

for the PE regimen was 10.2 months and that for the IP regimen was 9.3 months (Hanna *et al*, 2006). The discrepancy between the Japanese and American trials may be explained by the different cisplatin dose schedules; cisplatin was delivered at a dose of 60 mg m⁻² on day 1 every 3 or 4 weeks in the Japanese trials, whereas cisplatin was delivered at a dose of 30 mg m⁻² on days 1 and 8 every 3 weeks in the American one. A platinum agent administered at divided doses was associated with poor survival in patients with ED-SCLC in our previous randomised phase II study (Sekine *et al*, 2003).

The issue of adding further agents to the standard doublet regimen has been investigated in patients with ED-SCLC. The addition of ifosfamide or cyclophosphamide and epirubicin to the cisplatin and etoposide combination produced a slight survival benefit, but at the expense of greater toxicity (Loehrer *et al*, 1995; Pujol *et al*, 2001). Phase III trials of cisplatin and etoposide with or without paclitaxel showed unacceptable toxicity with 6–13% toxic deaths in the paclitaxel-containing arm (Mavroudis *et al*, 2001; Niell *et al*, 2005). The results in these studies and the current study are consistent in the increased toxicity despite the G-CSF support and no definite survival benefit in the three or four drug combinations over the standard doublet in patients with ED-SCLC.

In conclusion, the IPE regimen was marginally more effective than the IP regimen, but was too toxic despite the administration of prophylactic G-CSF.

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Enhancement of the antitumor activity of ionising radiation by nimotuzumab, a humanised monoclonal antibody to the epidermal growth factor receptor, in non-small cell lung cancer cell lines of differing epidermal growth factor receptor status

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The expression and activity of the epidermal growth factor receptor (EGFR) are determinants of radiosensitivity in several tumour types, including non-small cell lung cancer (NSCLC). However, little is known of whether genetic alterations of EGFR in NSCLC cells affect the therapeutic response to monoclonal antibodies (mAbs) to EGFR in combination with radiation. We examined the effects of nimotuzumab, a humanised mAb to EGFR, in combination with ionising radiation on human NSCLC cell lines of differing EGFR status. Flow cytometry revealed that H292 and Ma-1 cells expressed high and moderate levels of EGFR on the cell surface, respectively, whereas H460, H1299, and H1975 cells showed a low level of surface EGFR expression. Immunoblot analysis revealed that EGFR phosphorylation was inhibited by nimotuzumab in H292 and Ma-1 cells but not in H460, H1299, or H1975 cells. Nimotuzumab augmented the cytotoxic effect of radiation in H292 and Ma-1 cells in a clonogenic assay *in vitro*, with a dose enhancement factor of 1.5 and 1.3, respectively. It also enhanced the antitumor effect of radiation on H292 and Ma-1 cell xenografts in nude mice, with an enhancement factor of 1.3 and 4.0, respectively. Nimotuzumab did not affect the radioresponse of H460 cells *in vitro* or *in vivo*. Nimotuzumab enhanced the antitumor efficacy of radiation in certain human NSCLC cell lines *in vitro* and *in vivo*. This effect may be related to the level of EGFR expression on the cell surface rather than to EGFR mutation.

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Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is abnormally upregulated and activated in a variety of tumours (Baselga, 2002). Deregulation of receptor tyrosine kinases as a result of overexpression or activating mutations is frequently associated with human cancers and leads to the promotion of cell proliferation or migration, inhibition of cell death, or the induction of angiogenesis (Gschwind *et al*, 2004). The epidermal growth factor receptor has thus been identified as an important target in cancer therapy (Baselga and Arteaga, 2005). Several agents, including small-molecule inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKIs) and monoclonal antibodies (mAbs) specific for EGFR, have been designed to block EGFR signalling selectively (Ettinger, 2006; Harari and Huang, 2006; Imai and Takaoka, 2006). Among EGFR-TKIs, gefitinib and erlotinib have been extensively evaluated in non-small cell lung cancer (NSCLC),

and sensitivity to these drugs has been associated with the presence of somatic mutations in the EGFR kinase domain or with EGFR amplification (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005). Various mAbs to EGFR are also undergoing preclinical and clinical trials of their efficacy as anticancer agents. However, biological markers able to predict the response to such antibodies have remained elusive.

The possibility of combining chemotherapy or radiation therapy with anti-EGFR mAb treatment has generated much interest, because the cellular targets for these agents and their mechanisms of action are different (Baumann and Krause, 2004). Studies have thus been undertaken to determine whether inhibition of EGFR signalling improves the response to chemotherapy or radiation therapy. Preclinical studies have shown that the anti-EGFR mAb cetuximab markedly increases the cytotoxic effect of chemotherapy or radiation therapy in various EGFR-expressing tumour cell lines (Huang *et al*, 1999; Milas *et al*, 2000; Buchsbaum *et al*, 2002; Prewett *et al*, 2002; Raben *et al*, 2005; Ettinger, 2006). A phase III clinical trial also showed that the combination of cetuximab with

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radiation therapy resulted in a significant improvement in local control and survival compared with radiation therapy alone, without an increase in radiation-induced side effects, in patients with locally advanced head and neck cancer (Bonner *et al*, 2006).

Nimotuzumab (also known as h-R3) is a humanised anti-EGFR mAb, which is currently undergoing clinical evaluation. In a preclinical study, nimotuzumab showed marked antiproliferative, proapoptotic, and antiangiogenic effects in tumours that overexpress EGFR (Crombet-Ramos *et al*, 2002). In early clinical trials, nimotuzumab has shown a longer half-life and a greater area under the curve (AUC) in comparison with other anti-EGFR antibodies (Crombet *et al*, 2003). A phase I/II trial showed that nimotuzumab was well tolerated and enhanced the curative potential of radiation in patients with advanced head and neck cancer (Crombet *et al*, 2004). Given that little is known of the antitumor action of nimotuzumab in NSCLC, we investigated the growth-inhibitory effects of this mAb alone and in combination with radiation in NSCLC cell lines with differing patterns of EGFR expression. We also examined whether genetic alterations of EGFR affect the antitumor action of combined treatment with nimotuzumab and radiation.

