Addition of S-1 to the Epidermal Growth Factor Receptor Inhibitor Gefitinib Overcomes Gefitinib Resistance in Non–small cell Lung Cancer Cell Lines with *MET* Amplification

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Abstract

Purpose: Most non–small cell lung cancer (NSCLC) tumors with activating mutations in the epidermal growth factor receptor (EGFR) are initially responsive to EGFR tyrosine kinase inhibitors (EGFR-TKI) such as gefitinib and erlotinib, but they almost invariably develop resistance to these drugs. A secondary mutation in *EGFR* (T790M) and amplification of the *MET* proto-oncogene have been identified as mechanisms of such acquired resistance to EGFR-TKIs. We have now investigated whether addition of the oral fluoropyrimidine derivative S-1 to gefitinib might overcome gefitinib resistance in NSCLC cell lines.

Experimental Design: The effects of gefitinib on EGFR signaling and on the expression both of thymidylate synthase and of the transcription factor E2F-1 in gefitinib-resistant NSCLC cells were examined by immunoblot analysis. The effects of S-1 (or 5-fluorouracil) and gefitinib on the growth of NSCLC cells were examined *in vitro* as well as in nude mice.

Results: Gefitinib induced down-regulation of thymidylate synthase and E2F-1 in gefitinib-resistant NSCLC cells with *MET* amplification but not in those harboring the T790M mutation of *EGFR*. The combination of 5-fluorouracil and gefitinib synergistically inhibited the proliferation of cells with *MET* amplification, but not that of those with the T790M mutation of *EGFR, in vitro*. Similarly, the combination of S-1 and gefitinib synergistically inhibited the growth only of NSCLC xenografts with *MET* amplification.

Conclusions: Our results suggest that the addition of S-1 to EGFR-TKIs is a promising strategy to overcome EGFR-TKI resistance in NSCLC with *MET* amplification.

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is abnormally amplified or activated in a variety of tumors, including non–small cell lung cancer (NSCLC; refs. 1–3), and it has therefore been identified as an important target in cancer treatment. Two inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI), gefitinib and erlotinib, both of which compete with ATP for binding to the tyrosine kinase pocket of the receptor, have been extensively studied in patients with NSCLC (4–7). Somatic mutations in the kinase domain of EGFR are associated with the response to EGFR-TKIs in a subset of NSCLC patients (8–15). Deletions in exon 19 of the EGFR gene (*EGFR*) and replacement of leucine with arginine at codon 858 (L858R) account for ~90% of these mutations (16–18). Despite the benefits of gefitinib and erlotinib in treatment of NSCLC associated with *EGFR* mutations, most, if not all, patients ultimately develop resistance to these drugs. In ~50% of these individuals, acquired resistance is associated with a secondary mutation, T790M, in *EGFR* (19–21). A recent study further suggested that ~20% of patients who become resistant to gefitinib do so as a result of acquired amplification of the proto-oncogene *MET* (22). The identification of strategies or agents capable of overcoming acquired resistance to EGFR-TKIs is thus an important clinical goal.

S-1 is an oral fluoropyrimidine derivative consisting of tegafur (FT) and two modulators, 5-chloro-2,4-dihydroxypyridine (gimeracil, CDHP) and potassium oxonate (oteracil, oxo), in a molar ratio of 1:0.4:1 (23, 24). S-1 is currently under evaluation for the treatment of NSCLC both as a single agent and in combination with other drugs (25–27). We have recently shown that combined treatment with S-1 and gefitinib has a synergistic antiproliferative effect on NSCLC cells regardless of the absence or presence of *EGFR* mutations (28). The gefitinib-induced down-regulation of thymidylate synthase (TS), likely mediated by down-regulation of the transcription factor E2F-1, was implicated in the synergistic antitumor effect

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Translational Relevance

Most non–small cell lung cancer (NSCLC) tumors with activating mutations in the epidermal growth factor receptor (EGFR) are initially responsive to EGFR tyrosine kinase inhibitors (EGFR-TKI) such as gefitinib and erlotinib, but they almost invariably develop resistance to these drugs. S-1 is an oral fluoropyrimidine derivative that has exhibited marked antitumor activity in recent clinical trials including patients with NSCLC. We have investigated whether the addition of S-1 to gefitinib might overcome gefitinib resistance in NSCLC cells. Gefitinib induced down-regulation of both thymidylate synthase and the transcription factor E2F-1 in gefitinib-resistant NSCLC cells with *MET* amplification but not in those harboring the T790M mutation of *EGFR*. The combination of S-1 and gefitinib exerted a synergistic antitumor effect only in gefitinib-resistant cells with *MET* amplification both *in vitro* and *in vivo*. Our preclinical findings indicate that the addition of S-1 to EGFR-TKIs is a promising strategy to overcome EGFR-TKI resistance.

