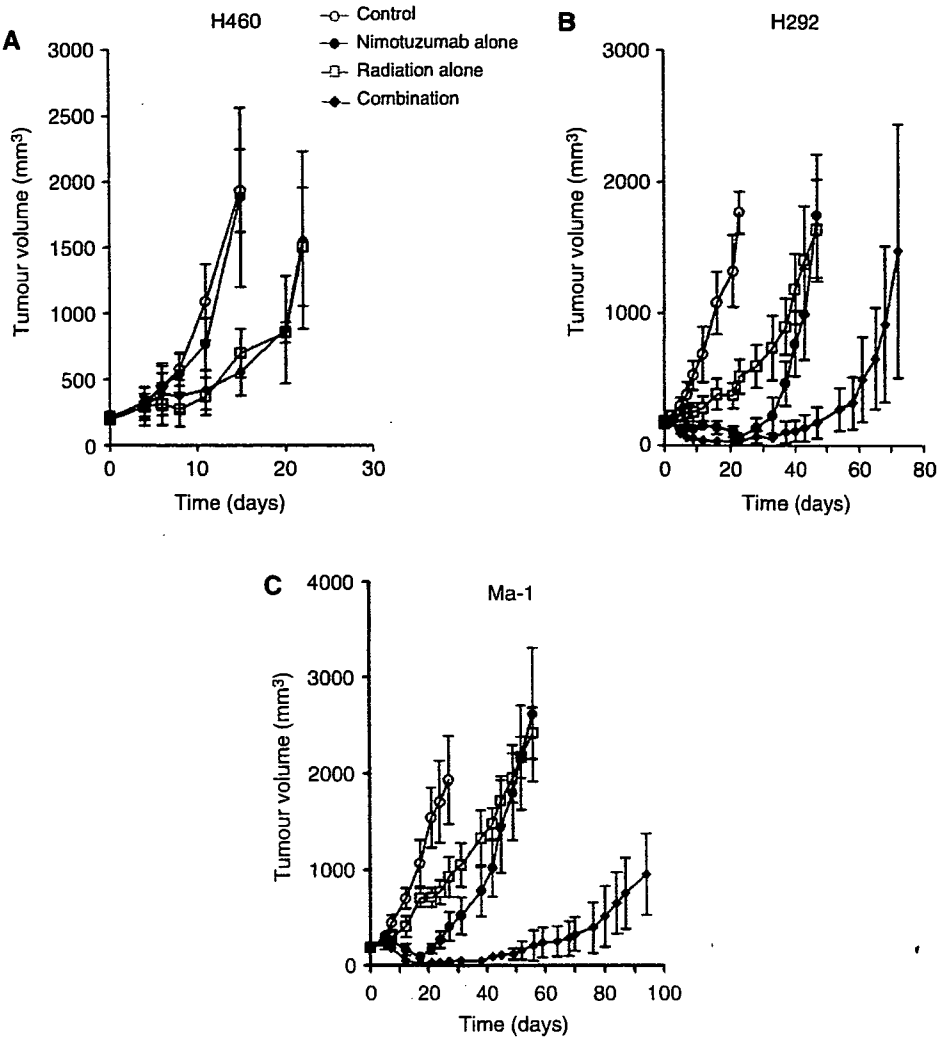


**Figure 4** Effect of nimotuzumab on the response of NSCLC cells to radiation *in vivo*. H460 (**A**), H292 (**B**), or Ma-1 (**C**) cells were injected subcutaneously in athymic nude mice. Treatment was initiated when tumours in each group achieved an average volume of approximately 170–200 $mm^3$. Mice were treated with a single dose of nimotuzumab (1.0 mg per mouse) intraperitoneally, a single dose of $\gamma$-radiation (10 Gy), or neither (control) or both modalities, and tumour volume was determined at the indicated time points thereafter. Data are means ± s.d. for seven to eight mice per group.

**Table 2** Tumour growth delay in nude mice treated with nimotuzumab, radiation, or both modalities

| | H460 | | H292 | | Ma-1 | |
|---|---|---|---|---|---|---|
| **Treatment** | **Days[a]** | **GD[b]** | **Days** | **GD** | **Days** | **GD** |
| Control | 10.4 | | 13.2 | | 15.1 | |
| Nimotuzumab alone | 11.8 | 1.4 | 40.4 | 27.2 | 41.8 | 26.7 |
| Radiation alone | 20.4 | 10.0 | 32.8 | 19.6 | 28.1 | 13.0 |
| Nimotuzumab+radiation | 20.5 | 10.1 | 66.8 | 53.6 | 93.4 | 78.3 |
| Enhancement factor | 0.86 | | 1.3 | | 4.0 | |

GD = growth delay [a]Time required for xenografts in each group to achieve a fivefold increase in volume. [b]The additional time (days) required for xenografts in each treatment group to achieve a fivefold increase in volume relative to the corresponding time for xenografts in the control group.

than additive. No pronounced tissue damage or toxicities such as diarrhoea or a decrease in body weight of >10% were observed in mice in any of the four treatment groups. These results thus suggested that nimotuzumab potentiated the antitumor activity of radiation in H292 and Ma-1 cells *in vivo* as well as *in vitro*.

## DISCUSSION

Somatic mutations in the EGFR kinase domain and *EGFR* amplification have been associated with a better response to EGFR-TKIs, such as gefitinib and erlotinib, in patients with NSCLC (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005). Given that little is known of the relation between such *EGFR* alterations and the response to treatment with anti-EGFR mAbs, we investigated the antitumor effect of combined treatment with the anti-EGFR mAb nimotuzumab and radiation in NSCLC cell lines of differing *EGFR* status.

The antitumor effect of EGFR-specific mAbs has been thought to result from inhibition of ligand binding to EGFR and consequent inhibition of EGFR activation (Li *et al*, 2005; Marshall, 2006). We, therefore, examined the effect of nimotuzumab on EGF-dependent EGFR signalling. Nimotuzumab inhibited the EGF-induced or constitutive phosphorylation of EGFR in H292 and Ma-1 cells (with high and moderate levels of surface EGFR expression, respectively), consistent with the mode of action of this antibody. However, nimotuzumab did not block EGF-induced or constitutive EGFR phosphorylation in H460, H1299, or H1975 cells (all with a

low level of surface EGFR expression). These observations suggest that the inhibitory effect of nimotuzumab on EGFR signalling depends on the expression level of EGFR on the cell surface. A clonogenic cell survival assay revealed that nimotuzumab enhanced the cytotoxic effect of radiation in H292 and Ma-1 cells, but not that in H460, H1299, or H1975 cells. These findings support the notion that the inhibition of EGFR signalling by nimotuzumab is responsible, at least in part, for the enhancement of the cytotoxic effect of radiation by this antibody. Irradiation of tumour cells has been shown to activate EGFR via ligand-independent and ligand-dependent mechanisms, possibly accounting for radiation-induced acceleration of tumour cell repopulation and the development of radioresistance (Schmidt-Ullrich *et al*, 1997, 2003; Dent *et al*, 2003). Such radiation-induced activation of EGFR-dependent processes may represent a rationale for combined treatment with radiation and EGFR inhibitors. It remains to be determined whether nimotuzumab is able to block radiation-induced activation of EGFR.

Consistent with our *in vitro* results, we found that nimotuzumab enhanced the antitumor effect of radiation on H292 or Ma-1 cells in nude mice. Such enhancement was not apparent for tumours formed by H460 cells. Nimotuzumab alone also manifested a substantial antitumor effect for xenografts formed by H292 or Ma-1 cells but not for those formed by H460 cells. Together these results suggest that the efficacy of nimotuzumab monotherapy is a prerequisite for augmentation of radioresponse by this mAb. Nimotuzumab was previously shown to induce the regression of A431 tumour xenografts *in vivo* as a result of inhibition of both tumour cell proliferation and tumour angiogenesis (Crombet-Ramos *et al*, 2002). Immunohistochemical analysis of tumour specimens from head and neck cancer patients treated with the combination of nimotuzumab and radiation also showed evidence of antiproliferative and antiangiogenic effects (Crombet *et al*, 2004). These observations suggest that effects of nimotuzumab on both NSCLC cell proliferation and tumour angiogenesis might contribute to the enhancement of the antitumor efficacy of radiation by this antibody observed in the present study. Enhancement of the anticancer effect of radiation by the anti-EGFR mAb cetuximab was previously shown to be increased by transfection of cells to upregulate the level of EGFR expression, suggesting that potentiation of the antitumor efficacy of radiation by anti-EGFR mAbs is related to the absolute level of EGFR expression (Liang *et al*, 2003; Bonner *et al*, 2004). This finding is consistent with our present results showing that potentiation of the antitumor activity of radiation by nimotuzumab was related to the level of surface EGFR expression. The nimotuzumab-resistant cell line H460 harbours a mutant form of *KRAS* (Balko *et al*, 2006) that has been associated with resistance to cetuximab (Lievre *et al*, 2006). However, we found that nimotuzumab also failed to inhibit EGF-induced EGFR phosphorylation and to enhance the cytotoxic effect of radiation in H1299 cells, which harbour wild-type *KRAS* (Coldren *et al*, 2006). These observations thus support the notion that a low level of EGFR expression at the cell surface is related to resistance to combined treatment with nimotuzumab and radiation, irrespective of *KRAS* status.