MATERIALS AND METHODS

Cell lines and reagents

The human NSCLC cell lines NCI-H292 (H292), NCI-H460 (H460), Ma-1, NCI-H1299 (H1299), and NCI-H1975 (H1975) were obtained as previously described (Okabe *et al*, 2007) and were maintained under a humidified atmosphere of 5% CO₂ in air at 37.0°C in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Nimotuzumab was provided by Daiichi Sankyo Co Ltd (Tokyo, Japan), and gefitinib was obtained from AstraZeneca (Macclesfield, UK).

Flow cytometric analysis of surface EGFR expression

Cells (1.0×10^6) were stained for 2 h at 4°C with an R-phycoerythrin-conjugated mAb to EGFR (BD Biosciences, San Jose, CA, USA) or an isotype-matched control mAb (BD Biosciences). The cells were washed three times before measurement of fluorescence with a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA, USA).

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 (Okabe *et al*, 2007). Primary antibodies to phosphorylated EGFR (pY1068) were obtained from Cell Signaling Technology (Beverly, MA, USA), and those to EGFR were from Zymed (South San Francisco, CA, USA). Horseradish peroxidase-conjugated goat secondary antibodies were obtained from Amersham Biosciences (Little Chalfont, UK).

Clonogenic assay

Exponentially growing cells in 25-cm² flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities and plated in triplicate in 25-cm² flasks containing 10 ml of medium supplemented with 1% fetal bovine serum in the absence or presence of 700 nM nimotuzumab. After incubation for 24 h, the cells were exposed to various doses of

γ -radiation with a ⁶⁰Co irradiator at a rate of approximately 0.82 Gy min⁻¹ and at room temperature. The cells were then washed with phosphate-buffered saline, cultured in drug-free medium for 10–14 days, fixed with methanol:acetic acid (10:1, v/v), and stained with crystal violet. Colonies containing > 50 cells were counted. The surviving fraction was calculated as (mean number of colonies)/(number of inoculated cells \times plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for control cultures not exposed to nimotuzumab or radiation. The surviving fraction for combined treatment was corrected by that for nimotuzumab treatment alone. The dose enhancement factor was then calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.5 for vehicle-treated cells divided by that for nimotuzumab-treated cells (after correction for drug toxicity).

Antitumor activity of nimotuzumab with or without radiation *in vivo*

Animal experiments were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University, and they met the requirements of the UKCCCR guidelines (Workman *et al*, 1998). Tumour cells (2×10^6) were injected subcutaneously into the right hind leg of 7-week-old female athymic nude mice. Tumour volume was determined from caliper measurement of tumour length (*L*) and width (*W*) according to the formula $LW^2/2$. Treatment was initiated when tumours in each group achieved an average volume of approximately 170–200 mm³. Treatment groups consisted of control, nimotuzumab alone, radiation alone, and the combination of nimotuzumab and radiation, with each group containing seven or eight mice. Nimotuzumab was administered intraperitoneally in a single dose of 1.0 mg per mouse; mice in the control and radiation-alone groups were injected with vehicle (physiological saline). Tumours in the right hind leg of mice were exposed to 10 Gy of γ -radiation with a ⁶⁰Co irradiator at a rate of approximately 0.32 Gy min⁻¹ beginning 6 h after drug treatment. Growth delay (GD) was calculated as the time required for treated tumours to achieve a fivefold increase in volume minus the corresponding time required for control tumours. The enhancement factor was then determined as $(GD_{\text{combination}} - GD_{\text{nimotuzumab}}) / (GD_{\text{radiation}})$.

RESULTS

Surface EGFR expression in NSCLC cell lines of differing EGFR status

We first examined the surface expression of EGFR in five NSCLC cell lines by flow cytometry. The EGFR status for the cell lines was determined in our previous study (Okabe *et al*, 2007). Three cell lines (H460, H292, and H1299) possess wild-type EGFR alleles, whereas the other two cell lines (Ma-1 and H1975) harbour EGFR mutations (Table 1). Ma-1 cells have an in-frame deletion in

Table 1 Characteristics of NSCLC cell lines

Cell line	EGFR surface expression	EGFR status
H460	Low	Wild type
H292	High	Wild type
H1299	Low	Wild type
Ma-1	Moderate	del(E746–A750)
H1975	Low	L858R/T790M

EGFR = epidermal growth factor receptor; NSCLC = non-small cell lung cancer

exon 19 (E746–A750). H1975 cells harbour the L858R mutation in exon 21 and a secondary mutation in exon 20 (T790M). Activating mutations in exons 19 and 21 are associated with sensitivity to EGFR-TKIs (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005), whereas the T790M mutation contributes to the development of resistance to these drugs (Kobayashi *et al*, 2005;

Pao *et al*, 2005). Our flow cytometric analysis demonstrated that H292 and Ma-1 cells express high and moderate levels of EGFR on the cell surface, whereas H460, H1299, and H1975 cells showed a low level of surface EGFR expression (Figure 1).

Effect of nimotuzumab on EGFR phosphorylation

Next, we determined whether nimotuzumab inhibits ligand-induced EGFR phosphorylation in the five NSCLC cell lines. The cells were deprived of serum overnight, exposed to various concentrations of nimotuzumab, or to gefitinib, for 15 min, and then stimulated with EGF for 15 min. In the NSCLC cells that harbour wild-type *EGFR* (H460, H292, and H1299), phosphorylation of EGFR was undetectable in the absence of EGF, but was markedly induced on exposure of the cells to this growth factor. The EGF-induced phosphorylation of EGFR in these cells was completely inhibited by the EGFR-TKI gefitinib. Nimotuzumab also inhibited the EGF-induced EGFR phosphorylation in a concentration-dependent manner in H292 cells (which have a high level of surface EGFR expression), whereas it did not substantially affect such phosphorylation in H460 or H1299 cells (both of which have a low level of surface EGFR expression) (Figure 2A–C). We previously showed that the basal level of EGFR phosphorylation was increased in the *EGFR* mutant NSCLC cell lines Ma-1 and H1975, indicative of constitutive activation of the EGFR tyrosine kinase (Okabe *et al*, 2007). The phosphorylation of EGFR in EGF-treated Ma-1 cells (which have a moderate level of surface EGFR expression) was inhibited by gefitinib as well as by nimotuzumab in a concentration-dependent manner (Figure 2D). In contrast, the constitutive activation of EGFR in H1975 cells (which have a low level of surface EGFR expression) was inhibited partially by gefitinib but was unaffected by nimotuzumab (Figure 2E). These results suggested that the inhibition of EGFR phosphorylation by nimotuzumab may be related to the surface expression level of EGFR rather than to the mutational status of *EGFR*.