of combined treatment with this EGFR-TKI and S-1. We have now examined whether treatment with gefitinib induces down-regulation of TS in gefitinib-resistant cells with *MET* amplification or the T790M mutation of *EGFR*. Moreover, we investigated the possibility that the addition of S-1 to gefitinib might overcome gefitinib resistance in NSCLC cells both *in vitro* and *in vivo*.

Materials and Methods

Cell lines and reagents. The human NSCLC cell lines HCC827, HCC827 GR5, HCC827 GR6, PC-9, PC-9/ZD, and H1975 were obtained as described previously (22, 29–31). HCC827, PC-9, PC-9/ZD, and H1975 cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. HCC827 GR5 and HCC827 GR6 cells were cultured in RPMI 1640 supplemented 10% fetal bovine serum and 1 μmol/L gefitinib. Gefitinib was obtained from AstraZeneca, S-1 was provided by Taiho Pharmaceutical Co. Ltd., and 5-fluorouracil (5-FU) was from Wako.

Immunoblot analysis. Cell lysates were fractionated by SDS-PAGE on 7.5% or 12% (TS) gels, 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 phosphorylated EGFR (pY1068), to extracellular signal-regulated kinase (ERK), to phosphorylated AKT, and to AKT were obtained from Cell Signaling Technology; those to EGFR were from Zymed; those to phosphorylated ERK and to E2F-1 were from Santa Cruz Biotechnology; those to TS were from Taiho; 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 (Amersham Biosciences) and by subsequent exposure to enhanced chemiluminescence reagents (Perkin-Elmer).

***Growth inhibition assay* in vitro.** Cells (2.0×10^3) were plated in 96-well flat-bottomed plates and cultured for 24 h before incubation for 72 h in the presence of various concentrations of 5-FU and gefitinib either alone or together at a ratio of 1:5, respectively. 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. Data were analyzed by the median-effect method (CalcuSyn software; Biosoft) to determine the drug concentrations resulting in 50% growth inhibition (IC_{50}). The Chou and Talalay combination index (CI), a well-established index reflecting the interaction of two drugs (32), was calculated at different levels of growth inhibition with the use of CalcuSyn software. The CI for 50% growth inhibition (IC_{50}) was calculated as follows:

$$\text{CI at } IC_{50} = \frac{IC_{50}\ (5-\text{FU combination})}{IC_{50}\ (5-\text{FU alone})} + \frac{IC_{50}(\text{gefitinib combination})}{IC_{50}\ (\text{gefitinib alone})}$$

CI values of <1, 1, and >1 indicate synergistic, additive, and antagonistic effects, respectively.

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 Co. Ltd.

***Growth inhibition assay* in vivo.** Cubic fragments of tumor tissue (~2 by 2 by 2 mm) were implanted s.c. into the axilla of 5- to 6-wk-old male athymic nude mice. Treatment was initiated when tumors in each group achieved an average volume of 50 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 per kilogram of body mass) and gefitinib (3 or 50 mg/kg) were administered by oral gavage daily for 28 d; control animals received 0.5% (w/v) aqueous solution of 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 twice or thrice per week.

Statistical analysis. Data are presented as means ± SE as indicated and were analyzed by Student's t test. A P value of <0.05 was considered statistically significant.