We demonstrated that nimotuzumab inhibited EGFR phosphorylation and enhanced the antitumor effect of radiation in *EGFR* mutant Ma-1 cells (with a moderate level of surface EGFR expression) but not in *EGFR*-mutant H1975 cells (with a low level of surface EGFR expression). Nimotuzumab also potentiated the cytotoxic effect of radiation in H292 cells, which harbour wild-type *EGFR* alleles and have a high level of surface EGFR expression. These findings support the notion that *EGFR* mutation is not the major determining factor for enhancement of the antitumor effect of radiation by nimotuzumab, consistent with previous observations with cetuximab (Barber *et al*, 2004; Tsuchihashi *et al*, 2005). However, the mechanisms underlying such enhancement of the antitumor effect of radiation may differ between NSCLC cells harbouring wild-type or mutant *EGFR* alleles. We and others have previously shown that mutations in the tyrosine kinase domain of EGFR are associated with increased ligand-independent tyrosine kinase activity of EGFR (Lynch *et al*, 2004) and aberrant EGFR signalling (Amann *et al*, 2005; Okabe *et al*, 2007). Given that cell-cycle checkpoints activated by ionising radiation are defective in *EGFR*-mutant NSCLC cell lines (Das *et al*, 2006), the constitutive activity of EGFR in such cells may result in unchecked DNA synthesis and in apoptosis on exposure to ionising radiation. It is possible that these defects in *EGFR*-mutant cells affect the enhancement of the antitumor efficacy of radiation by nimotuzumab.

In summary, we have shown that nimotuzumab enhanced the antitumor efficacy of radiation *in vitro* and *in vivo*, providing a rationale for future clinical investigations of the therapeutic efficacy of nimotuzumab in combination with radiotherapy. Our data suggest that potentiation of the antitumor activity of radiation by nimotuzumab may be related to the level of EGFR expression at the cell surface rather than to *EGFR* mutation. The preselection of patients on the basis of genetic factors that predict treatment sensitivity or resistance may thus be required for the combination therapy with nimotuzumab and radiation.

## ACKNOWLEDGEMENTS

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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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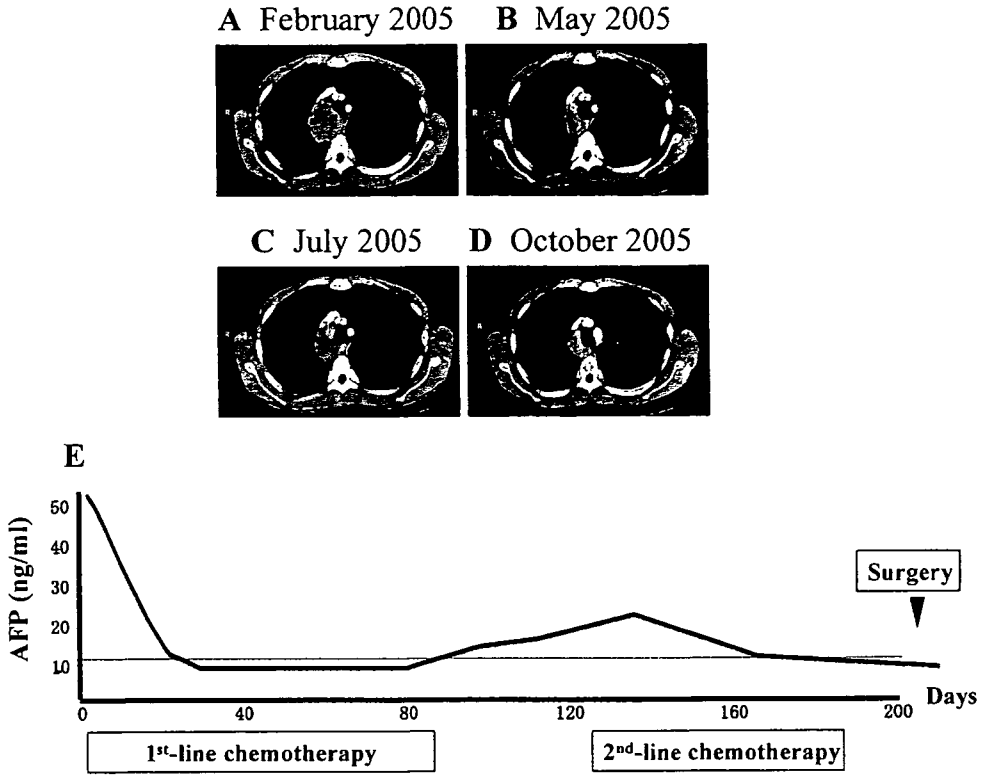


**FIGURE 1.** Chest computed tomography (CT) findings and serum AFP levels in the patient. *A–D*, Chest CT findings. A mass in the middle mediastinum was initially detected (*A*). The tumor had shrunk after three cycles of neoadjuvant chemotherapy (*B*), but its progression had resumed after the fourth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

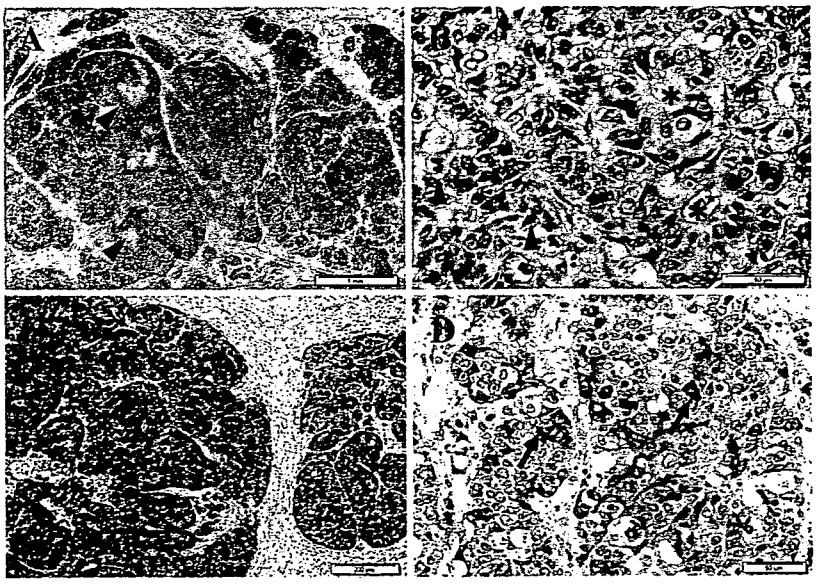


**FIGURE 2.** Histology and immunohistochemical analysis of the tumor specimen obtained at surgery. *A*, Hematoxylin-eosin staining revealed solid tumor nests with areas of necrosis (arrow heads). Note the homogeneous appearance of the tumor. *B*, High-power magnification of the tumor stained as in (*A*), 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 $\mu$.

## 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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# Synergistic antitumor effect of S-1 and the epidermal growth factor receptor inhibitor gefitinib in non-small cell lung cancer cell lines: role of gefitinib-induced down-regulation of thymidylate synthase

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## Abstract

**Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are associated with the therapeutic response to EGFR tyrosine kinase inhibitors (TKI) in patients with advanced non-small cell lung cancer (NSCLC). The response rate to these drugs remains low, however, in NSCLC patients with wild-type *EGFR* alleles. Combination therapies with EGFR-TKIs and cytotoxic agents are considered a therapeutic option for patients with NSCLC expressing wild-type *EGFR*. We investigated the antiproliferative effect of the combination of the oral fluorouracil S-1 and the EGFR-TKI gefitinib in NSCLC cells of differing *EGFR* status. The combination of 5-fluorouracil and gefitinib showed a synergistic antiproliferative effect *in vitro* in all NSCLC cell lines tested. Combination chemotherapy with S-1 and gefitinib *in vivo* also had a synergistic antitumor effect on NSCLC xenografts regardless of the absence or presence of *EGFR* mutations. Gefitinib inhibited the expression of the transcription factor E2F-1, resulting in the down-regulation of thymidylate synthase at the mRNA and protein levels. These observations suggest that gefitinib-induced down-regulation of thymidylate synthase is responsible, at least in part, for the synergistic antitumor effect of combined treatment with S-1 and gefitinib and provide a basis for clinical evaluation of combination chemotherapy with S-1 and EGFR-TKIs in patients with solid tumors. [Mol Cancer Ther 2008;7(3):599–606]**

## Introduction

Targeted therapy in the treatment of cancer has made substantial progress over the last few years. The ErbB family of receptor tyrosine kinases includes the epidermal growth factor receptor (EGFR; ErbB1), ErbB2 (HER2/*neu*), ErbB3, and ErbB4 and is important for normal development as a result of its roles in cell proliferation and differentiation (1–3). Aberrant expression of EGFR has been detected in a wide range of human epithelial malignancies, including non-small cell lung cancer (NSCLC), and is correlated with poor prognosis and reduced survival time (4, 5). Agents that specifically target EGFR are therefore under development as anticancer drugs. Indeed, two inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI), gefitinib and erlotinib, both of which compete with ATP for binding to the catalytic pocket of the receptor, have been extensively studied in individuals with NSCLC (6–9). Somatic mutations in the region of *EGFR* that encodes the tyrosine kinase domain have been associated with tumor responsiveness to EGFR-TKIs in a subset of NSCLC patients (10–17). In contrast, achievement of a clinical benefit of these drugs in NSCLC patients who express wild-type *EGFR* has been problematic.

S-1 (Taiho Pharmaceutical) is an oral anticancer agent composed of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate in a molar ratio of 1:0.4:1 (18). Tegafur is a prodrug that generates 5-fluorouracil (5-FU) in blood largely as a result of its metabolism by cytochrome *P*450 in the liver. CDHP increases the plasma concentration of 5-FU through competitive inhibition of dihydropyrimidine dehydrogenase (DPD), which catalyzes 5-FU catabolism (19). Oxonate reduces the gastrointestinal toxicity of 5-FU (20). A response rate of 22% and a median survival time of 10.2 months were obtained in a clinical trial of S-1 in patients with advanced NSCLC not subjected previously to chemotherapy (21). Few severe gastrointestinal or hematologic adverse events were reported. Moreover, a phase II trial of S-1 plus cisplatin in NSCLC patients revealed a 47% response rate and an acceptable safety profile (22).

