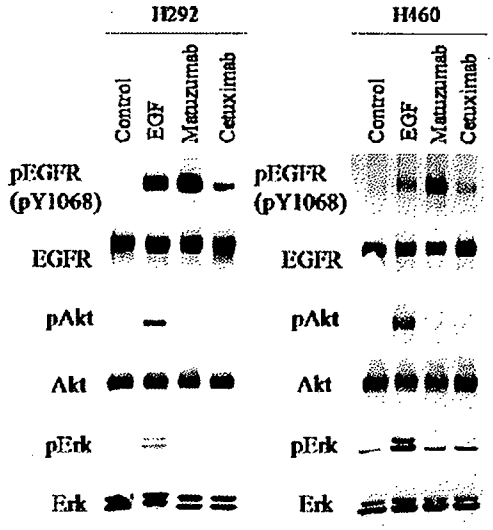

FIGURE 2 – Failure of matuzumab or cetuximab to activate Akt or Erk. H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies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

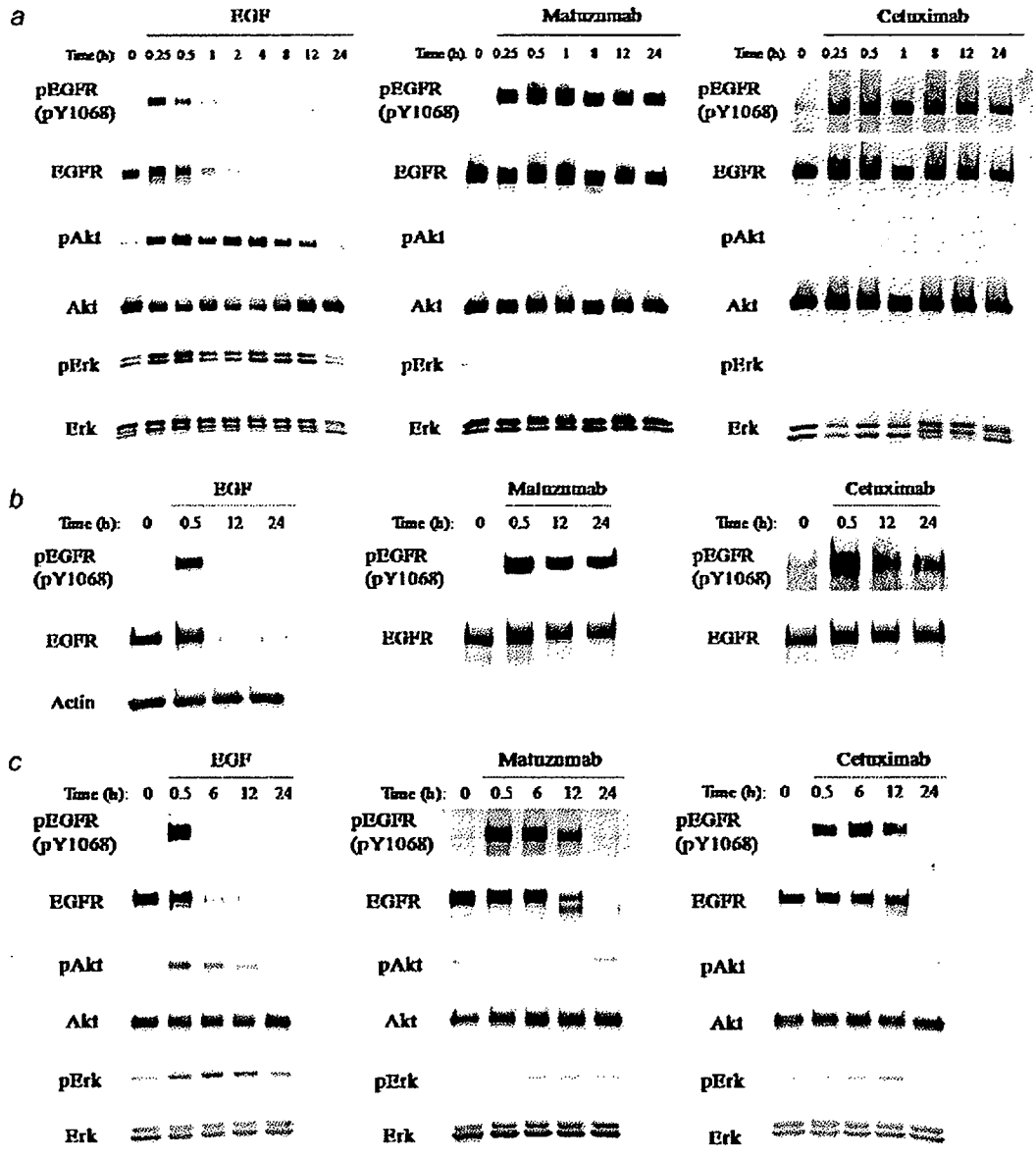

FIGURE 3 – Lack of EGFR turnover in cells treated with matuzumab or cetuximab. (*a*) H292 cells were deprived of serum overnight and then incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), respectively. Cell lysates were subjected to immunoblot 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 theY1068-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