Results

Effects of gefitinib on TS expression in gefitinib-resistant cell lines with* MET *amplification. TS is an important target enzyme for 5-FU (33, 34), with a reduced level of TS expression having been associated with a higher rate of response to 5-FU–based chemotherapy (35, 36). We first examined the effects of gefitinib on the expression of E2F-1 and TS as well as on the phosphorylation of EGFR and downstream signaling molecules in three cell lines (HCC827, HCC827 GR5, and HCC827 GR6) by immunoblot analysis. HCC827 cells harbor the E746_A750 deletion in exon 19 of *EGFR*; HCC827 GR5 and HCC827 GR6 cells are clones of HCC827 that developed resistance to gefitinib as a result of exposure to increasing concentrations of the drug and which exhibit *MET* amplification. Gefitinib (5 μmol/L) completely inhibited both the phosphorylation of EGFR, of the protein kinase AKT, and of ERK as well as the expression of E2F-1 and TS in the parental HCC827 cells in a time-dependent manner (Fig. 1). In the resistant cells, gefitinib substantially inhibited the phosphorylation of EGFR, but it had no effect on that of AKT or ERK (Fig. 1), consistent with previous observations (22). Gefitinib induced a time-dependent decrease in the amounts of E2F-1 and TS in the resistant cells (Fig. 1). These data thus showed that gefitinib induced the down-regulation of TS expression, likely as a result of a decrease in the abundance of E2F-1, in gefitinib-resistant cells with *MET* amplification.

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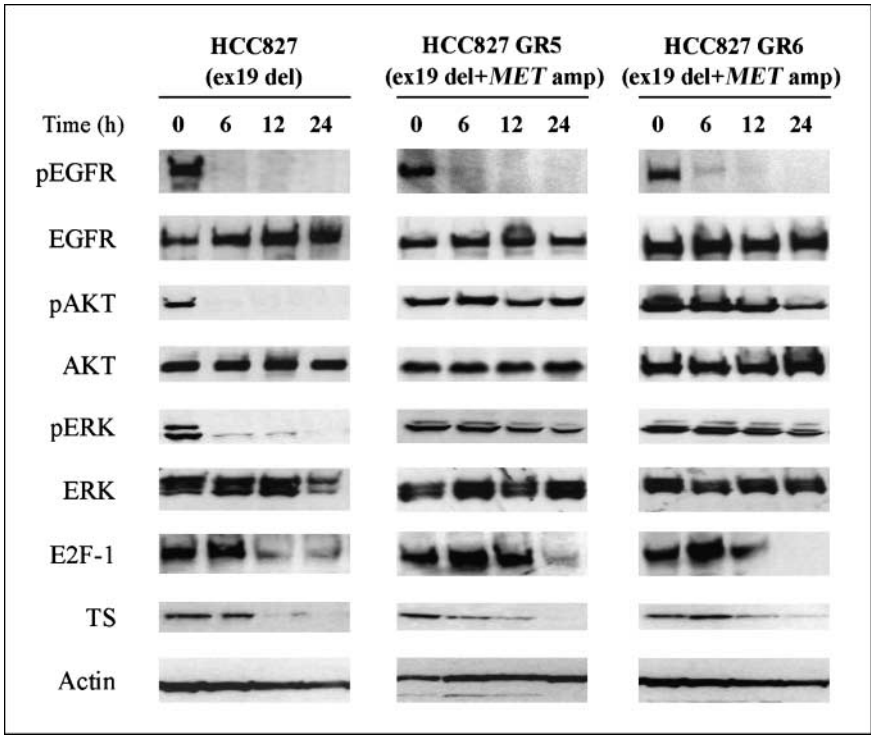

Fig. 1. Effects of gefitinib on EGFR, AKT, and ERK phosphorylation as well as on E2F-1 and TS expression in gefitinib-resistant NSCLC cells with *MET* amplification. Parental HCC827 cells and gefitinib-resistant clones with *MET* amplification (HCC827 GR5 and HCC827 GR6) were incubated with gefitinib (5 μmol/L) for the indicated times in medium containing 10% serum, after which cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR, AKT, and ERK as well as with those to E2F-1, TS, and β-actin (loading control).

***Effects of gefitinib on TS expression in gefitinib-resistant cell lines with the T790M mutation of* EGFR.** We next investigated whether gefitinib might inhibit TS expression in gefitinib-resistant cells harboring the T790M mutation of *EGFR*. We examined three cell lines: PC-9, PC-9/ZD, and H1975. PC-9 cells contain the E746_A750 deletion in exon 19 of *EGFR*, whereas PC-9/ZD cells are a gefitinib-resistant clone of PC-9 and also harbor the T790M mutation of *EGFR*; H1975 cells possess both L858R and T790M mutations of *EGFR*. Gefitinib completely or almost completely inhibited the phosphorylation of EGFR, AKT, and ERK as well as the expression of E2F-1 and TS in PC-9 cells in a time-dependent manner (Fig. 2). In contrast, phosphorylation of EGFR, AKT, and ERK as well as the expression of E2F-1 and TS were maintained in PC-9/ZD and H1975 cells incubated in the presence of gefitinib (Fig. 2). These findings thus showed that gefitinib failed to inhibit the expression of TS in gefitinib-resistant cells with a secondary T790M mutation of *EGFR*.