Based on this background, we examined the anticancer effect of the combination of S-1 and gefitinib in NSCLC cell lines of differing *EGFR* status. We found that the combination of S-1 (or 5-FU) and gefitinib exhibited a marked and synergistic antiproliferative effect both *in vivo*

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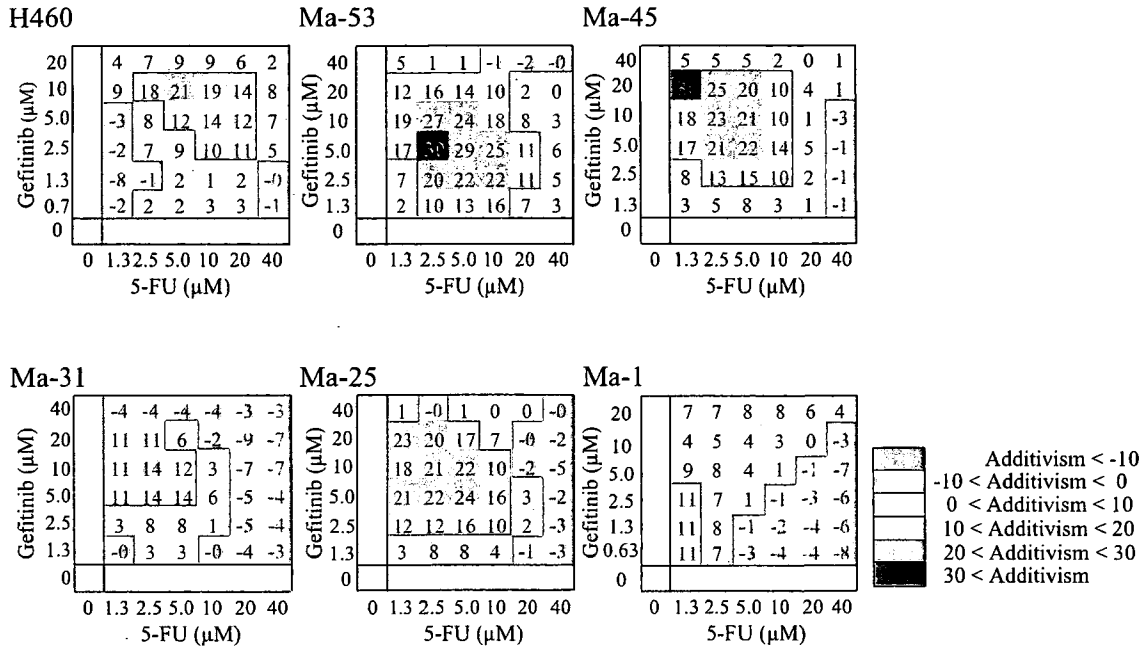


**Figure 1.** Inhibition of NSCLC cell growth by the combination of 5-FU and gefitinib *in vitro*. Cells with wild-type (H460, Ma-53, Ma-45, Ma-31, and Ma-25) or mutant (Ma-1) EGFR alleles were exposed for 72 h to 5-FU and gefitinib at the indicated concentrations, after which cell viability was measured with a colorimetric assay. The observed excess inhibition (%) relative to that predicted by the Bliss additivism model is shown color-coded in a drug concentration matrix for each cell line. Yellow, orange, pink, and red, synergy; light and dark blue, antagonism. Mean of triplicates from a representative experiment.

and *in vitro* in cells regardless of the absence or presence of *EGFR* mutations. Furthermore, we assessed the effects of gefitinib on the expression of enzymes that function in 5-FU metabolism, including thymidylate synthase (TS), DPD, and orotate phosphoribosyltransferase (OPRT), to gain insight into the mechanism underlying the synergistic effect of combination therapy with S-1 and gefitinib.

## Materials and Methods

### Cell Lines and Reagents

The human NSCLC cell lines NCI-H460 (H460), Ma-1, Ma-25, Ma-31, Ma-45, and Ma-53 were obtained as described previously (23). MiaPaca-2 cells were obtained from Japan Health Sciences Foundation. These cell lines were cultured under a humidified atmosphere of 5% $CO_2$ at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. Gefitinib was provided by AstraZeneca. S-1 and CDHP were provided by Taiho Pharmaceutical. 5-FU was obtained from Wako.

### Growth Inhibition Assay *In vitro*

Cells ($2.0 \times 10^3$) were plated in 96-well flat-bottomed plates and cultured for 24 h before the addition of various concentrations of 5-FU and gefitinib and incubation for an additional 72 h. Cell Counting Kit-8 solution (Dojindo) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 450 nm. Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of 5-FU or gefitinib resulting in 50% growth inhibition ($IC_{50}$) was calculated. The effect of combining 5-FU and gefitinib was classified as additive, synergistic, or antagonistic with the Bliss additivism model (24–26). A theoretical curve was calculated for combined inhibition with the equation: $E_{bliss} = E_A + E_B - (E_A \times E_B)$, where $E_A$ and $E_B$ are the fractional inhibitory effects of drug A alone and drug B alone at specific concentrations. $E_{bliss}$ is then the fractional inhibition that would be expected if the effect of the combination of the two drugs was exactly additive. In this study, the Bliss variable is expressed as percentage decrease in cell growth above what would be expected for the combination. Bliss = 0 indicates that the effect of the combination is additive; Bliss > 0 is indicative of synergy; and Bliss < 0 indicates antagonism.

### Animals

Male athymic nude mice were exposed to a 12-h light, 12-h dark cycle and provided with food and water *ad libitum* in a barrier facility. All experiments were done in compliance with the regulations of the Animal Experimentation Committee of Taiho Pharmaceutical.

### Growth Inhibition Assay *In vivo*

Cubic fragments of tumor tissue (~2 × 2 × 2 mm) were implanted s.c. into the axilla of 5- to 6-week-old male athymic nude mice. Treatment was initiated when tumors in each group achieved an average volume of 100 to 150 $mm^3$. Treatment groups consisted of control, S-1 alone, gefitinib alone, and the combination of S-1 and gefitinib. Each treatment group contained seven mice. S-1 (10 mg/kg body mass) and gefitinib (50 or 3 mg/kg) were administered by oral gavage once a day for 14 days; control animals

received 0.5% (w/v) hydroxypropylmethylcellulose as vehicle. Tumor volume was determined from caliper measurements of tumor length ($L$) and width ($W$) according to the formula $LW^2$ / 2. Both tumor size and body weight were measured two or three times per week.

### Immunoblot Analysis

Cell lysates were fractionated by SDS-PAGE on 12% gels (NuPAGE Bis-Tris Gels; Invitrogen), and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites with 5% skim milk, the membrane was incubated overnight at room temperature with primary antibodies. Antibodies to DPD, OPRT, and TS were obtained from Taiho Pharmaceutical; those to E2F-1 were from Santa Cruz Biotechnology; and those to β-actin (loading control) were from Sigma. Immune complexes were detected by incubation of the membrane for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to mouse or rabbit immunoglobulin and by subsequent exposure to enhanced chemiluminescence reagents (Pierce).

### Immunoprecipitation Analysis

Immunoprecipitation of EGFR was done according to standard procedures. Whole-cell lysates (800 μg protein) were incubated overnight at 4°C with antibodies to EGFR (Santa Cruz Biotechnology), after which Protein G Plus/Protein A-Agarose Suspension (Calbiochem) was added and the mixtures were incubated for an additional 1 h at 4°C. Immunoprecipitates were isolated, washed, resolved by SDS-PAGE on a 7.5% gel (Bio-Rad), and subjected to immunoblot analysis with antibodies to phosphotyrosine (PY20) and EGFR (Zymed).

### Reverse Transcription and Real-time PCR Analysis

Total RNA (1 μg) extracted from cells with the use of an RNeasy Mini Kit (Qiagen) was subjected to reverse transcription with the use of a SuperScript Preamplification System (Invitrogen Life Technologies). The resulting cDNA was then subjected to real-time PCR analysis with the use of a TaqMan PCR Reagent Kit and a Gene Amp 5700 Sequence Detection System (Applied Biosystems). The forward and reverse primers and TaqMan probe for TS cDNA were 5-GCCTCGGTGTGCCTTTCA-3 and 5-CCCGTGATGTGCGCAAT-3 and 6-FAM-5'-TCGCCA-GCTACGCCCTGCTCA-3'-TAMRA, respectively. Glyceraldehyde-3-phosphate dehydrogenase mRNA were used as an internal standard.

### Statistical Analysis

Data are presented as mean ± SE and were analyzed by the Aspin-Welch $t$ test. $P < 0.05$ was considered statistically significant.