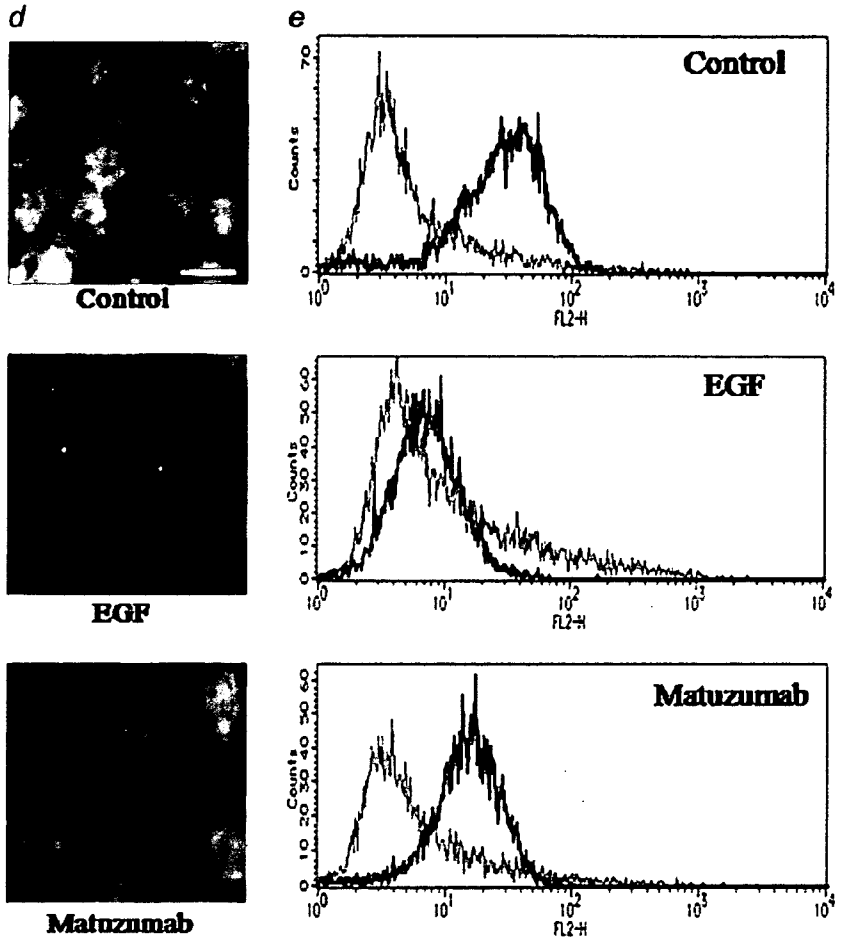

FIGURE 3 – CONTINUED

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

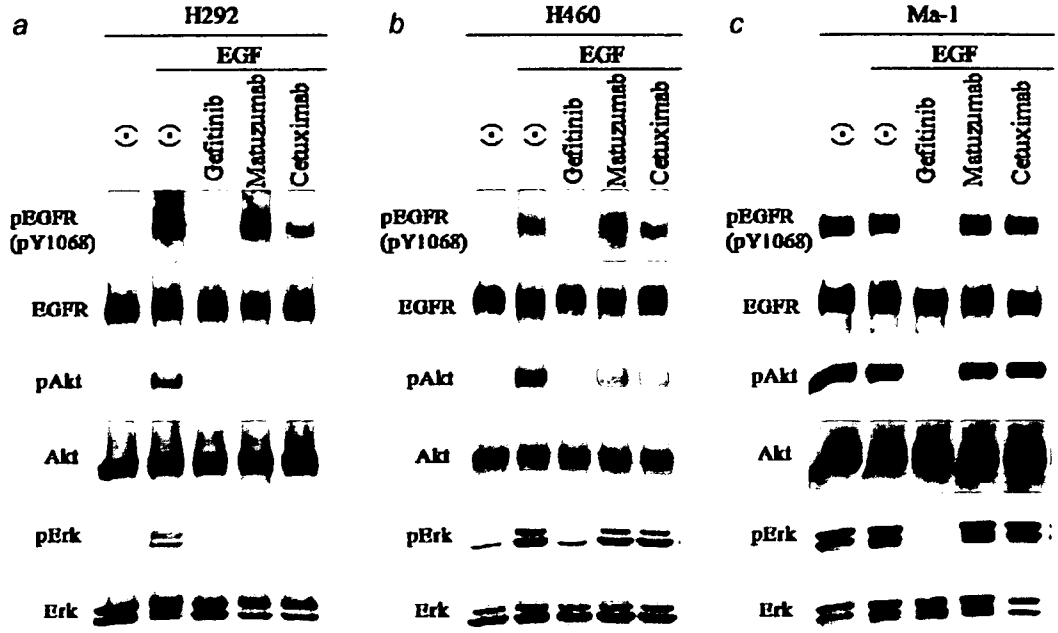

FIGURE 4 – Effects of matuzumab and cetuximab on EGF-induced EGFR signaling. H292 (*a*), H460 (*b*) and Ma-1 (*c*) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or gefitinib (10 μM) and then for an additional 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.