***Effects of the combination of 5-FU and gefitinib on the growth of gefitinib-resistant cell lines* in vitro.** We next investigated whether the down-regulation of TS expression induced by gefitinib in gefitinib-resistant cells with *MET* amplification would render these cells sensitive to the synergistic antiproliferative effect of the combination of S-1 and gefitinib. We therefore first examined the antiproliferative activity of the combination of 5-FU and gefitinib in the four gefitinib-resistant cell lines (HCC827 GR5, HCC827 GR6, PC-9/ZD, and H1975) *in vitro*. We used 5-FU instead of S-1 for *in vitro* experiments because tegafur, which is a component of S-1, is metabolized to 5-FU primarily in the liver. The combined effect of 5-FU and gefitinib was evaluated on the basis of the CI. The combination of 5-FU and gefitinib induced a synergistic growth-inhibitory effect (CI < 1) in cells with *MET* amplification, yielding CI values of 0.87 and 0.78 at 50% growth inhibition for HCC827 GR5 and HCC827 GR6 cells, respectively (Table 1; Fig. 3). In contrast, an antagonistic interaction (CI > 1) between 5-FU and gefitinib was apparent for cells harboring the T790M mutation of *EGFR*, with CIs of 1.10 and 1.42 at 50% growth inhibition for PC-9/ZD and H1975 cells, respectively (Table 1; Fig. 3). These results thus showed that the combination of 5-FU and gefitinib had a synergistic effect in gefitinib-resistant cells with *MET* amplification but not in those with the T790M mutation of *EGFR*.

***Effects of combined treatment with S-1 and gefitinib on the growth of gefitinib- resistant cell lines* in vivo.** We next investigated whether combined treatment with S-1 and gefitinib exhibited a synergistic effect on the growth of gefitinib-resistant cells with *MET* amplification *in vivo*. Doses of the two agents were selected to ensure moderate independent effects on tumor growth. When the tumors become palpable (50-150 mm^3), mice were divided into four groups and treated with vehicle, S-1, gefitinib, or the combination of both drugs by oral gavage for 4 wk. Combination therapy with S-1 and gefitinib inhibited the growth of tumors formed by PC-9 cells to a significantly greater extent than did treatment with S-1 or gefitinib alone (Fig. 4*A*). In contrast, no such synergistic effect was observed with tumors formed by PC-9/ZD (Fig. 4*B*) or H1975 (Fig. 4*C*) cells. Given that TS expression was inhibited by gefitinib in PC-9 cells but not in the gefitinib-resistant clone PC-9/ZD or in H1975 cells, these data suggested that the down-regulation of TS by gefitinib was responsible, at least in part, for the synergistic antitumor effect of S-1 and gefitinib. We then examined the effects of S-1 and gefitinib on the growth of HCC827 GR5 tumor xenografts with *MET* amplification. Neither S-1 nor gefitinib alone had a substantial effect on tumor growth (Fig. 4*D*). In contrast, administration of the two agents together resulted in a synergistic and almost complete inhibition of tumor growth (Fig. 4*D*). All of the treatments were well-tolerated, with no signs of toxicity or