## Results

### Effect of the Combination of 5-FU and Gefitinib on NSCLC Cell Growth *In vitro*

Tegafur, which is a component of S-1, is metabolized to 5-FU in the liver and exerts antitumor effects. We first examined the antiproliferative activity of the combination of 5-FU and gefitinib in six NSCLC cell lines. Five of the cell lines (H460, Ma-53, Ma-45, Ma-31, and Ma-25) possess wild-type *EGFR* alleles, whereas Ma-1 cells harbor an *EGFR* mutation (E746_A750del) that is associated with a high response rate to the EGFR-TKIs gefitinib and erlotinib in individuals with advanced NSCLC. We assessed

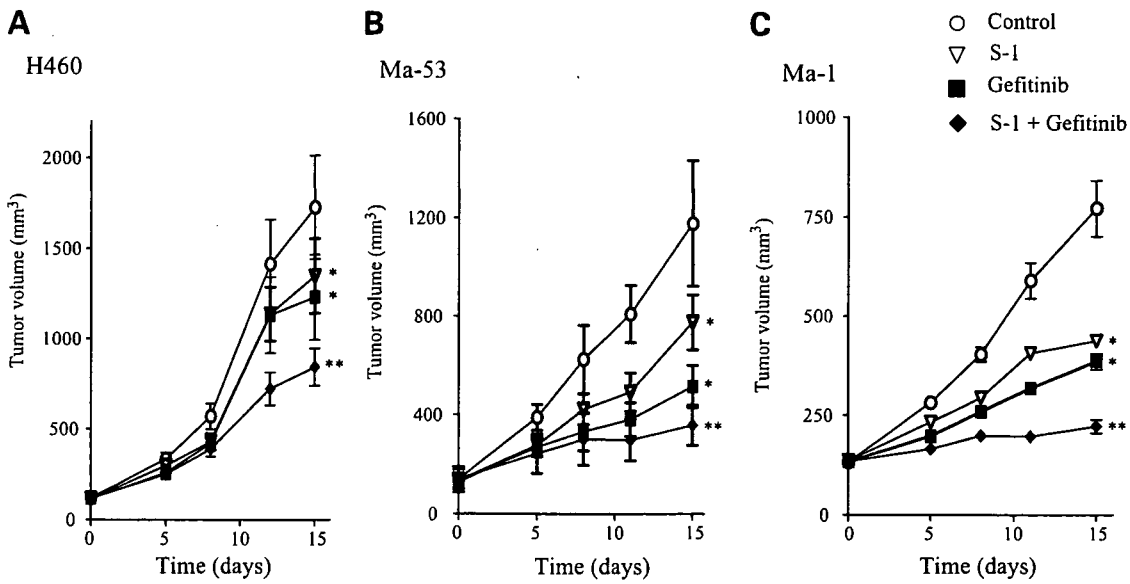


**Figure 2.** Antitumor activity of the combination of S-1 and gefitinib *in vivo*. **A** and **B**, nude mice with tumor xenografts established by s.c. implantation of NSCLC cells (H460 and Ma-53) possessing wild-type EGFR were treated daily for 2 wk with vehicle (control), S-1 (10 mg/kg), gefitinib (50 mg/kg), or both drugs by oral gavage. **C**, nude mice with tumor xenografts derived from NSCLC cells (Ma-1) expressing mutant EGFR were treated daily for 2 weeks with vehicle (control), S-1 (10 mg/kg), gefitinib (3 mg/kg), or both drugs by oral gavage. Tumor volume in all animals was determined at the indicated times after the onset of treatment. Mean ± SE of values from seven mice per group. *, $P < 0.05$ versus control; **, $P < 0.05$ versus S-1 or gefitinib alone for values 15 d after treatment onset (Aspin-Welch $t$ test).

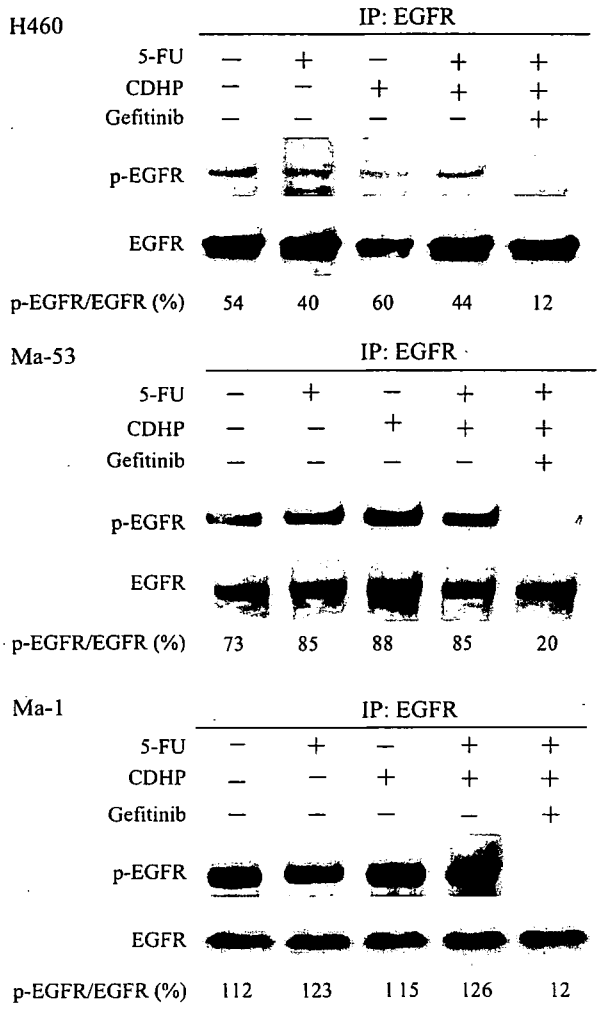


**Figure 3.** Lack of effect of 5-FU and CDHP on EGFR phosphorylation in NSCLC cell lines. NSCLC cells (H460, Ma-53, and Ma-1) were incubated for 24 h in medium supplemented with 2% fetal bovine serum and with 5-FU (10 μmol/L), CDHP (3 μmol/L), or gefitinib (5 μmol/L). Cell lysates were then prepared and subjected to immunoprecipitation (*IP*) with antibodies to EGFR, and the resulting precipitates were subjected to immunoblot analysis with antibodies to phosphotyrosine (for detection of phosphorylated EGFR) and with antibodies to EGFR. The intensity of the phosphorylated EGFR band relative to that of the EGFR band was determined by densitometry and is expressed as a percentage below each lane.

whether 5-FU and gefitinib showed additivity, synergy, or antagonism based on the Bliss additivism model (24–26). We chose this model rather than isobologram or combination index analysis because it would allow us to evaluate the nature of drug interactions even in instances in which the maximal inhibition by 5-FU or gefitinib alone was too low to obtain a reliable $IC_{50}$ value. The six test concentrations for each agent were chosen after first determining the corresponding $IC_{50}$ values. The $IC_{50}$ values for 5-FU chemosensitivity were not associated with *EGFR* status and ranged from 7 to 11 μmol/L. The effect of combined treatment with 5-FU and gefitinib on the proliferation of the six NSCLC cell lines was tested in triplicate in a 6 × 6 concentration matrix. Calculation of the percentage inhibition in excess of that predicted by the Bliss additivism model revealed synergistic effects of Bliss > 0 for 5-FU and gefitinib in all of the six cell lines tested (Fig. 1). These results suggested that 5-FU and gefitinib act synergistically to inhibit cell growth in NSCLC cells.

### Effect of Combined Treatment with S-1 and Gefitinib on NSCLC Cell Growth *In vivo*

We therefore next investigated whether combined treatment with S-1 and gefitinib might also exert a synergistic effect on NSCLC cell growth *in vivo*. Doses of both agents were selected so that their independent effects on tumor growth would be moderate. Nude mice were implanted s.c. with H460, Ma-53, or Ma-1 tumor fragments to establish tumor xenografts. When the H460 or Ma-53 tumors, which harbor wild-type *EGFR*, became palpable (100-150 $mm^3$), the mice were divided into four groups for daily treatment with vehicle, S-1 (10 mg/kg), gefitinib (50 mg/kg), or both drugs by oral gavage over 2 weeks. For xenografts formed by H460 or Ma-53 cells, combination therapy with S-1 and gefitinib resulted in a significant reduction in tumor size compared with that apparent in animals treated with S-1 or gefitinib alone (Fig. 2*A* and *B*). Mice bearing Ma-1 tumors, which express mutant *EGFR*, were treated with vehicle, S-1 (10 mg/kg), gefitinib (3 mg/kg), or both agents daily over 2 weeks. Combination treatment with S-1 and gefitinib significantly inhibited the growth of Ma-1 xenografts relative to that apparent in mice treated with either agent alone (Fig. 2C). None of the drug treatments induced a weight loss of >20% during the 2-week period, and no signs of overt drug toxicity were apparent (data not shown). These results thus suggested that combination chemotherapy with S-1 and gefitinib *in vivo* had a synergistic antitumor effect on NSCLC xenografts regardless of the absence or presence of *EGFR* mutations, consistent with our results *in vitro*.

### Effects of 5-FU and CDHP on EGFR Phosphorylation in NSCLC Cell Lines

To investigate the mechanism responsible for the observed interaction between S-1 and gefitinib, we examined the effect of 5-FU on EGFR signal transduction in NSCLC cells expressing wild-type (H460 and Ma-53) or mutant (Ma-1) *EGFR*. Immunoprecipitation analysis revealed that exposure of H460 or Ma-53 cells to 5-FU (10 μmol/L) for 24 h had no effect on the basal level of EGFR phosphorylation (Fig. 3). We have shown previously that EGFR is constitutively phosphorylated in Ma-1 cells maintained in serum-free medium (23). Exposure of Ma-1 cells to 5-FU for 24 h did not affect this constitutive level of EGFR phosphorylation (Fig. 3). We next examined the effects of both CDHP, which is a component of S-1, and the combination of CDHP and 5-FU on EGFR phosphorylation in H460, Ma-53, and Ma-1 cells. Neither CDHP alone nor the combination of CDHP and 5-FU affected the level of EGFR phosphorylation in any of these three cell lines (Fig. 3). These results thus indicated that 5-FU and CDHP have no effect on EGFR signal transduction.

### Effects of Gefitinib on the Expression of DPD, OPRT, and TS in NSCLC Cell Lines

We next investigated whether gefitinib might affect the expression of DPD, OPRT, or TS, enzymes that are major determinants of the sensitivity of cells to 5-FU. We first examined the abundance of these enzymes in the NSCLC cell lines H460, Ma-53, and Ma-1 by immunoblot analysis. The expression of DPD was detected in MiaPaca-2 cells (positive control) but not in H460, Ma-53, or Ma-1 cells (Fig. 4*A*). In contrast, OPRT and TS were detected in all three NSCLC cell lines and their abundance did not appear related to *EGFR* status (Fig. 4*A*). Treatment of H460, Ma-53, or Ma-1 cells with gefitinib (5 μmol/L) for up to 48 h resulted in a time-dependent decrease in the amount of TS, whereas that of OPRT or DPD remained unaffected (Fig. 4*B*). A reduced level of TS expression in tumors has been associated previously with a higher response rate to 5-FU-based chemotherapy (27, 28). Our data thus suggested that the suppression of TS expression by gefitinib might increase the sensitivity of NSCLC cells to 5-FU.