Augmentation of the cytotoxic effect of radiation in NSCLC cells by nimotuzumab *in vitro*

We examined whether nimotuzumab might enhance the anticancer effect of γ -radiation in the five NSCLC cell lines with the use of a clonogenic assay. Tumour cells were incubated with or without nimotuzumab for 24 h, exposed to various doses of γ -radiation, and then allowed to form colonies in drug-free medium for 10–14 days. Survival curves revealed that, whereas nimotuzumab had no effect on the radiation sensitivity of H460, H1299, or H1975 cells, it enhanced the cytotoxic effect of radiation in H292 and Ma-1 cells, with a dose enhancement factor of 1.5 and 1.3, respectively (Figure 3). These results showed that nimotuzumab increased the radiosensitivity of the NSCLC cell lines with high or moderate levels of surface EGFR expression, consistent with the inhibitory effects of this antibody on EGFR signalling.

Augmentation of the antitumor effect of radiation in NSCLC cells by nimotuzumab *in vivo*

To determine whether the nimotuzumab-induced potentiation of the response of NSCLC cells to radiation observed *in vitro* might also be apparent *in vivo*, we injected three of the cell lines into nude mice to elicit the formation of solid tumours. The mice were then treated with nimotuzumab, radiation, or both modalities. In the H460 xenograft model, tumour growth was inhibited by radiation alone but not by nimotuzumab alone, and the effect of radiation was not promoted by nimotuzumab (Figure 4A). In contrast, radiation and nimotuzumab each inhibited the growth of tumours formed by H292 (Figure 4B) or Ma-1 (Figure 4C) cells during the first few weeks after treatment. Thereafter, the rate of

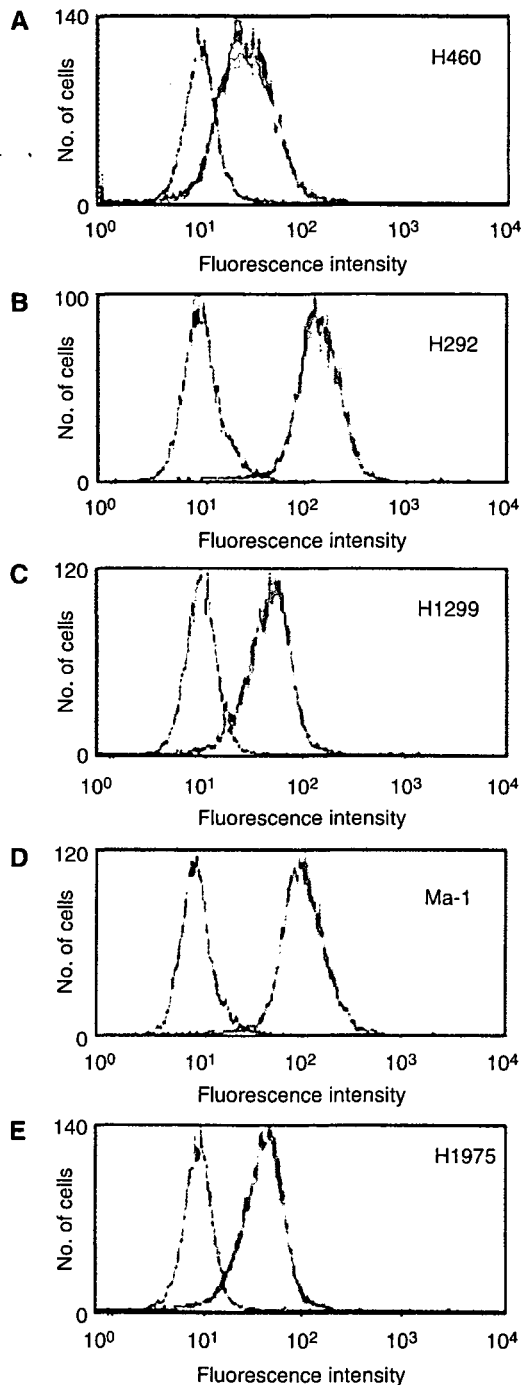


Figure 1 Expression of EGFR on the surface of NSCLC cells. Surface expression of EGFR on H460 (A), H292 (B), H1299 (C), Ma-1 (D), and H1975 (E) cells was determined by flow cytometry. Representative histograms of cells stained with an anti-EGFR mAb (red peak) or with an isotype-matched control mAb (black peak) are shown.

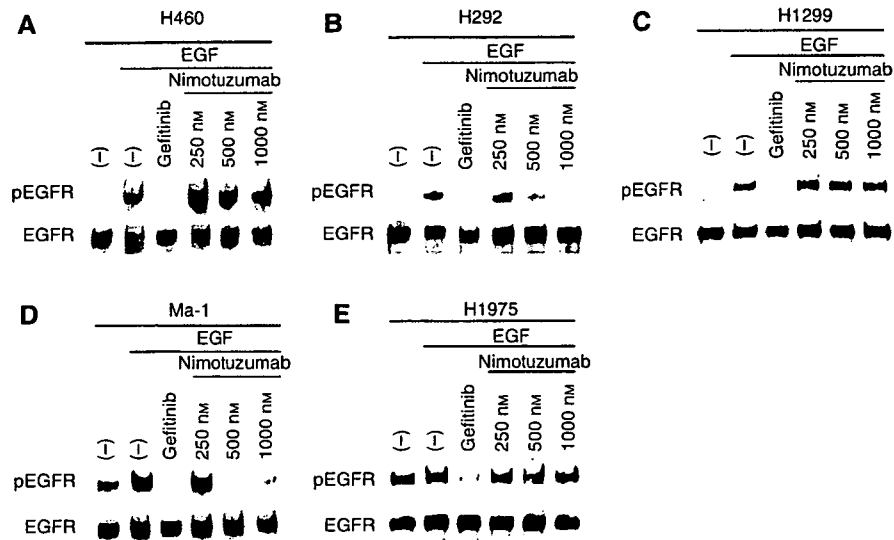


Figure 2 Effect of nimotuzumab on EGFR phosphorylation in NSCLC cells. H460 (A), H292 (B), H1299 (C), Ma-1 (D), and H1975 (E) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of the indicated concentrations of nimotuzumab or gefitinib (10 μM) and then for an additional 15 min in the additional absence or presence of EGF (100 ng ml⁻¹). Cell lysates were then subjected to immunoblot analysis with antibodies to the Tyr1068-phosphorylated form of EGFR (pEGFR) as well as with those to total EGFR.

