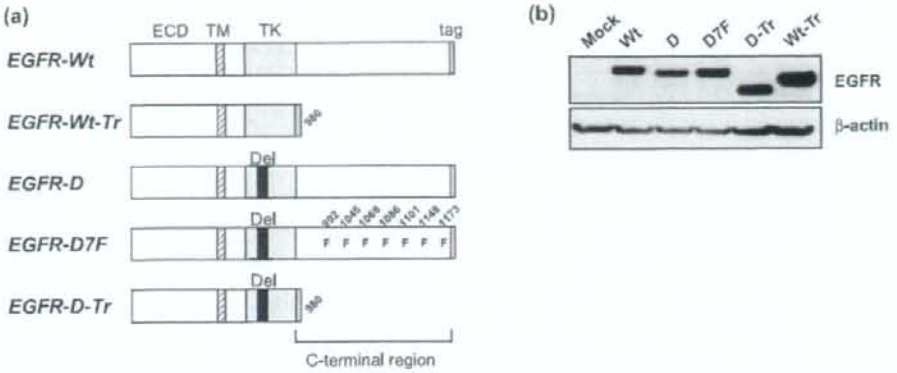

Fig. 1. Epidermal growth factor receptor (EGFR) constructs and their expression. (a) Structures of the various EGFR mutants. EGFR-Wt, wild-type human EGFR; EGFR-Wt-Tr, wild-type kinase domain of EGFR with C-terminal truncation at amino acid 980; EGFR-D, EGFR with a 15-bp deletion from the tyrosine kinase domain (delE746_A750); EGFR-D7F, 15-bp deletion of EGFR (delE746_A750) and substitution of seven tyrosine residues to phenylalanine (Y992F, Y1068F, Y1045F, Y1068F, Y1086F, Y1148F, Y1173F); and EGFR-D-Tr, 15-bp deletion of EGFR (delE746_A750) with C-terminal truncation at amino acid 980. EGFR-Wt, EGFR-D, EGFR-D7F, and EGFR-D-Tr contained a myc-tag. EGFR-Wt-Tr contained a flag-tag. ECD, extracellular domain; TK, tyrosine kinase; TM, transmembrane. (b) Stable transfectants were lysed and cell lysates containing equal amounts of protein were immunoblotted with anti-EGFR antibody recognizing the extracellular domain of EGFR. A band with a molecular weight of ~170 kDa was detected in the HEK293/Wt, HEK293/D, and HEK293/D7F cells, and a band of lower molecular weight was detected in the HEK293/D-Tr and HEK293/Wt-Tr cells. Mock, HEK293/Mock; Wt, HEK293/Wt; Wt-Tr, HEK293/Wt-Tr; D, HEK293/D; D7F, HEK293/D7F; and D-Tr, HEK293/D-Tr.

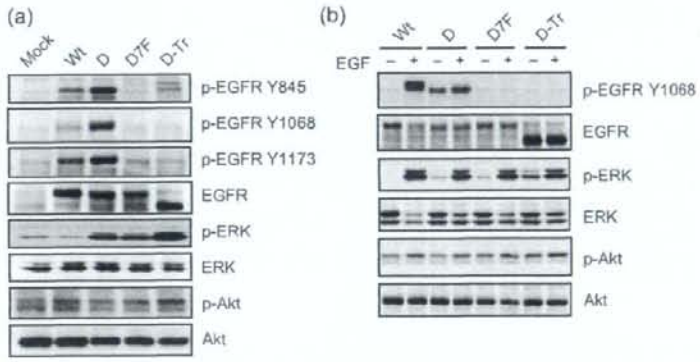

Fig. 2. Epidermal growth factor receptor (EGFR) lacking C-terminal autophosphorylation sites retains EGFR signal transduction. (a) The HEK293/Mock, HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were lysed, and the cell lysates were immunoblotted with anti-phospho-EGFR (p-EGFR Y845, Y1068, Y1173), anti-EGFR (recognizing the extracellular domain), anti-phospho-ERK, anti-ERK, anti-phospho-Akt, and anti-Akt antibodies. (b) The HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were incubated in 1% serum starve medium for 12 h, followed by treatment with 10 ng/mL epidermal growth factor for 10 min at 37°C. The cell lysates were immunoblotted. Mock, HEK293/Mock; Wt, HEK293/Wt; D, HEK293/D; D7F, HEK293/D7F; and D-Tr, HEK293/D-Tr.

and HEK293/D-Tr cells, as well as the HEK293/Wt and HEK293/D cells. These results indicate that EGFR lacking the C-terminal autophosphorylation sites (EGFR-D-Tr and EGFR-D7F) retained signal transduction ability.