The transcription factor E2F-1 regulates expression of the TS gene (29–31). We therefore examined the possible effect of gefitinib on E2F-1 expression in NSCLC cell lines. Incubation of H460, Ma-53, or Ma-1 cells with gefitinib for up to 48 h also induced a time-dependent decrease in the amount of E2F-1 (Fig. 4*B*), suggesting that this effect might contribute to the down-regulation of TS expression by gefitinib in these cell lines.

### Effect of Gefitinib on TS mRNA Abundance in NSCLC Cell Lines

The abundance of TS mRNA would be expected to be decreased if the down-regulation of E2F-1 expression by gefitinib was responsible for the reduced level of TS. We determined the amount of TS mRNA in H460, Ma-53, or Ma-1 cells at various times after exposure to gefitinib with the use of reverse transcription and real-time PCR analysis. Gefitinib indeed induced a time-dependent decrease in the amount of TS mRNA in all three NSCLC cell lines (Fig. 5), suggesting that the down-regulation of TS expression by gefitinib occurs at the transcriptional level and may be due to suppression of E2F-1 expression.

## Discussion

The recent identification of activating somatic mutations of *EGFR* in NSCLC and their relevance to prediction of the therapeutic response to EGFR-TKIs such as gefitinib and erlotinib have had a major effect on NSCLC treatment (10–17). The response rate to these drugs remains low, however, in NSCLC patients with wild-tye *EGFR* alleles. Combination therapy with EGFR-TKIs and cytotoxic agents is a potential alternative strategy for NSCLC expressing wild-type *EGFR*. In the present study, we have evaluated the potential cooperative antiproliferative effect of combined treatment with the EGFR-TKI gefitinib and the new oral fluorouracil S-1 in NSCLC cell lines of differing *EGFR* status. We found that S-1 (or 5-FU) and gefitinib exert a synergistic antiproliferative effect on NSCLC cells both *in vivo* and *in vitro* regardless of the absence or presence of *EGFR* mutation. We chose a gefitinib dose of 50 mg/kg for treatment of mice bearing H460 or Ma-53 tumors. The median effective dose of gefitinib was shown previously to be ~50 mg/kg in athymic nude mice bearing A431 cell-derived xenografts (32). A gefitinib dose of 50 mg/kg has therefore subsequently been widely used in tumor xenograft studies (33–36). The U.S. Food and Drug Administration recommends that drug doses in animals be converted to those in humans based on body surface area (37). According to this guideline, a gefitinib dose of 50 mg/kg in mouse xenograft models is approximately equivalent to the therapeutic dose (250 mg/d) of the drug in humans. In addition, the tumor concentrations of gefitinib in NSCLC xenografts of mice treated with this drug (50 mg/kg) ranged from 9.7 to 13.3 μg/g, values that were similar to the

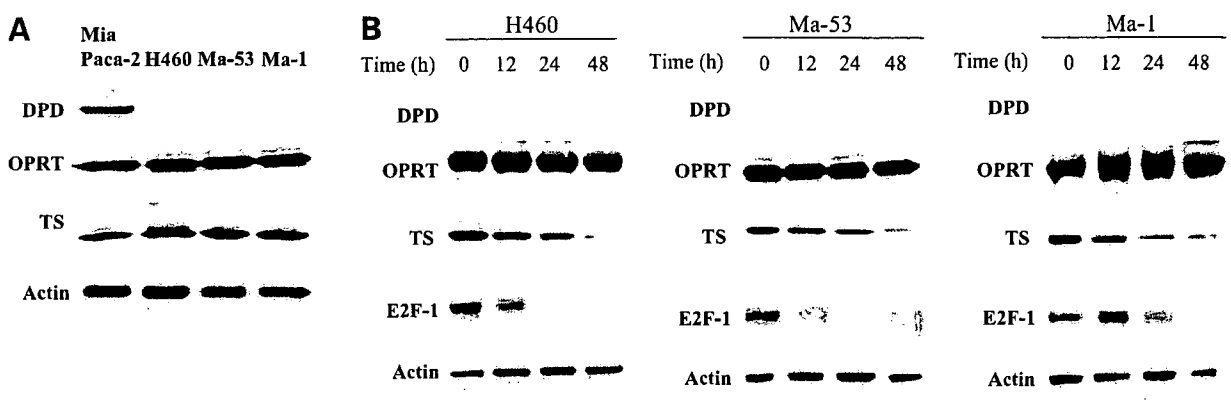


**Figure 4.** Effects of gefitinib on the expression of E2F-1, DPD, OPRT, and TS in NSCLC cell lines. **A**, lysates of H460, Ma-53, or Ma-1 cells were subjected to immunoblot analysis with antibodies to DPD, OPRT, TS, or β-actin (loading control). MiaPaca-2 cells were also examined as a positive control for DPD expression. **B**, NSCLC cells were incubated with gefitinib (5 μmol/L) for the indicated times in medium containing 10% serum, after which cell lysates were subjected to immunoblot analysis as in **A**, with the addition that E2F-1 expression was also examined.

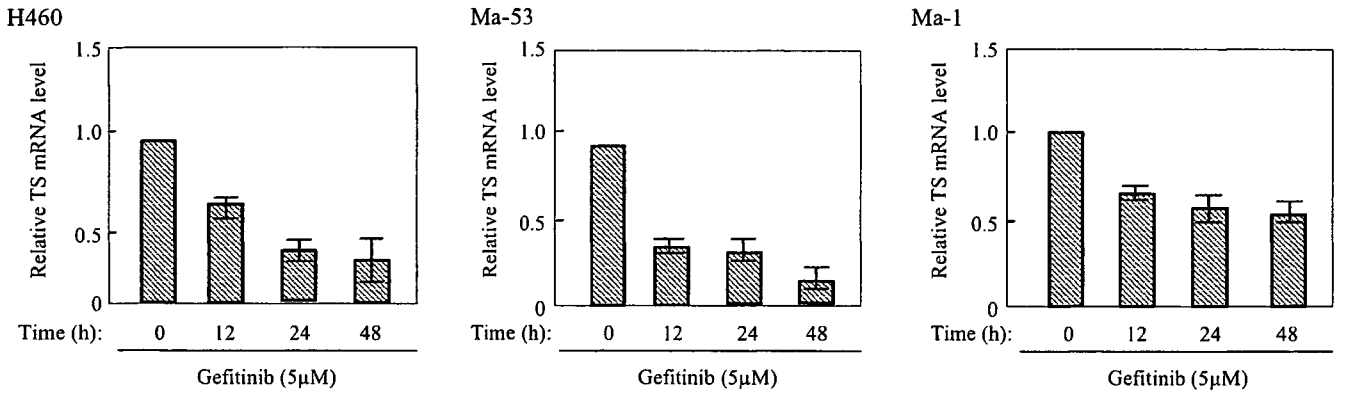


**Figure 5.** Down-regulation of TS mRNA by gefitinib in NSCLC cell lines. H460, Ma-53, or Ma-1 cells were incubated with gefitinib (5 μmol/L) for the indicated times in medium containing 10% serum, after which total RNA was extracted from the cells and subjected to reverse transcription and real-time PCR analysis of TS mRNA. The amount of TS mRNA was normalized by that of glyceraldehyde-3-phosphate dehydrogenase mRNA. Mean ± SE of values from three separate experiments.

achievable concentrations of gefitinib in tumor tissues of treated humans (34). These observations suggest that a gefitinib dose of 50 mg/kg in mouse xenograft models is appropriate for mimicking the therapeutic dose in humans.

EGFR-TKIs have been shown previously to act synergistically with radiation or cytotoxic agents such as cisplatin, paclitaxel, and irinotecan (38–40). These cytotoxic agents and radiation have been shown to increase the phosphorylation level of EGFR, possibly reflecting the activation of prosurvival signaling, and this effect is blocked by EGFR-TKIs, resulting in the synergistic antitumor effects of the combination therapies. Such a synergistic effect of 5-FU and gefitinib was attributed to 5-FU-induced EGFR phosphorylation in colorectal cancer cells (41). In contrast, we found that 5-FU had no effect on the level of EGFR phosphorylation in NSCLC cell lines. Further examination of different concentrations of 5-FU and different exposure times also failed to reveal an effect of 5-FU on EGFR phosphorylation in these cells (data not shown). These findings indicate that NSCLC cell lines respond differently to 5-FU than do colorectal cancer cells and that the synergistic antiproliferative effect of 5-FU and gefitinib in NSCLC cells is not mediated at the level of EGFR phosphorylation.

Our results indicate that the synergistic interaction of 5-FU (or S-1) and gefitinib is attributable, at least in part, to down-regulation of TS expression by gefitinib. The active metabolite of 5-FU, FdUMP, forms a covalent ternary complex with 5,10-methylenetetrahydrofolate and TS, resulting in inhibition of DNA synthesis (42). TS is thus an important therapeutic target of 5-FU. The amount of TS in neoplastic cells has been found to increase after exposure to 5-FU, resulting in the maintenance of free enzyme in excess of that bound to 5-FU (43–47). Such an increase in TS expression and activity has been viewed as a mechanistic driver of 5-FU resistance in cancer cells (48–50). The development of a new therapeutic strategy that reduces TS expression would therefore be of interest. Indeed, preclinical studies have shown that the down-regulation of TS by antisense oligonucleotides or other means enhances the efficacy of 5-FU (51–54). Down-regulation of TS would be expected to enhance the cytotoxicity of 5-FU as a result of the decrease in the amount of its protein target (55). Consistent with these preclinical data, an inverse relation between TS expression and 5-FU sensitivity has been shown in various human solid tumors (27, 28, 56–60). We have now shown that gefitinib alone induced down-regulation of TS expression, suggesting that this effect of gefitinib contributes to its synergistic interaction with 5-FU (or S-1) in NSCLC cell lines.