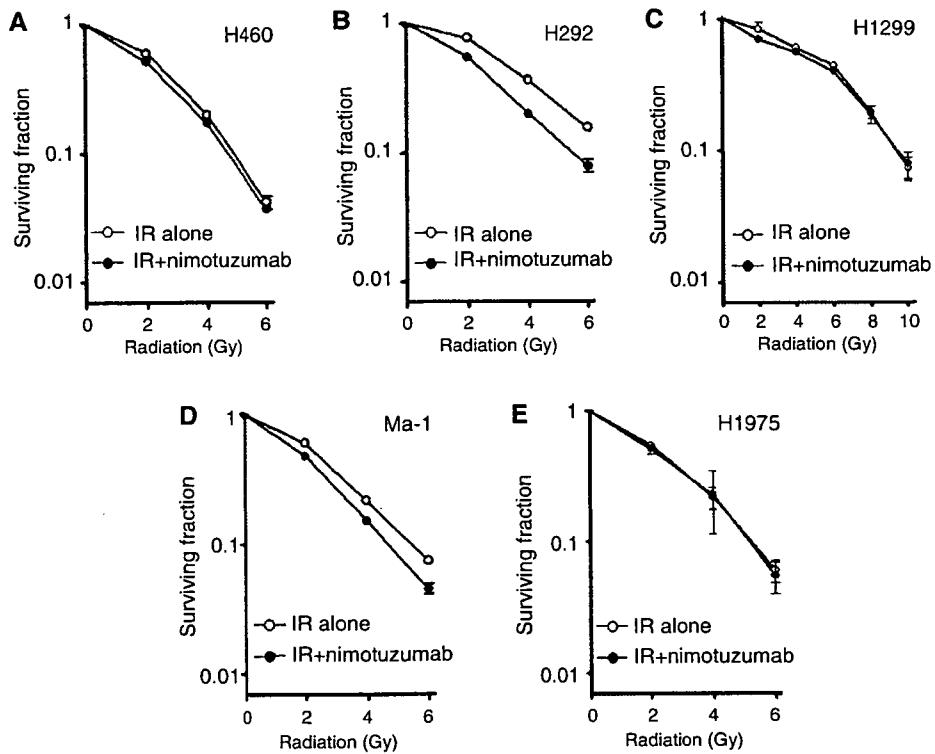


Figure 3 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vitro*. H460 (A), H292 (B), H1299 (C), Ma-1 (D), and H1975 (E) cells were incubated with or without 700 nM nimotuzumab in medium supplemented with 1% fetal bovine serum for 24 h, exposed to the indicated doses of γ -radiation, and then incubated in drug-free medium supplemented with 10% serum for 10–14 days for determination of colony-forming ability. Survival curves were generated after correction of colony formation observed for combined treatment with ionising radiation (IR) and nimotuzumab by that apparent for treatment with nimotuzumab alone. Data are means \pm s.d. of triplicates from a representative experiment.

tumour growth increased to a value similar to that seen in control animals. Combined treatment with radiation and nimotuzumab resulted in a substantial delay in tumour growth and subsequent inhibition of the growth rate of H292 and Ma-1 xenografts. The growth delay after treatment with nimotuzumab alone, radiation

alone, or both nimotuzumab and radiation was thus 27.2, 19.6, and 53.6 days, respectively, for H292 cells and 26.7, 13.0, and 78.3 days, respectively, for Ma-1 cells (Table 2). The enhancement factor for the effect of nimotuzumab on the efficacy of radiation was 1.3 for H292 cells and 4.0 for Ma-1 cells, revealing the effect to be more

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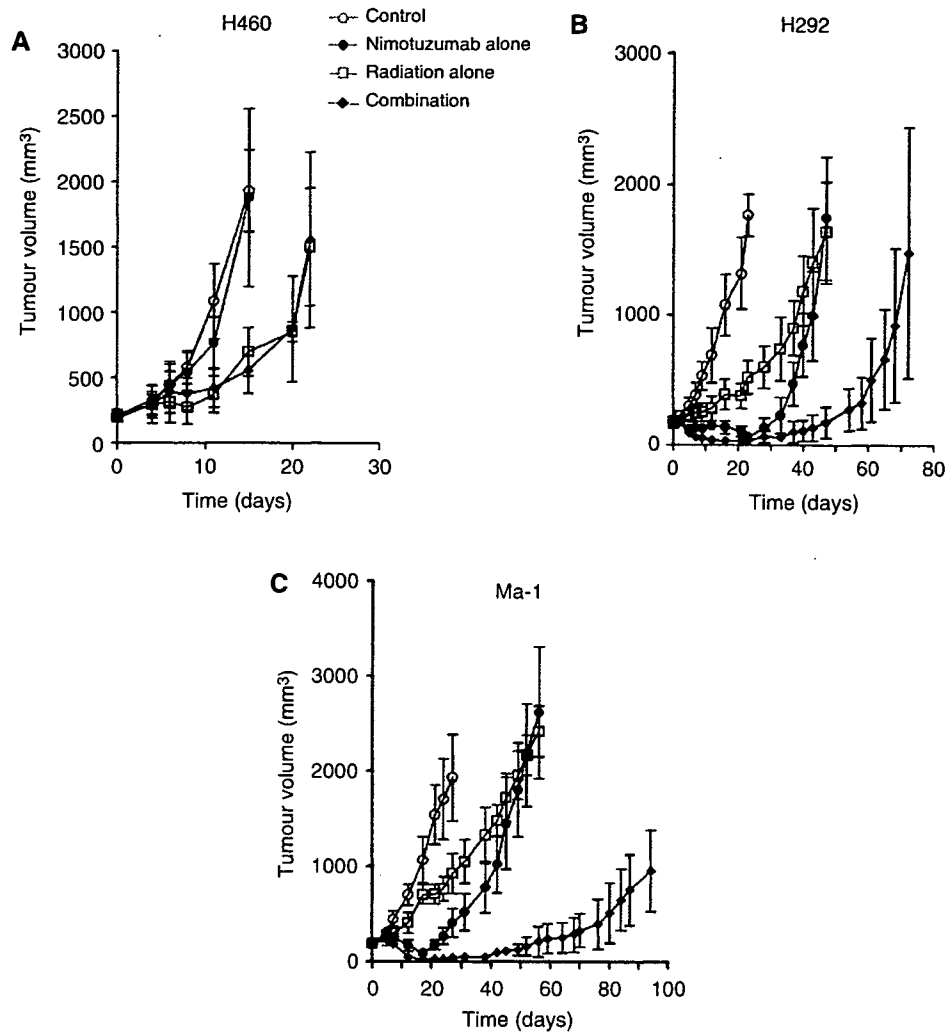