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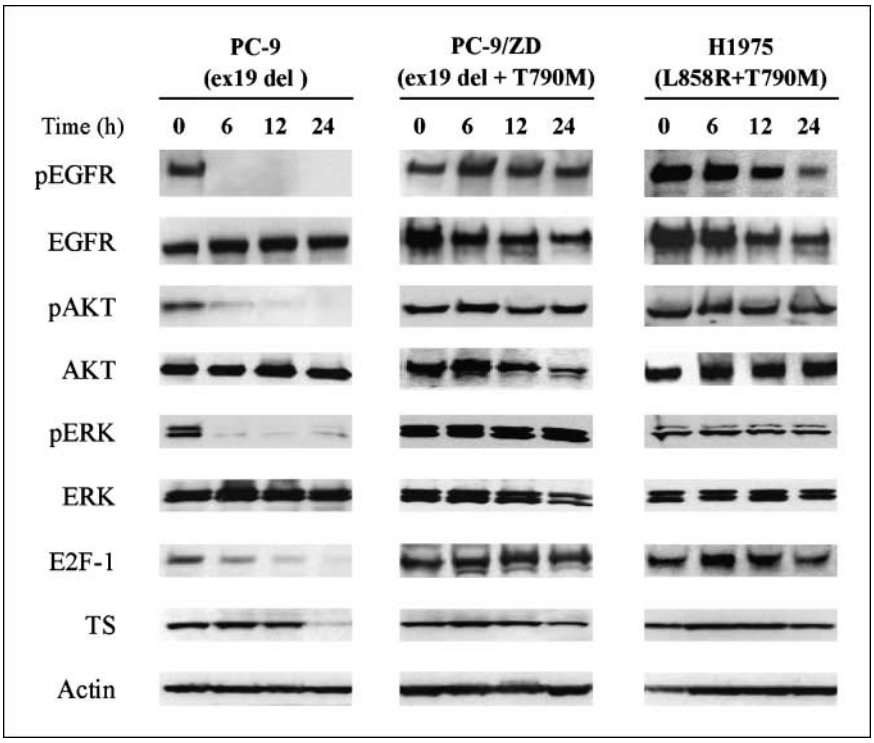

Fig. 2. Effects of gefitinib on EGFR, AKT, and ERK phosphorylation as well as on E2F-1 and TS expression in gefitinib-resistant NSCLC cells with the T790M mutation of *EGFR*. PC-9 cells as well as the gefitinib-resistant lines PC-9/ZD and H1975 harboring the T790M mutation of *EGFR* were incubated with gefitinib (5 μmol/L) for the indicated times in medium containing 10% serum, after which cell lysates were subjected to immunoblot analysis as in Fig. 1.

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weight loss during therapy (data not shown). These findings suggested that combination treatment with S-1 and gefitinib had a synergistic antitumor effect *in vivo* with gefitinib-resistant xenografts manifesting *MET* amplification, but not with those harboring the T790M mutation of *EGFR*, consistent with the results obtained *in vitro*.

Discussion

We have previously shown that combination treatment with S-1 and gefitinib had a synergistic antiproliferative effect on NSCLC cells regardless of the absence or presence of *EGFR* mutations, with down-regulation of TS by gefitinib contributing to its synergistic interaction with S-1 (28). In the present study, we examined whether gefitinib induces down-regulation of TS expression in NSCLC cell lines with different mechanisms of resistance to EGFR-TKIs. We used a gefitinib concentration of 5 μmol/L for these *in vitro* experiments. The concentration of gefitinib in tumor xenografts was previously shown to be 5 to 14 times that in plasma of mouse hosts treated with this drug (37). Daily oral administration of gefitinib (250 mg) in patients also gave rise to a drug concentration in tumor tissue that was substantially higher (by a mean factor of 42) than that in plasma (37). We previously showed that the maximal concentration of gefitinib in plasma of patients with advanced solid tumors had a mean value of 0.76 μmol/L at a daily dose of 225 mg (38). On the basis of these observations, a gefitinib concentration of 5 μmol/L is similar to the achievable concentration in tumor tissue of treated humans. We found that gefitinib inhibited TS expression in association with E2F-1 down-regulation in gefitinib-resistant cells with *MET* amplification but not in those with the T790M mutation of *EGFR*. One possible explanation for this difference in response between cells with *MET* amplification and those with the T790M mutation of *EGFR* is that gefitinib inhibited EGFR phosphorylation in the former cells but not in the latter. The T790M mutation is thought to inhibit the ability of gefitinib or erlotinib to bind to the ATP-binding pocket of the catalytic

Table 1. IC_{50} and CI values for the antiproliferative effects of gefitinib and 5-FU, alone or combined, on the growth of NSCLC cells *in vitro*

	Alone IC_{50} (μmol/L)		Combination IC_{50} (μmol/L)*		CI at IC_{50}
	Gefitinib	5-FU	Gefitinib	5-FU	
HCC827 GR5	11.64	2.83	5.56	1.11	0.87
HCC827 GR6	14.44	4.90	7.11	1.42	0.78
PC9/ZD	9.13	8.66	8.33	1.67	1.10
H1975	34.82	11.67	30.98	6.20	1.42