We further explored the molecular mechanism by which gefitinib induces down-regulation of TS expression in NSCLC cells. Given that EGFR signal transduction has been shown to be involved in activity of E2F-1 that regulates the expression of several genes including TS (61, 62), which controls the expression of several genes including that for TS, we examined the possible effects of gefitinib on E2F-1 expression and on the abundance of TS mRNA. Gefitinib induced down-regulation of E2F-1 in NSCLC cell lines harboring wild-type *EGFR*, consistent with previous observations (63), as well as in those expressing mutant *EGFR*. In addition, gefitinib reduced the amount of TS mRNA in NSCLC cells, consistent with the notion that the suppression of TS expression by gefitinib is attributable to inhibition of gene transcription as a result of down-regulation of E2F-1. For our experiments examining the effects of gefitinib on TS and E2F-1 expression, we used a drug concentration of 5 μmol/L. The concentration of gefitinib in tumor xenografts was shown previously to be 5 to 14 times that in the plasma concentration of the mouse hosts (34). Daily oral administration of gefitinib (250 mg) in patients also gave rise to a drug concentration in tumor tissue that was substantially higher (mean, 42-fold) than that in plasma concentration (34). We showed previously that the maximal concentration of gefitinib in the plasma of patients with advanced solid tumors had a mean value of 0.76 μmol/L at a daily dose of 225 mg (64). Based on these data, we considered that a gefitinib concentration of 5 μmol/L was appropriate for our

analyses of TS and E2F-1 expression. Together, our present findings suggest that down-regulation of E2F-1 and consequently that of TS by gefitinib is responsible, at least in part, for the synergistic antitumor effect of combined treatment with S-1 and gefitinib.

Somatic mutations of *EGFR* have been associated with sensitivity to EGFR-TKIs in patients with advanced NSCLC (13–16). However, although most NSCLCs with *EGFR* mutations initially respond to EGFR-TKIs, the vast majority of these tumors ultimately develop resistance to the drug. In the present study, the synergistic effect of combination chemotherapy with S-1 and gefitinib was observed even in *EGFR* mutant cells. Our findings thus suggest that the addition of S-1 (or 5-FU) to EGFR-TKIs might overcome chemoresistance to EGFR-TKIs and that exploration of the effect of such combination therapy in cells resistant to EGFR-TKIs is warranted. *EGFR* mutations appear to be largely limited to lung cancer, with few such mutations having been detected in other types of cancer (65–67). 5-FU is widely used as an anticancer agent and is considered a key drug in chemotherapy for solid tumors such as gastrointestinal and cervical cancer (68–70). Our present results show that gefitinib suppressed the expression of TS in NSCLC cell lines regardless of the absence or presence of *EGFR* mutations, suggesting that the addition of EGFR-TKIs to a 5-FU-containing regimen might increase the effectiveness of such treatment for solid cancers without *EGFR* mutations. Oral combined chemotherapy with drugs, such as S-1 and gefitinib, may also prove to be of low toxicity and therefore maintain quality of life. Our preclinical results provide a basis for future clinical investigations of combination chemotherapy with S-1 and EGFR-TKIs in patients with solid tumors.

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ORIGINAL PAPER

# Epidermal growth factor receptor gene amplification and gefitinib sensitivity in patients with recurrent lung cancer

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**Abstract** To evaluate the epidermal growth factor receptor (EGFR) protein expression, gene mutations and amplification as predictors of clinical outcome in patients with non-small-cell lung cancer (NSCLC) receiving gefitinib, we have performed fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC). We investigated the *EGFR* amplification and EGFR protein expression statuses in 27 surgically treated non-small-cell lung cancer (NSCLC) cases. These patients experienced relapse after surgery and received gefitinib 250 mg/day. The presence or absence of *EGFR* mutations of kinase domains was analyzed by genotyping analysis and sequences, and already reported. *EGFR* mutations were found from 15/27 lung cancer patients. *EGFR* mutation status was significantly correlated with better prognosis (log-rank test $P = 0.0023$). Smoking status (never smoker vs. smoker, $P = 0.0032$), and pathological subtypes (adenocarcinoma vs. non-adenocarcinoma, $P = 0.0011$), but not *EGFR* amplification ($P = 0.1278$), were correlated with survival of lung cancers. EGFR IHC results were correlated with FISH results ($P = 0.0125$), but not correlated with prognosis ($P = 0.7921$). Thus, the *EGFR* gene amplification or protein expression is not a predictor of gefitinib efficacy in Japanese patients with NSCLC. We have also evaluated the *EGFR* mutation status and clinico-pathological features for 27 NSCLC patients who had undergone surgery followed by treatment with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center. The *EGFR* mutation status, especially exon19 mutation was correlated with good response to gefitinib than exon 21 point mutation.

**Keywords** *EGFR* · Lung cancer · Mutations · Amplification · Exon19

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## Introduction

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy (Ginsberg et al. 1993). There are much accumulated evidences that epidermal growth factor receptor (*EGFR*) and its family member are strongly implicated in the development and progression of numerous human tumors, including lung cancer (Nicolson et al. 2001; Onn et al. 2004). The *EGFR* tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of non-small-cell lung cancer (NSCLC) since 2002. Phase II and III trials have shown partial responses in 8–12% of unselected patients with progressive NSCLC after chemotherapy (Kris et al. 2003; Thatcher et al. 2005), especially higher response in never smokers, females and Asian ethnicity (more than 20%) (Fukuoka et al. 2003; Miller et al. 2004). Two original reports showed that *EGFR* mutations status at ATP binding pockets in NSCLC patients was correlated with the clinico-pathological features related

to good response to gefitinib (Paez et al. 2004; Lynch et al. 2004). These *EGFR* mutations were predominantly found in Japanese lung cancer patients (about 25–40%) (Paez et al. 2004; Kosaka et al. 2004; Shigematsu et al. 2005; Tokumo et al. 2005; Endo et al. 2005) when compared to USA patients (about 8–10%) (Paez et al. 2004; Lynch et al. 2004; Shigematsu et al. 2005; Pao et al. 2004) or European patients (Shigematsu et al. 2005; Marchetti et al. 2005). Actually, *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy (Paez et al. 2004; Lynch et al. 2004; Pao et al. 2004; Mitsudomi et al. 2005). On the other hands, Cappuzzo et al. (2005) reported that *EGFR* amplification by fluorescence in situ hybridization (FISH) and high *EGFR* protein expression has been associated with responsiveness to gefitinib. Takano et al. (2005) showed that both *EGFR* gene mutation and increased copy numbers predicted gefitinib sensitivity in patients with recurrent NSCLC. However, this Japanese report is based on polymerase chain reaction (PCR) assay.

To determine the *EGFR* amplification and *EGFR* mutation statuses and correlation with clinico-pathological features in Japanese gefitinib-treated lung carcinoma, we retrospectively performed FISH and immunohistochemistry. The findings were compared to the clinico-pathologic features of lung cancer.

## Materials and methods

### Patients and samples

This was a retrospective study and the study group included 27 lung cancer patients who were treated with gefitinib for their recurrent diseases after they had undergone surgery at the Department of Surgery II, Nagoya City University Medical School. Written informed consent was obtained and the institutional ethics committee of the Nagoya City University Medical School approved the study. We have also investigated *EGFR* mutation status for 27 NSCLC patients who were treated with gefitinib for their recurrent diseases after they had undergone surgery at the National Hospital Organization, Kinki-chuo Chest Medical Center (Endo et al. 2005). The lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan. All tumor samples were immediately frozen and stored at −80°C until assayed. The clinical and pathological characteristics of the 27 lung cancer patients are as follows; 14 (67.7%) were male and 13 were female. Twenty-two (63%) were diagnosed as adenocarcinoma, and five were diagnosed as other types of carcinoma. Fourteen (52%) were never smokers and 13 were smokers.

### PCR assays for *EGFR* and *K-ras* mutations

Genomic DNA was extracted using Wizard SV Genomic DNA purification Systems (Promega) according to the manufacturers' instructions. The primers and TaqMan MGB probe were designed with Primer Express 2.0 software (Applied Biosystems). The sequences of 13 allele-specific probes and primer sets used in the TaqMan PCR assay are already shown (Endo et al. 2005). The results of TaqMan PCR assays were already reported (Endo et al. 2005). *K-ras* codon 12/13 mutation status was investigated by direct sequencing using the primers reported by Krypuy et al. (2006). Total RNA was extracted from the lung cancer tissues using Isogen kit (Nippon gene, Tokyo, Japan) according to the manufacturers' instructions. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD, USA) with 0.5 μg oligo $(dT)_{12-16}$ (Amersham Pharmacia Biotech Inc. Piscataway, NJ, USA). The direct sequencing for *EGFR* genes was performed from genomic DNA (Paez et al. 2004) or cDNA (Sasaki et al. 2006). Some cases were genotyped using LightCycler (Sasaki et al. 2005) and confirmed.