Figure 4 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vivo*. H460 (A), H292 (B), or Ma-1 (C) cells were injected subcutaneously in athymic nude mice. Treatment was initiated when tumours in each group achieved an average volume of approximately 170–200 mm³. Mice were treated with a single dose of nimotuzumab (1.0 mg per mouse) intraperitoneally, a single dose of γ -radiation (10 Gy), or neither (control) or both modalities, and tumour volume was determined at the indicated time points thereafter. Data are means \pm s.d. for seven to eight mice per group.

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

Treatment	H460		H292		Ma-1	
	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 ^aTime required for xenografts in each group to achieve a fivefold increase in volume. ^bThe 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.

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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 1A). 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/m²) on days 1 to 5, and cisplatin (20 mg/m²) 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 1B, E). However, the AFP concentration started to increase thereafter, and progression of the tumor was confirmed after the fourth cycle of chemotherapy (Figure 1C, E). The patient then received second-line chemotherapy with cisplatin (80 mg/m²) on day 1 and paclitaxel (200 mg/m²) on day 1 every 21 days for three cycles before surgery. The serum AFP level again decreased in association with tumor shrinkage (Figure 1D, 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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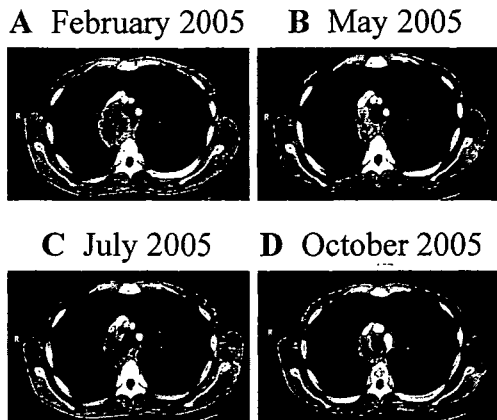
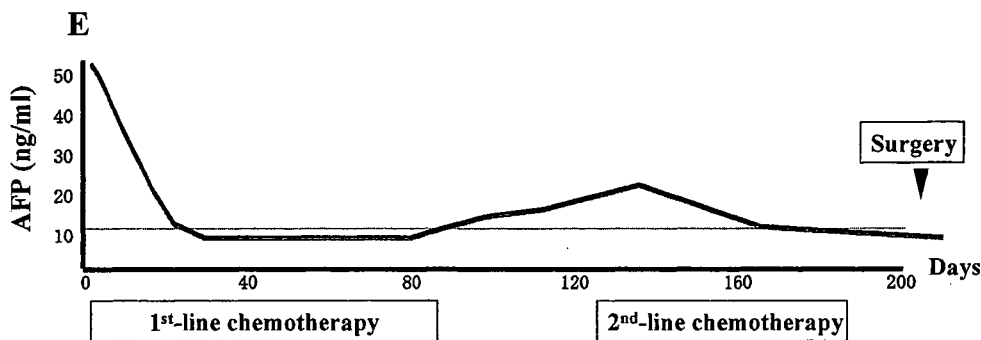


FIGURE 1. Chest computed tomography (CT) findings and serum AFP levels in the patient. A–D, Chest CT findings. A mass in the middle mediastinum was initially detected (A). The tumor had shrunk after three cycles of neoadjuvant chemotherapy (B), but its progression had resumed after the fourth cycle (C). 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 2A, 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 2C), and synaptophysin as well as negative for CD5, CD30, human chorionic gonadotropin, placental alkaline phosphatase, hepatocyte antigen, and thyroid transcription factor-1. No re-

gions 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 2D). 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.

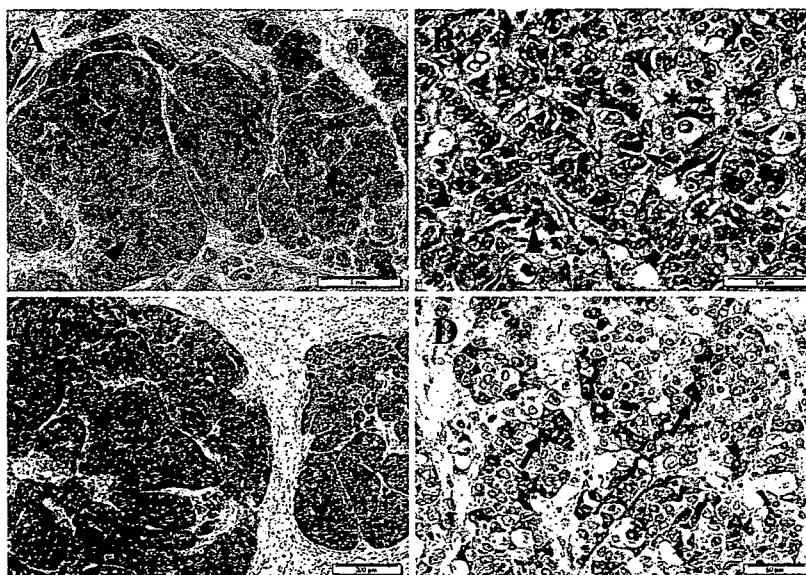


FIGURE 2. Histology and immunohistochemical analysis of the tumor specimen obtained at surgery. A, Hematoxylin-eosin staining revealed solid tumor nests with areas of necrosis (arrow heads). Note the homogeneous appearance of the tumor. B, High-power magnification of the tumor stained as in (A), showing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