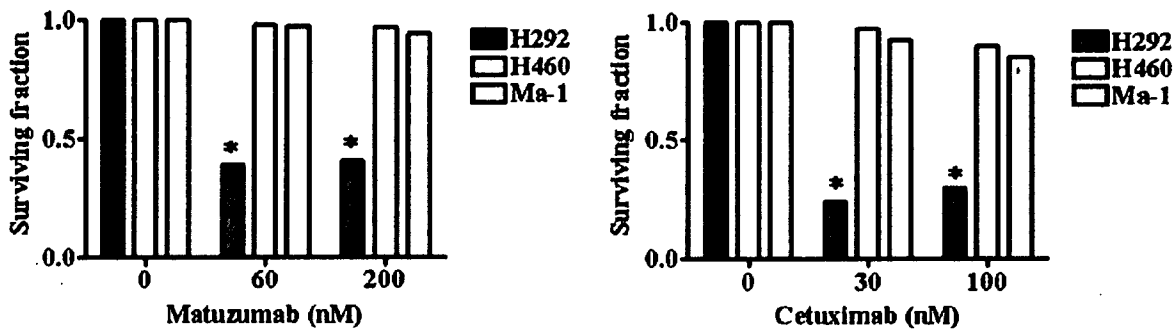

FIGURE 5 – Effects of matuzumab and cetuximab on cell survival. H292, H460 or Ma-1 cells were plated at a density of 200 cells per 25-cm^2 flask in triplicate and cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated with medium alone 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.

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