### FISH analysis

Tumor specimens were obtained at surgical operation and embedded in paraffin. Serial sections (6 μm) containing representative malignant cell were stained with hematoxylin and eosin. Gene copy number per cell was investigated by FISH using the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probe (Vysis, Abbott laboratories, IL, USA) according to a published protocol (Hirsch et al. 2003). Sections were incubated at 56°C overnight, deparaffinized and dehydrated. After incubation in 2× saline sodium citrate buffer (2× SSC; pH 7.0) at 75°C for 15–25 min, sections were digested with protein K (0.25 mg/ml in 2× SSC; pH 7.0) at 37°C for 15–25 min, rinsed in 2× SSC at room temperature for 5 min, and dehydrated using ethanol in a series of increasing concentrations. The EGFR/CEP 7 probe set was applied per the manufacture's instructions onto the selected area based on the presence of tumor foci on each slide. The slides were incubated at 80°C for 8–10 min for codenaturation of chromosomal and probe DNA and then placed in a humidified chamber at 37°C for 20–24 h to allow hybridization to occur. Post hybridization washes were performed in 1.5 M urea and 0.1× SSC at 45°C for 30 min and in 2× SSC for 2 min at room temperature. Pathologist who was blinded to the patients' clinical characteristics and all other molecular variables performed FISH analysis independently. Patients were classified according to the Cappuzzo et al. (2005) criteria with ascending number of copies of the *EGFR* gene

per cell and the frequency of tumor cells with specific number of copies of the *EGFR* gene and chromosome 7 centromere: high polysomy (≥4 copies in ≥40% of cells) and gene amplification (defined by presence of tight *EGFR* gene clusters and a ratio of *EGFR* gene to chromosome of ≥2 or ≥copies of *EGFR* per cell in ≥10% of analyzed cells) were considered as FISH positive. Disomy (≤2 copies in >90% of cells); low trisomy (≤2 copies in ≥40% of cells, 3 copies in 10–40% of cells, 4 ≥copies in <10% of cells); high trisomy (≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells) and low polysomy (≥4 copies in 10–40% of cells) were considered as FISH negative.

### Immunohistochemistry

EGFR protein expression was evaluated by immunohistochemistry using the mouse anti-human EGFR, clone 2-18C9 monoclonal antibody (Dako NorthAmerica, Inc., Via Real, Carpinteria, CA, USA). Four micrometer sections were made from paraffin tissue blocks from lung tumors. The slides were treated with xylenes, and then dehydrated in alcohol. After treated with proteinase K for 5 min, endogenous peroxidase was blocked with Peroxidase ($H_2O_2$) Block. After washed with Wash Buffer (Dako NorthAmerica Inc., USA), the slides were incubated with the monoclonal antibody against EGFR (ready-to use) for 30 min at room temperature. Labeled Polymer, HRP (30 min) and 3,3-diaminobenzidine (DAB) substrate (10 min) were used to visualize the antibody binding, and the sections were counterstained with hematoxylin. The intensity score was defined according to Cappuzzo et al. (2005); 1 = barely detectable, 2 = readily appreciable brown staining, 3 = dark brown staining, 4 = very strong staining. The total score was calculated by multiplying the intensity score and the fraction score (positive cells; 0–100%). Scores of 201–400 were considered positive.

### Statistical methods

Statistical analyses were done using the Mann–Whitney *U* test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between the variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and $\chi^2$ test. The overall survival of lung cancer patients was examined by the Kaplan–Meier methods, and differences were examined by the log-rank test. All analyses were done using the Stat-View software package (Abacus Concepts Inc. Berkeley, CA, USA), and were considered significant when the *P*-value was less than 0.05.

## Results

### *EGFR* gene copy number and clinical outcome

First we assessed *EGFR* copy number by FISH according to Cappuzzo et al. criteria (2005). High polysomy for the *EGFR* gene was present in 44.4% ($n = 12$), and low polysomy in 11.1% ($n = 3$) (Fig.1). However no association was observed between gene amplification and clinical characteristics (Table 1). Smoking status (never smoker vs. smoker, $P = 0.1283$), pathological subtypes (adenocarcinoma vs. non-adenocarcinoma, $P = 0.6280$), or gender (male vs. female, $P = 0.2519$) did not correlate with the *EGFR* amplification status. FISH positive results were obtained in 40% of the patients with *EGFR* mutations. Three other patients with *EGFR* mutations had low polysomy.

A partial response (PR) was achieved in 14 patients, 5 patients had stable disease (SD), and 8 had progressive disease (PD). *EGFR* amplification status was not associated with gefitinib response ($P = 0.7036$). *EGFR* amplification status was not significantly correlated with prognosis (log-rank test, $P = 0.1278$; Breslow–Gehan–Wilcoxon test, $P = 0.0528$) (Fig. 2).

### EGFR protein expression and clinical outcome

EGFR protein expression was evaluated by immunohistochemisry (Fig. 3) and the outcome of patients according to the protein score is shown in Fig. 2. Patients with EGFR immunohistochemistry positive ($n = 13$) did not have any advantage for outcomes after treated with gefitinib therapy ($P = 0.7921$).

### *EGFR* gene mutation status in Japanese lung cancer patients

Among 27 patients, 15 had *EGFR* mutations, including four deletion 1a type mutations (2235–2249 del GGAATTAA GAGAAGC), two other types of exon 19 deletion mutations and six L858R mutations. Interestingly, exon 20 insertion mutant patients experienced progressive disease (manuscript submitted). We also compared associations between *EGFR* mutation status, FISH status, and protein expression in each tumor with patient's outcome. Summarized data are shown in Table 2. The overall survival of 27 gefitinib treated-lung cancer patients from Nagoya City University, with follow-up through 30 April 2007, was studied in reference to *EGFR* mutation status. *EGFR* mutations were not associated with FISH+ status, and high protein expression (wild type; 57.1% vs. $P > 0.9999$). Gene mutations were statistically significantly associated with better response ($P = 0.0018$) and longer survival. Patients

**Table 1** Clinico-pathological data of 27 lung cancer patients

| EGFR gene status | | | |
|---|---|---|---|
| Factors | FISH positive patients | FISH negative patients | *P* value |
| Mean age (years) 64.0 ± 11.9 | 12 | 15 | |
| Pathological subtypes | | | |
| Adeno | 9 (40.9%) | 13 (59.1%) | 0.6260 |
| Non-adeno | 3 (60.0%) | 2 (40.0%) | |
| Gender | | | |
| Male | 8 (57.1%) | 6 (42.9%) | 0.2519 |
| Female | 4 (30.8%) | 9 (69.2%) | |
| Smoking status | | | |
| Never smoker | 4 (28.6%) | 10 (71.4%) | 0.1283 |
| Smoker | 8 (61.5%) | 5 (38.5%) | |
| Differentiation | | | |
| Well | 6 (35.3%) | 11 (64.7%) | 0.2566 |
| Moderately or poorly or Others | 6 (60.0%) | 4 (40.0%) | |
| Gefitinib response | | | |
| Responder | 7 (50.0%) | 7 (50.0%) | 0.7036 |
| Non-responder | 5 (38.5%) | 8 (61.5%) | |
| EGFR mutations | | | |
| Wild type | 6 (50.0%) | 6 (50.0%) | 0.8052 |
| Mutant | 6 (40.0%) | 9 (60.0%) | |
| IHC | | | |
| Positive | 9 (69.2%) | 4 (30.8%) | 0.0213 |
| Negative | 3 (21.4%) | 11 (78.6%) | |

*IHC* immunohistochemistry, *Adeno* adenocarcinoma

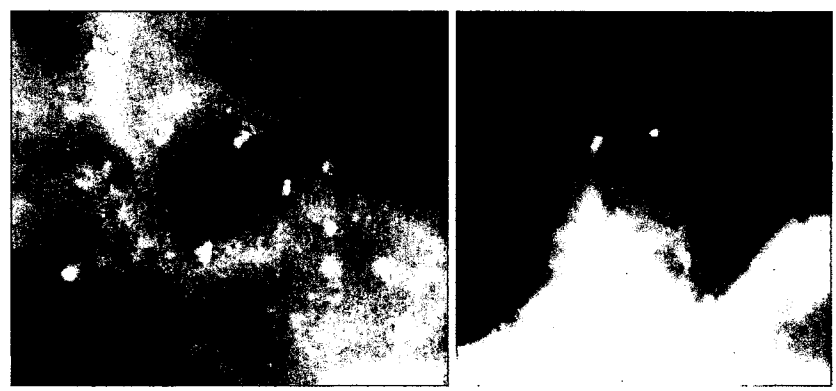

**Fig. 1** FISH analysis for lung cancer tissues. *Left* high polysomy case (4 copy numbers in cells >40%), *right* disomy case

with *EGFR* mutations were significantly better in prognosis than the patients with wild type (log-rank test $P = 0.0023$, Breslow–Gehan–Wilcoxon test, $P = 0.0012$) (Fig. 4). Smoking status (never smoker vs. smoker, log-rank test $P = 0.0032$; Breslow–Gehan–Wilcoxon test, $P = 0.0012$), and pathological subtypes (adenocarcinoma vs. non-adenocarcinoma, log-rank test $P = 0.0011$, Breslow–Gehan–Wilcoxon test, $P = 0.0019$), but neither gender (male vs. female, log-rank test $P = 0.0709$, Breslow–Gehan–Wilcoxon test, $P = 0.0353$), nor response (log-rank test $P = 0.2465$, Breslow–Gehan–Wilcoxon test, $P = 0.0588$) were correlated with better prognosis. Using the Cox hazard regression model, *EGFR* mutations ($P = 0.0208$) and smoking status ($P = 0.0218$) were independent prognostic factors, but not pathological subtypes (0.1121). In this analysis, only one *K-ras* codon 12 mutation was found among 27 patients. This patient was wild type for *EGFR* and did not respond to gefitinib therapy.