NOTE: Data are means of triplicates from a representative experiment.
*The concentrations of the two drugs needed to inhibit cell growth by 50% when gefitinib and 5-FU are combined.

domain of the receptor (19, 20), with the result that these agents are not able to suppress the phosphorylation of EGFR. *MET* amplification confers EGFR-TKI resistance by activating ErbB3 signaling in an EGFR-independent manner (22). Given that the increased affinity of EGFR for gefitinib conferred by primary *EGFR* mutations is maintained in cells with *MET* amplification, gefitinib is still able to inhibit EGFR phosphorylation in such cells (22). These observations raise the possibility that gefitinib-induced down-regulation of TS is determined by modulation of EGFR phosphorylation. On the other hand, the phosphorylation of AKT and ERK was not blocked by gefitinib in cells with the T790M mutation of *EGFR* or those with *MET* amplification, suggesting that the expression of TS might be regulated by an EGFR signaling pathway other than that mediated by AKT and ERK.

We evaluated the effects of combined treatment with S-1 and gefitinib on the proliferation of NSCLC cells with the two different types of gefitinib resistance mechanism. We found that S-1 (or 5-FU) and gefitinib exerted a synergistic antiproliferative effect in NSCLC cells with *MET* amplification both *in vitro* and *in vivo*, but that no such effect was apparent with cells harboring the T790M mutation of *EGFR*. These observations were consistent with the gefitinib-induced down-regulation of TS observed in the former cells but not in the latter. The active metabolite of 5-FU, fluoro-dUMP, forms a covalent complex with TS, resulting in inhibition of DNA synthesis (33, 34). TS is thus an important therapeutic target of 5-FU. An increase in TS expression and activity has been viewed as a mechanistic driver of 5-FU resistance in cancer cells (39–41). Down-regulation of TS would thus be expected to enhance the cytotoxicity of 5-FU as a result of the decrease in the amount of its protein target (42). Indeed, preclinical studies have shown that the down-regulation of TS by antisense oligonucleotides or other means enhances the efficacy of 5-FU (43–46), supporting the notion that gefitinib-induced down-regulation of TS contributes to its synergistic interaction with S-1 in gefitinib-resistant NSCLC cells with *MET* amplification.

A recent clinical study showed that most patients with acquired resistance to gefitinib or erlotinib manifested a worsening of lung cancer symptoms and an increase in tumor size after discontinuation of these agents (47). However, most of these individuals showed stabilization or improvement in symptoms and a decrease in tumor size on resumption of EGFR-TKI treatment (47). These clinical findings suggest the possibility that tumors with acquired resistance to EGFR-TKIs continue to require signaling through EGFR for their survival. Indeed, a preclinical study found that the combination of gefitinib and an inhibitor of the tyrosine kinase activity of MET, but neither agent alone, induced substantial growth inhibition in gefitinib-resistant NSCLC cells with *MET* amplification (22). These observations support the notion that the continuation of treatment with gefitinib or erlotinib might be of value even after the development of acquired resistance to these drugs. Our present results indicate that the addition of S-1 to EGFR-TKIs might overcome EGFR-TKI resistance in patients whose resistance is attributable to *MET* amplification.

The T790M mutation of *EGFR* and *MET* amplification account for ~60% to 70% of all cases of acquired resistance to gefitinib or erlotinib (19–22). A signaling pathway dependent on the insulin-like growth factor receptor was also recently implicated in resistance to EGFR-TKIs (48). The mechanisms of EGFR-TKI resistance other than that mediated by the T790M mutation of *EGFR* may thus be dependent on the activation of receptor tyrosine kinases that are not directly targeted by these drugs. Given that gefitinib would be expected to inhibit EGFR phosphorylation in all gefitinib-resistant cells with such a mechanism of resistance, treatment with this drug might also be expected to induce the down-regulation of TS