ing 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-dihydropyridine (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 P450 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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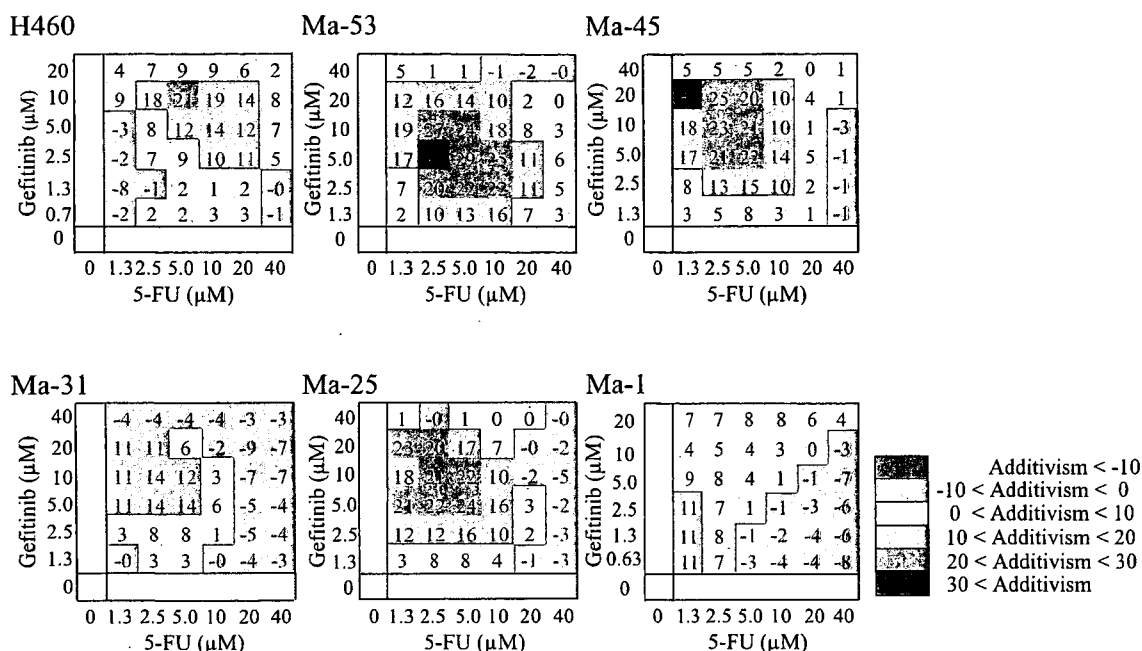


Figure 1. Inhibition of NSCLC cell growth by the combination of 5-FU and gefitinib *in vitro*. Cells with wild-type (H460, Ma-53, Ma-45, Ma-31, and Ma-25) or mutant (Ma-1) EGFR alleles were exposed for 72 h to 5-FU and gefitinib at the indicated concentrations, after which cell viability was measured with a colorimetric assay. The observed excess inhibition (%) relative to that predicted by the Bliss additivity 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% CO₂ 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 (2.0×10^3) 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 (IC₅₀) was

calculated. The effect of combining 5-FU and gefitinib was classified as additive, synergistic, or antagonistic with the Bliss additivity model (24–26). A theoretical curve was calculated for combined inhibition with the equation: $E_{\text{bliss}} = E_A + E_B - (E_A \times E_B)$, 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 ($\sim 2 \times 2 \times 2$ 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 mm³. 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 μ g 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 μ g) 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-GCCTCGGTGTGCCTTCA-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 \pm 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

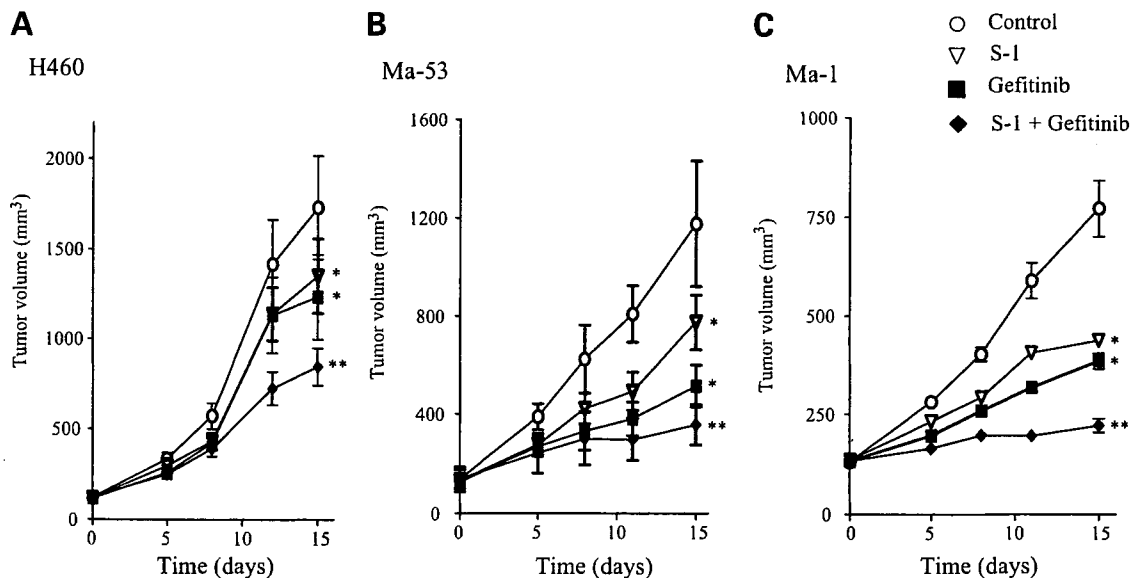


Figure 2. Antitumor activity of the combination of S-1 and gefitinib *in vivo*. **A** and **B**, nude mice with tumor xenografts established by s.c. implantation of NSCLC cells (H460 and Ma-53) possessing wild-type EGFR were treated daily for 2 wk with vehicle (control), S-1 (10 mg/kg), gefitinib (50 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 \pm 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).