Transfectants with EGFR lacking C-terminal autophosphorylation sites retain their hypersensitivity to EGFR TKI. EGF stimulation increased the growth of HEK293/Wt cells significantly but did not affect their sensitivity to AG1478 (data not shown). To examine the role of the C-terminal region of EGFR in cellular sensitivity to EGFR TKI, the sensitivity of these transfectants was examined by growth-inhibition assay (Fig. 3a). HEK293/Wt and HEK293/Wt-Tr cells with normal EGFR in relation to the kinase domain were relatively resistant to EGFR TKI, with IC_{50} values of 3.0 ± 0.97 and 8.1 ± 0.99 μM. On the other hand, HEK293/D (0.028 ± 0.018 μM), HEK293/D7F (0.047 ± 0.030 μM), and HEK293/D-Tr (0.017 ± 0.017 μM) cells were ~100 times more sensitive to AG1478 compared to HER293/Wt cells (Fig. 3a), suggesting that the cells transfected with EGFR lacking C-terminal phosphorylation sites retained hypersensitivity to EGFR TKI. There were no differences in the proliferation rates of these cell lines under the absence of drug exposure (data not shown).

To elucidate the effect of EGFR TKI on the EGFR-triggered signal cascade, the phosphorylation status of EGFR and ERK was examined in the transfectants treated with AG1478 under the 1% serum starve medium (Fig. 3b). AG1478 at a concentration of 20 nM inhibited the phosphorylation of EGFR in HEK293/D cells, but not in the other cell lines. The increased phosphorylation of ERK observed in the HEK293/D, HEK293/D7F, and HEK293/D-Tr cells was inhibited by AG1478 at 20 nM. These results suggest that signal transduction from C-terminal-truncated EGFR to downstream molecules allows sensitivity to EGFR TKI to be retained, just like the deletion mutant of EGFR (delE746_A750).

Endogenous HER families are not involved in the dimerization of EGFR-D-Tr and EGFR-D7F. We hypothesized that the signals from EGFR lacking the C-terminal autophosphorylation sites were transduced through heterodimerization with endogenous EGFR, HER2, or HER3. No significant endogenous EGFR expression or its phosphorylation was observed in the HEK293/Mock cells (Fig. 4a). Very low levels of intrinsic HER2 or HER3 expression were detected in the HEK293 cells, and the expression levels seemed not to be involved in significant drug sensitivity nor increased signal transduction (Fig. 4b,c). Therefore, it is not likely that heterodimerization of

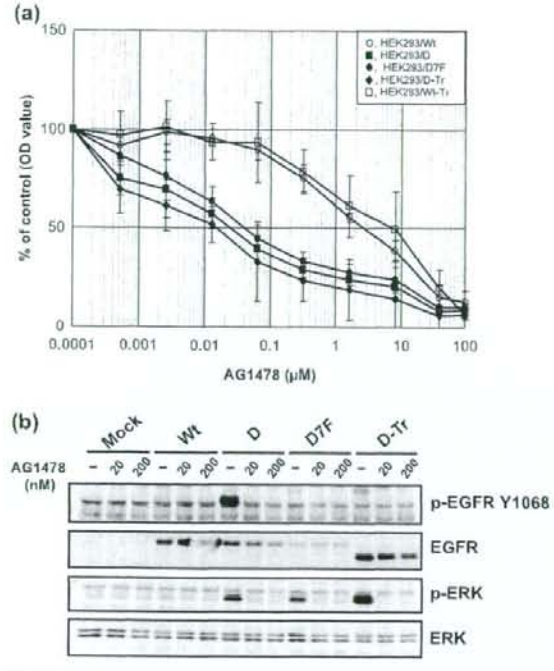

Fig. 3. Sensitivity of cell growth and downstream epidermal growth factor receptor (EGFR) signaling to AG1478 in the mutant EGFR transfectants. (a) The growth-inhibitory effect of AG1478 in HEK293/Wt, HEK293/Wt-Tr, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells. The seeded cells were exposed to AG1478 for 72 h and the cellular proliferative activity was determined by MTT assay. (b) The HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were incubated in 1% serum starve medium for 12 h, followed by exposure to 20 or 200 nM AG1478 for 3 h at 37°C. The cell lysates were immunoblotted with anti-phospho-EGFR (p-EGFR Y1068), anti-EGFR (recognizing the extracellular domain), anti-phospho-ERK, or anti-ERK antibodies. Mock, HEK293/Mock; Wt, HEK293/Wt; Wt-Tr, HEK293/Wt-Tr; D, HEK293/D; D7F, HEK293/D7F; D-Tr, HEK293/D-Tr.