We have sequenced 27 NSCLC patients who had undergone surgery followed by treatment with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center and already reported. We have added these data

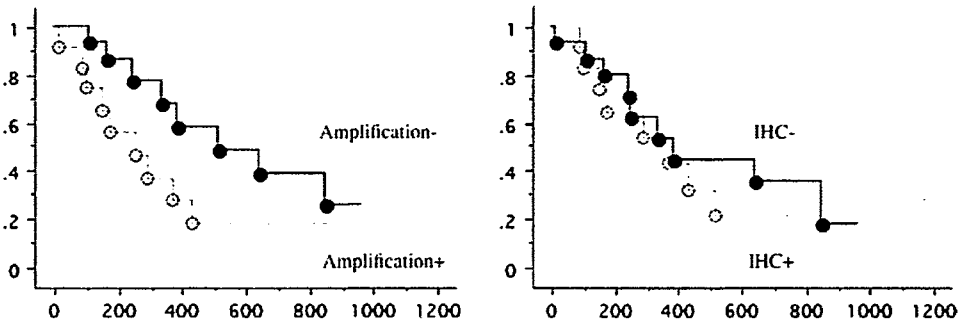


**Fig. 2** The overall survival of 27 gefitinib untreated lung cancer patients was studied in reference to the *EGFR* amplification status (*left*) and EGFR protein expression (*right*). Prognosis from patients with EGFR amplification ($n = 12$, 9 were dead) and without *EGFR* amplification ($n = 15$, 8 were dead) was not significantly different (log-rank test, $P = 0.1278$; Breslow–Gehan–Wilcoxon test, $P = 0.0528$). Prognosis from patients with positive EGFR expression ($n = 13$, 8 were dead) and without negative EGFR expression ($n = 14$, 9 were dead) was not significantly different (log-rank test, $P = 0.7921$; Breslow–Gehan–Wilcoxon test; $P = 0.9105$)

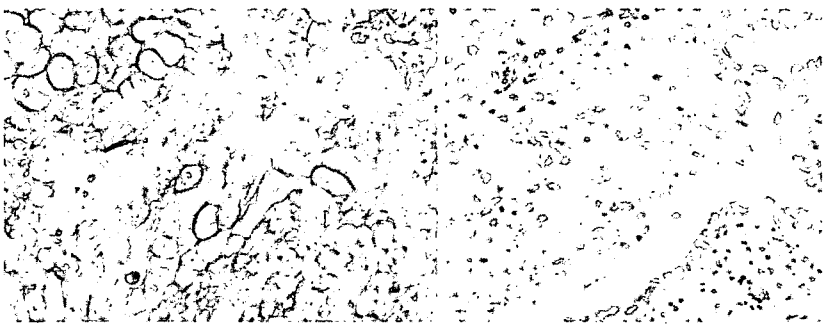

**Fig. 3** EGFR protein expression by immunohistochemistry. *Left* positive case, *right* negative case

(Table 3). Ten patients had *EGFR* mutations, including two L858R, one deletion type 1a, and one G719S at exon 18. Three patients had deletion 1b type mutation (2236–2250 del GAATTAAGAGAAGCA). Of 54 patients, 25 were male and 29 female. Twenty-eight were never smokers and 26 were smokers. Forty-eight patients had adenocarcinoma, four had squamous cell carcinoma and one had adenosquamous cell carcinoma. *EGFR* mutation status was significantly correlated with better prognosis (log-rank test $P = 0.0128$, Breslow–Gehan–Wilcoxon test $P = 0.0051$). Patients with *EGFR* mutation at exon 19 deletion 1 types had significantly better prognosis than wild type patients ($P = 0.0032$). However, the prognosis of patients with L858R mutation and wild type was not significantly different ($P = 0.2823$) (Fig. 5).

## Discussion

We obtained the findings that the *EGFR* amplification, detected by FISH according to Cappuzzo et al. criteria, was not associated with the response to gefitinib. *EGFR* mutations, smoking history, and pathological subtype of lung cancers were correlated with survival of gefitinib-treated patients. This was in agreement with the recent reports that *EGFR* gene mutations are prognostic factor for gefitinib therapy (Takano et al. 2005; Mitsudomi et al. 2005; Sone et al. 2007). In addition, our analysis also suggested that the deletion type *EGFR* mutation might be more correlated with the survival for gefitinib-treated patients.

Some limitations to the study must be taken into consideration. Our finding is so far based on a single retrospective study with a relatively small number of patients, and the data need to be verified in a large cohort of patients and prospectively. The *EGFR* status was determined on the tumor tissue at the time of primary diagnosis, and possible changes after chemotherapy were not determined in this study (Cappuzzo et al. 2007).

Previous report suggested that NSCLC patients with resected tumors carrying high *EGFR* gene copy number have a tendency to a shorter survival (Hirsch et al. 2003). This might affect the controversial results of Cappuzo et al. (2005) In our analysis, FISH positive population did not correlate with the gender, smoking status and pathological subtypes. The presence of *EGFR* gene amplification did not reach statistical significance. An interesting finding was the association between *EGFR* mutations and increased gene copy number, a phenomenon that was recently described in the human lung cancer cell line H3255 (Tracy et al. 2004) and is probably relevant to gefitinib sensitivity. In fact,

**Table 2** EGFR mutation and amplification statuses in 27 gefitinib treated patients

| Age | Gender | Smoking | Pathology | EGFR mutation | EGFR amplification | IHC score | Survival (day) |
|---|---|---|---|---|---|---|---|
| 71 | F | 0 | Adeno | Della | High polysomy | 220 | 1,080 (A) |
| 72 | M | 600 | Adeno | L858R | Low polysomy | 240 | 885 (A) |
| 76 | M | 800 | Adeno | WT | High polysomy | 90 | 248 (D) |
| 72 | M | 0 | Adeno | exon 20 ins V | Disomy | 80 | 660 (A) |
| 70 | M | 1,000 | Adeno | L858R | Disomy | 210 | 515 (D) |
| 61 | F | 0 | Adeno | WT | Disomy | 160 | 854 (D) |
| 51 | M | 500 | Adeno | Della | High polysomy | 220 | 286 (D) |
| 76 | F | 0 | Adeno | WT | Disomy | 30 | 640 (D) |
| 57 | M | 20 | Adeno | WT | High polysomy | 210 | 101 (D) |
| 77 | M | 1,200 | Adeno | WT | Disomy | 0 | 168 (D) |
| 38 | M | 300 | Adeno | L858R | High polysomy | 210 | 430 (D) |
| 73 | F | 0 | Adeno | G719S | Disomy | 180 | 339 (D) |
| 42 | F | 0 | Adeno | Del4 | High polysomy | 100 | 700 (A) |
| 76 | F | 920 | SCC | WT | High polysomy | 220 | 145 (D) |
| 56 | F | 0 | Adeno | L858R | High polysomy | 200 | 368 (D) |
| 56 | M | 1,200 | Adeno | WT | High polysomy | 200 | 85 (D) |
| 78 | M | 1200 | SCC | WT | High polysomy | 250 | 174 (D) |
| 42 | M | 400 | SCC | WT | Disomy | 120 | 110 (D) |
| 67 | M | 800 | Adeno | WT | Disomy | 80 | 384 (D) |
| 63 | M | 600 | Adsq | WT | High polysomy | 90 | 11 (D) |
| 47 | F | 0 | Adeno | Del5 | Disomy | 210 | 945 (A) |
| 62 | F | 0 | Adeno | L858R | Disomy | 80 | 245 (D) |
| 71 | F | 0 | Adeno | L861Q | Low polysomy | 210 | 210 (A) |
| 61 | F | 0 | Adeno | Della | Low polysomy | 120 | 180 (A) |
| 64 | F | 0 | Adeno | WT | Disomy | 180 | 230 (A) |
| 72 | M | 0 | Adeno | L858R | High polysomy | 210 | 110 (A) |
| 77 | F | 0 | Adsq | Della | Disomy | 60 | 210 (A) |

*F* Female, *M* male, *Adeno* adenocarcinoma, *SCC* squamous cell carcinoma, *Adsq* adenosquamous cell carcinoma, *WT* wild type, *IHC* immunohistochemistry, *A* alive, *D* death

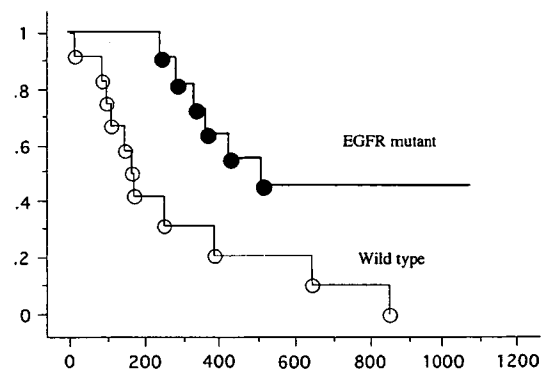


**Fig. 4** The overall survival of 27 gefitinib-treated lung cancer patients was studied in reference to the *EGFR* mutation status. Prognosis from patients with EGFR mutations ($n = 15$, 6 were dead) was significantly better than the patients without *EGFR* mutations ($n = 12$, 11 were dead) (log-rank test, $P = 0.0023$, Breslow–Gehan–Wilcoxon test; $P = 0.0012$)

among the 15 patients with *EGFR* mutations who responded to gefitinib therapy, six were also FISH positive (high polysomy) and three were low polysomy. However, between the two non-responding patients with *EGFR* mutations, both were FISH negative. Sone et al. (2007) reported that the *EGFR* mutations and not the gene amplifications were the predictors of gefitinib efficacy in Japanese lung cancers. They evaluated the biopsy specimens and 5/59 samples were small and inadequate for FISH analysis. Another possible explanation for the discrepancies between the findings from the studies described by Cappuzo et al. and our findings is the difference in *EGFR* mutation statuses according to ethnicity. Han et al. (2006) investigated *EGFR* gene mutations, gene amplification, *K-ras* mutation, and Akt phosphorylation in tumor samples from East-Asian patients with NSCLC and demonstrated that *EGFR* mutation was an independent predictor of response and survival