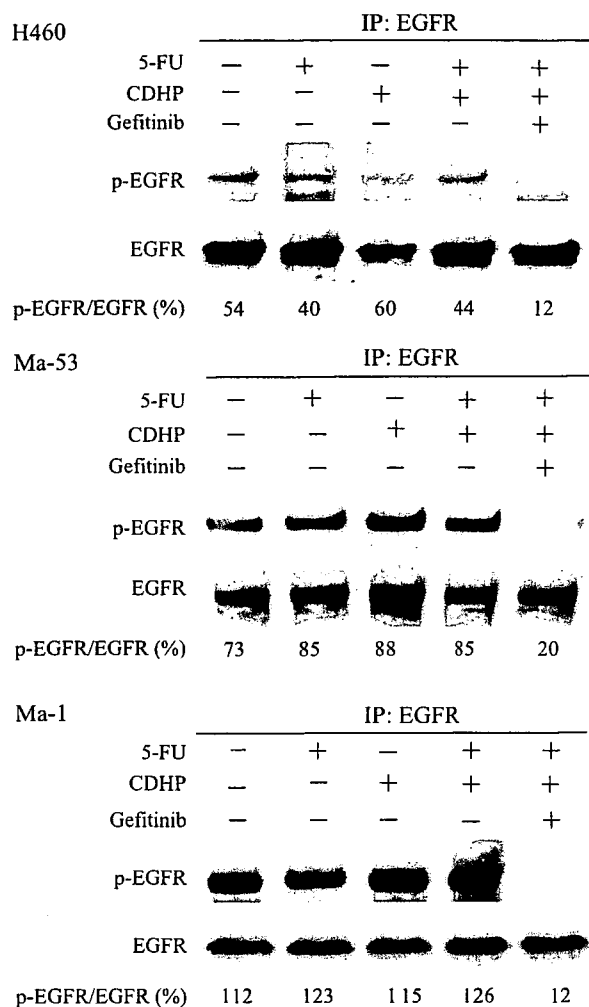


Figure 3. Lack of effect of 5-FU and CDHP on EGFR phosphorylation in NSCLC cell lines. NSCLC cells (H460, Ma-53, and Ma-1) were incubated for 24 h in medium supplemented with 2% fetal bovine serum and with 5-FU (10 $\mu\text{mol/L}$), CDHP (3 $\mu\text{mol/L}$), or gefitinib (5 $\mu\text{mol/L}$). Cell 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 $\mu\text{mol/L}$. 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. 2A 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 $\mu\text{mol/L}$) 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. 4A). In contrast, OPRT and TS were detected in all three NSCLC cell lines and their abundance did not appear related to *EGFR* status (Fig. 4A). Treatment of H460, Ma-53, or Ma-1 cells with gefitinib (5 $\mu\text{mol/L}$) 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. 4B). 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. 4B), 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-type *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 $\mu\text{g/g}$, values that were similar to the

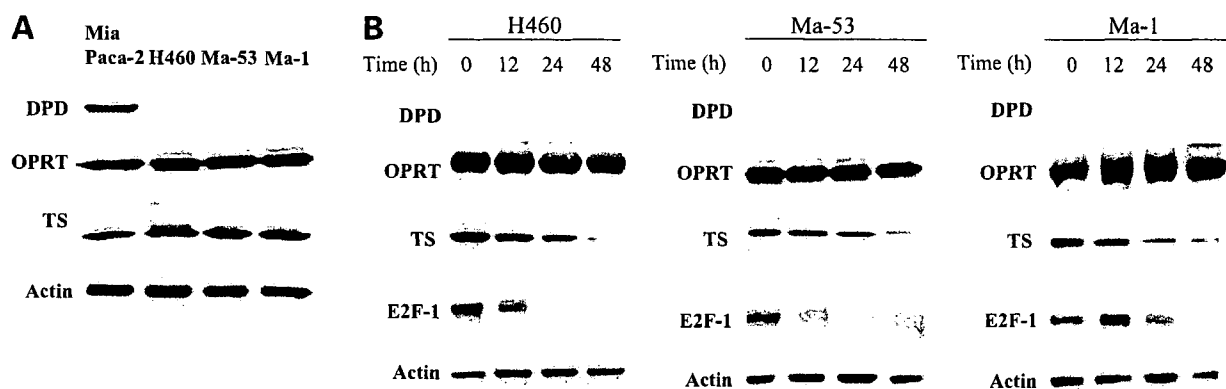


Figure 4. Effects of gefitinib on the expression of E2F-1, DPD, OPRT, and TS in NSCLC cell lines. **A**, lysates of H460, Ma-53, or Ma-1 cells were subjected to immunoblot analysis with antibodies to DPD, OPRT, TS, or β -actin (loading control). MiaPaca-2 cells were also examined as a positive control for DPD expression. **B**, NSCLC cells were incubated with gefitinib (5 $\mu\text{mol/L}$) 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.