EGFR lacking C-terminal autophosphorylation sites with endogenous HER receptors contributes to the signal transduction. It is thus speculated that homodimerization of EGFR lacking C-terminal autophosphorylation sites transduces the signals to downstream molecules. Indeed, the results of the chemical crosslinking assay revealed clear homodimerized bands in the HEK293/Wt, HEK293/D, and HEK293/D7F cells (Fig. 5a). In the HEK293/D-Tr cells, homodimerized bands with lower molecular weights (indicated by the black arrow) were detected (Fig. 5a) and these dimers were not phosphorylated (Fig. 5b). Taken together, we speculate that EGFR lacking C-terminal autophosphorylation sites form homodimers.

Despite a lack of C-terminal autophosphorylation sites, transfected cells retain their capacity for EGFR-dependent Shc phosphorylation. Binding of adaptor proteins to the C-terminal region of EGFR is essential for EGFR signal transduction. It is widely recognized that tyrosines 1068 and 1086 are most important for Sos and Grb2 activation; EGFR-D7F and EGFR-D-Tr lack these tyrosine residues. Sos and Grb2 were coprecipitated with EGFR in the HEK293/Wt and HEK293/D cells, but not in the HEK293/D7F or HEK293/D-Tr cells (Fig. 6a). The bands were confirmed by reblotting of the membranes used for immunoblotting (data not shown). Another adaptor protein, Shc, also binds to the C-terminal region of EGFR, and phosphorylation of Shc activates the ERK pathway. An increase in phosphorylated p46 and p52 Shc was observed in the HEK293/D, HEK293/D7F, and HEK293/D-Tr cells compared with the HEK293/Mock and HEK/Wt cells (Fig. 6b). The phosphorylation of Shc observed in the HEK293/D, HEK293/D7F, and HEK293/D-Tr cells was completely inhibited by 20 nM AG1478 (Fig. 6c). These results suggest that EGFR lacking C-terminal autophosphorylation sites activates Shc in a C-terminal-independent manner, and that Shc-mediated signals may be involved in the hypersensitivity to EGFR TKI of HEK293 cells expressing EGFR lacking C-terminal autophosphorylation sites.

Discussion

In the present study, we investigated the relationship between phosphorylation of tyrosine residues in the C-terminal region of EGFR and cellular sensitivity to EGFR TKI. Increased phosphorylation of Shc and ERK was observed in HEK293/D7F and HEK293/D-Tr cells, which expressed EGFR lacking autophosphorylation sites in the C-terminal region. Previous reports have demonstrated

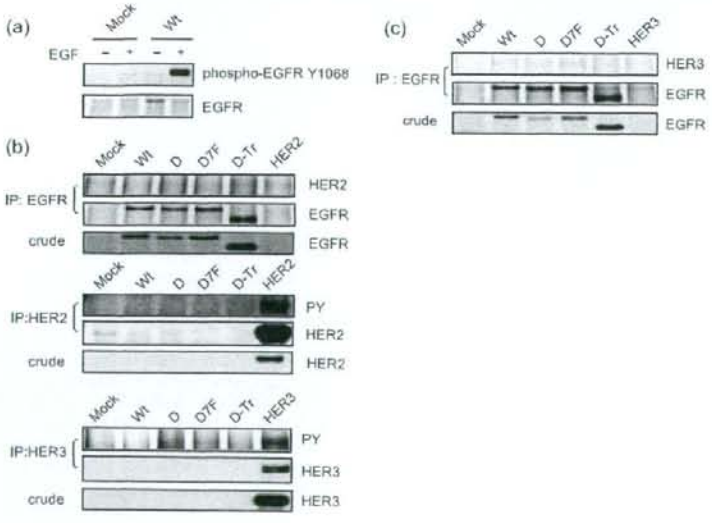

Fig. 4. Heterodimerization of mutant epidermal growth factor receptor (EGFR) with endogenous receptors of the HER family in mutant EGFR transfectants. Expression of endogenous EGFR and response to epidermal growth factor stimulation. HEK293/Mock, HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were incubated in 1% serum starve medium for 12 h followed by the addition of 10 ng/mL epidermal growth factor for 10 min at 37°C. (a) The whole-cell lysates of HEK293/Mock and HEK293/W+ cells containing equal amounts of protein were immunoblotted with anti-phospho-EGFR (p-EGFR Y1068) and anti-EGFR (recognized extracellular domain). (b,c) The lysates were immunoprecipitated with anti-EGFR, anti-HER2, or anti-HER3 antibodies, and immunoblotted with anti-EGFR, anti-HER2, anti-HER3, or anti-phosphotyrosine antibodies to detect the dimerization and phosphorylation of EGFR and endogenous HER2 or HER3. HER2, HER2-introduced HEK293 cells as a positive control; HER3, HER3-introduced HEK293 cells as a positive control.