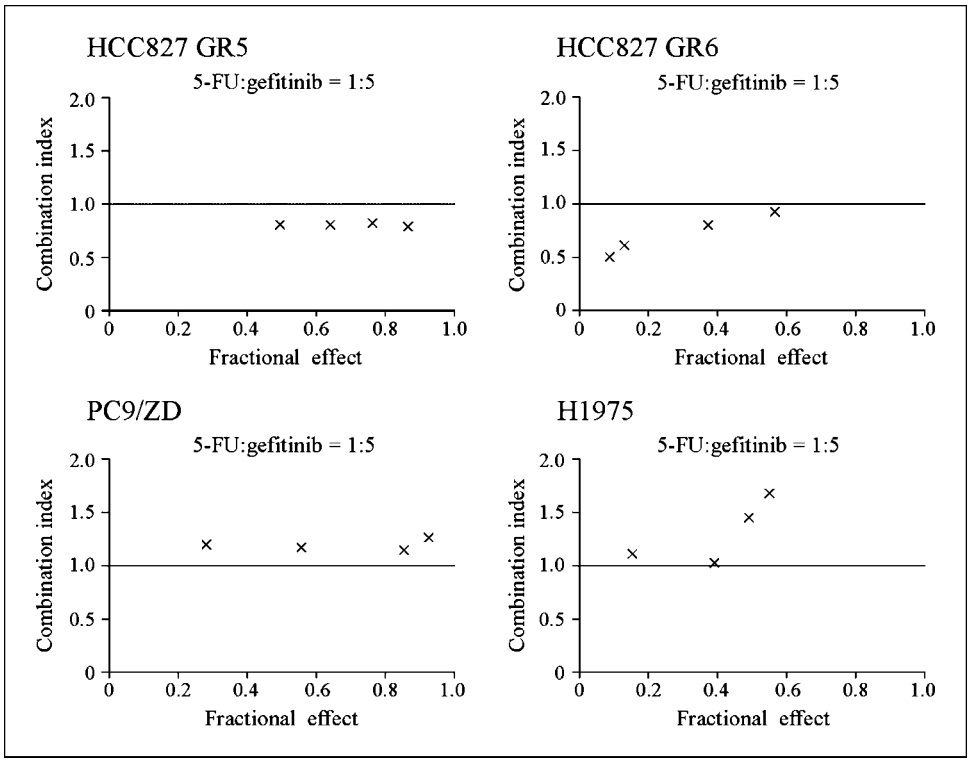

Fig. 3. Effects of the combination of 5-FU and gefitinib on the growth of gefitinib-resistant NSCLC cells *in vitro*. Cells with *MET* amplification (HCC827 GR5 and HCC827 GR6) or those harboring the T790M mutation of *EGFR* (PC-9/ZD and H1975) were incubated for 72 h with 5-FU or gefitinib alone or with both drugs at a fixed 5-FU:gefitinib molar ratio of 1:5, after which cell viability was measured. The interaction between 5-FU and gefitinib was evaluated on the basis of the CI, which is plotted against the fraction of growth inhibition. Data are means of triplicates from a representative experiment.