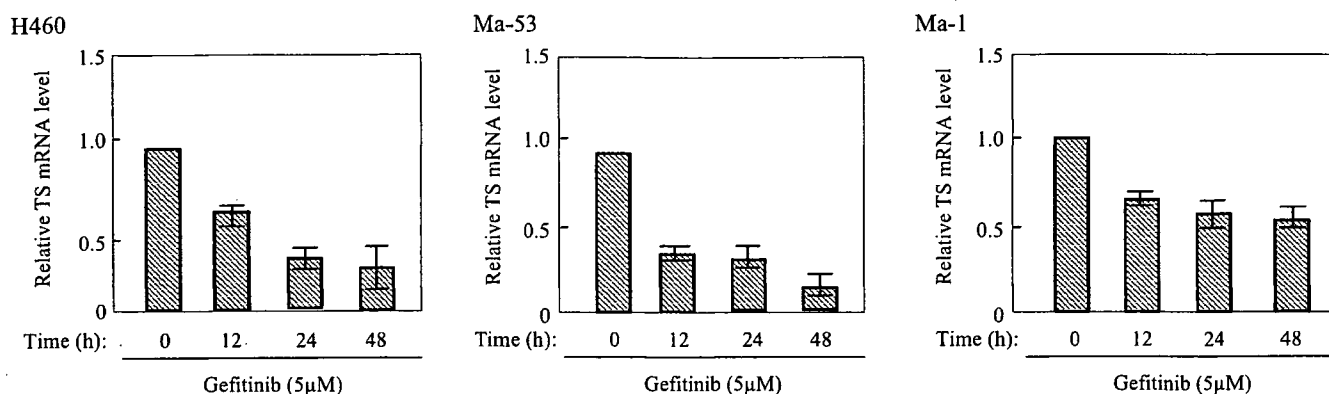


Figure 5. Down-regulation of TS mRNA by gefitinib in NSCLC cell lines. H460, Ma-53, or Ma-1 cells were incubated with gefitinib (5 μ mol/L) for the indicated times in medium containing 10% serum, after which total RNA was extracted from the cells and subjected to reverse transcription and real-time PCR analysis of TS mRNA. The amount of TS mRNA was normalized by that of glyceraldehyde-3-phosphate dehydrogenase mRNA. Mean \pm 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 pro-survival 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 μ mol/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 μ mol/L at a daily dose of 225 mg (64). Based on these data, we considered that a gefitinib concentration of 5 μ mol/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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Zinc and its transporter ZIP10 are involved in invasive behavior of breast cancer cells

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Zinc is an essential element, necessary for sustaining all life. Zinc deficiency causes taste impairments, immune deficiency, skin problems, and growth and mental retardation. Recent reports suggest that zinc is associated with an increased risk of cancer, although it is still unclear whether zinc or its transporters are involved in cancer progression. Here we show that zinc and its transporter ZIP10 are involved in the invasive behavior of breast cancer cells. The screening of clinical samples for ZIP10 mRNA expression suggested that ZIP10 was significantly associated with the metastasis of breast cancer to the lymph node. In addition, the expression of ZIP10 mRNA was higher in the invasive and metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-4355 than in less metastatic breast cancer cell lines, such as MCF7, T47D, ZR75-1 and ZR75-30. In *in vitro* cell migration assays, the depletion of zinc transporter ZIP10 and intracellular zinc inhibited the migratory activity of MDA-MB-231 and MDA-MB-4355 cells. These results showed that zinc and ZIP10 play an essential role in the migratory activity of highly metastatic breast cancer cells, and suggest ZIP10 as a possible marker for the metastatic phenotype of breast cancer and a promising target of novel treatment strategies. (*Cancer Sci* 2007; 98: 692–697)

Zinc is an essential heavy metal and a constituent of a great number of zinc metalloproteins in the human body.^(1,2) It is important in nucleic acid metabolism, cell replication, and tissue repair and growth, and it is required for tumor growth. Zinc deficiency is associated with diverse disorders, including impaired immunity, retarded growth, brain development disorders and delayed wound healing. Many reports have suggested an involvement of zinc in cancer development, and shown that the levels of zinc in the serum and malignant tissues decreases in patients with various malignancies, such as carcinoma of the liver, gallbladder, digestive tracts or prostate.^(3–7) In contrast with these observations, in breast cancer patients the zinc levels are lower in the serum and elevated in malignant tissues.^(3,6,8) In fact, studies of the role of zinc in malignant diseases have a long history of reporting contradictory and ill-defined biological processes.⁽⁹⁾

Eukaryotic cells have a remarkable ability to regulate their levels of intracellular zinc. Although cells are exposed to micromolar ranges of free zinc, the intracellular levels of free zinc that regulate the transcription of zinc influx, efflux or sequestration machinery are in the femtomolar range. Several proteins, including the ZRT IRT-like protein (ZIP) family zinc transporters, zinc transporter (ZnT) family zinc transporters and zinc-sequestering metallothioneins, maintain the intracellular zinc homeostasis.^(10–13) There are at least 14 human ZIP transporters, and they are believed to function in zinc influx into the cytosol.⁽¹⁰⁾ Some ZIP family members are reported to be involved in cancer progression: ZIP1 is reported to be a suppressor gene for prostate cancer⁽¹⁴⁾ and ZIP6/LIV1 to be involved in the metastasis of clinical breast cancer to the lymph node,⁽¹⁵⁾ although this issue is controversial.⁽¹⁶⁾ Nevertheless, the precise effects of human ZIP on tumor development and progression remain elusive.

Here we demonstrate that the migratory activity of metastatic breast cancer cells was inhibited by ZIP10 knockdown and by zinc chelation. Analysis of clinical samples showed that breast cancers with lymph-node metastasis had significantly higher ZIP10 zinc transporter expression than those without lymph-node metastasis. ZIP10 was recently shown to mediate zinc uptake and to act as a membrane transporter *in vivo*,⁽¹⁷⁾ but this is the first report describing a biological role for the ZIP10 zinc transporter in cancer progression. We also propose elevated ZIP10 as a candidate marker for the metastatic phenotype of breast cancer and a promising target of novel treatment strategies.

Materials and Methods

Clinical samples. The breast cancer samples used in the present study were obtained through a protocol approved by the Institutional Review Board, from 177 primary breast cancer patients who underwent breast-conserving surgery or mastectomy at the Department of Breast and Endocrine Surgery, Osaka University, from February 1998 to April 2005. The median age of patients was 54 years (range 28–87 years).

RNA extraction and reverse transcription. Total cellular RNA was extracted from frozen tumor specimens using TRIzol reagent, according to the protocol provided by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). Three micrograms of each total RNA sample was used for cDNA synthesis by Superscript II (Life Technologies, Rockville, MD, USA), with Oligo-(dT)₁₅ as the primer, and reaction conditions of 42°C for 90 min followed by heating at 70°C for 10 min.

Real-time polymerase chain reaction. Real-time polymerase chain reactions (PCR) were carried out using the ABI Prism 9700 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). For quantification, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcripts served as the quantitative control, and each sample was normalized to its *GAPDH* transcript level. The primer and probe mixtures for ZIP6, ZIP10 and *GAPDH* were purchased from Perkin-Elmer Applied Biosystems, and the PCR method was as recommended in the manufacturer's protocol.

Cell culture. Human breast cancer cell lines were obtained from American Type Culture Collection, Manassas, VA, USA. MDA-MB-231 and MDA-MB-4355 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), and MCF-7, T-47D, ZR-75-1 and ZR-75-30 cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin.

Conventional PCR. Conventional PCR amplification was carried out at 95°C for 1 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The primers used for PCR

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