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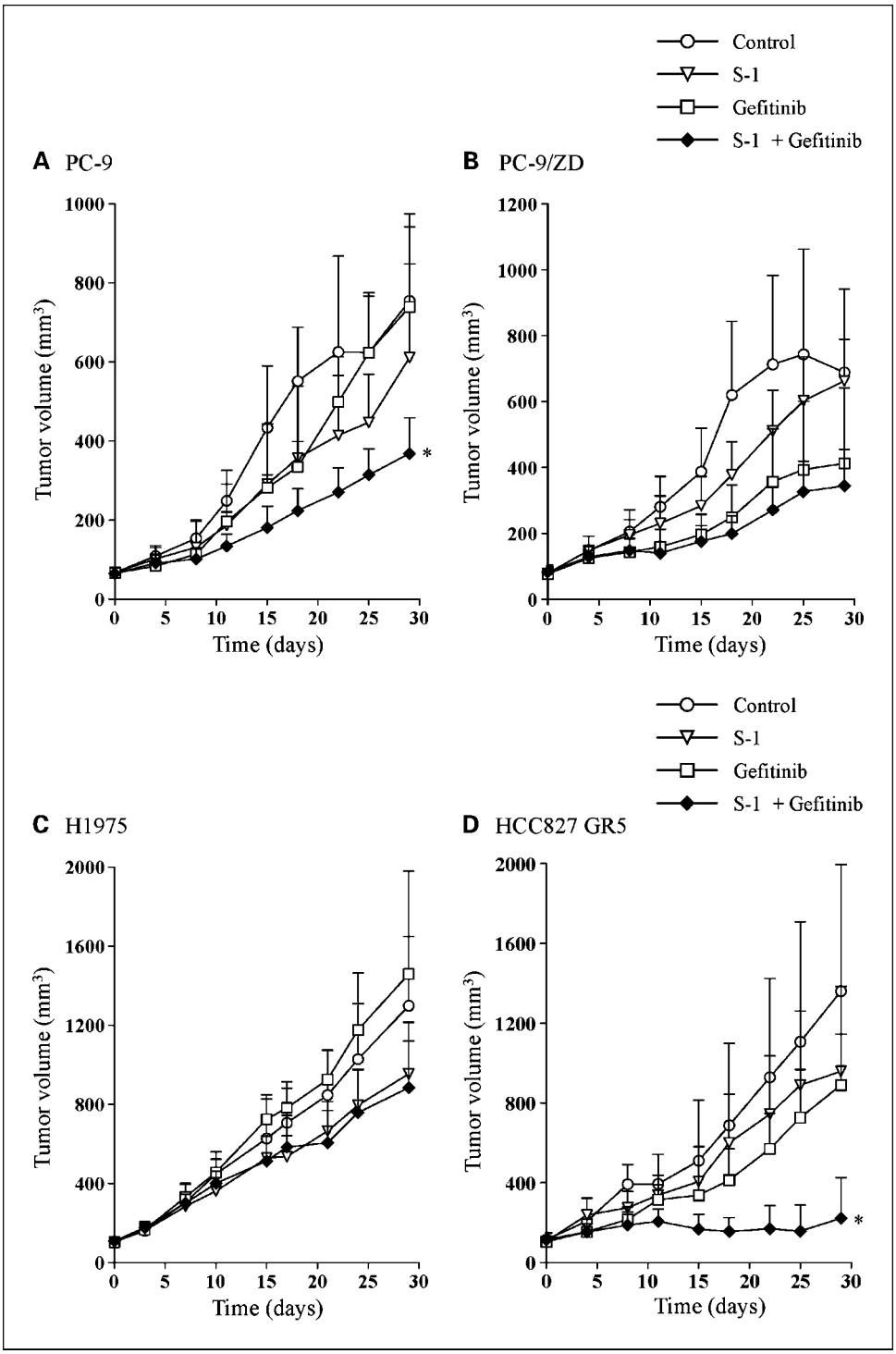

Fig. 4. Effects of the combination of S-1 and gefitinib on the growth of gefitinib-resistant NSCLC cells *in vivo*. *A,* nude mice with tumor xenografts established by s.c. implantation of PC-9 cells were treated daily for 4 wk with vehicle (control), S-1 (10 mg/kg), gefitinib (3 mg/kg), or both drugs by oral gavage. *B* to *D,* nude mice with tumor xenografts of NSCLC cells either harboring the T790M mutation of *EGFR* (PC-9/ZD and H1975) or exhibiting *MET* amplification (HCC827 GR5) were treated daily for 4 wk with vehicle (control), S-1 (10 mg/kg), gefitinib (50 mg/kg), or both drugs by oral gavage. Tumor volume was determined at the indicated times after the onset of treatment. *Points,* mean of values from seven mice per group; *bars,* SE. *, $P < 0.05$ for the combination of S-1 plus gefitinib versus control or either S-1 or gefitinib alone (Student's *t* test).

expression that facilitates the antitumor effect of S-1. Pemetrexed, an antifolate drug with multiple targets, has also shown antitumor activity mediated by TS inhibition in a broad range of tumors including NSCLC (49–53). The addition of S-1 or pemetrexed to gefitinib may thus prove effective in NSCLC patients whose gefitinib resistance is attributable to activation of a non-EGFR tyrosine kinase.

In conclusion, we have shown that the combination of S-1 and gefitinib had a synergistic antiproliferative effect in gefitinib-resistant NSCLC cells with *MET* amplification. The inhibition of EGFR phosphorylation and down-regulation of TS by gefitinib were associated with the synergistic interaction between gefitinib and S-1. Our preclinical results suggest that the addition of S-1 to gefitinib is a potential strategy for overcoming EGFR-TKI resistance and warrants clinical evaluation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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