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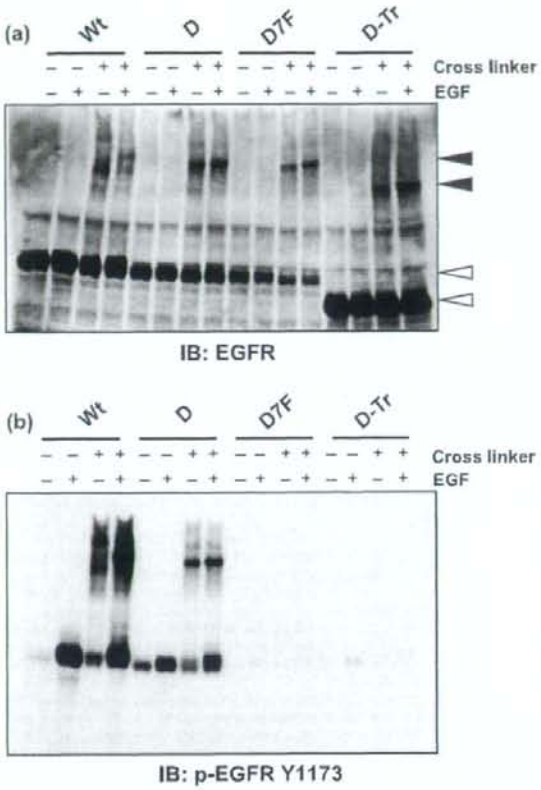

Fig. 5. The cells under 1% serum starved medium were allowed to react with 2 mM of the chemical crosslinking regent BS_3 before the crosslinking reaction was quenched. The cell lysates were immunoblotted with (a) anti-epidermal growth factor receptor (EGFR) (recognizing the extracellular domain) and (b) anti-phosphoEGFR (p-EGFR Y1173) antibodies to detect the dimerization and phosphorylation of wild-type and mutant EGFR. Black arrow, EGFR dimer; open arrow, EGFR monomer. Mock, HEK293/Mock; Wt, HEK293/Wt; D, HEK 293/D; D7F, HEK293/D7F; D-Tr, HEK293/D-Tr. EGF, epidermal growth factor.

that cells expressing EGFR lacking C-terminal autophospholylation sites retain EGF-induced mitogenic and transforming activity.[27,28] Our data and these previous reports suggest that there exist other EGFR signaling pathways besides those mediated by the C-terminal tyrosine residues. In addition, these signaling pathways are operative in the active EGFR mutant (delE746_A750) as well as wild-type EGFR.[28] The results of our growth-inhibition assay demonstrated the hypersensitivity of HEK293/D7F and HEK293/D-Tr cells to EGFR TKI, and phosphorylation of ERK and Shc in these cells was also inhibited. These results suggest that this EGFR signaling pathway contributes to tumor cell growth.

We demonstrated the hypersensitivity of the transfectants (HEK293/D7F and HEK293/D-Tr cells) to AG1478. We previously reported the hypersensitivity of transfectants carrying mutant EGFR to AG1478 as well as gefitinib, ZD6474, and erlotinib.[8,15,31] Therefore, it can be easily speculated that the HEK293/D7F and HEK293/D-Tr cells would also be hypersensitive to the clinically available EGFR TKI and AG1478.

Somatic EGFR mutation in lung cancer has been reported, and over 20 types of mutations have been reported.[10] The L858R point mutation in exon 21 of EGFR is a major point mutation (such as in delE746_A750) that contributes to EGFR TKI hypersensitivity.[32] Interestingly, we constructed cells that overexpressed EGFR-Wt-Tr and EGFR-D-Tr, and a mutant truncated form of EGFR similar to EGFR-Wt-Tr was previously found in patients with glioblastoma.[33] The mutant was truncated at amino acid 958 of EGFR and the frequency was relatively high in 7 of 48 patients. Therefore, it would be of interest to determine in future studies whether this C-terminal-truncated form of delE746_A750 EGFR, similar to EGFR-D-Tr, might be identifiable in human materials in the clinical setting.

We attempted to clarify the signaling pathway from the C-terminal region of EGFR. We observed the phosphorylation of ERK and Shc in HEK293/D7F and HEK293/D-Tr cells, and these phosphorylations were inhibited by exposure to AG1478. These phosphorylations were not observed in HEK293/Mock, HEK293/Wt, or HEK293/Wt-Tr cells. Our results suggest that the constitutively active mutant EGFR lacking C-terminal autophosphorylation sites is sufficient for activation of the downstream pathway. However, it remains unknown how signals are transduced from EGFR without a C-terminal region to Shc, as no direct binding of Grb2 or Shc with EGFR lacking the C-terminal region was detected in the HEK293/D7F and HEK293/D-Tr cells (Fig. 6a). We attempted to identify the mediator molecules binding to EGFR-D-Tr and EGFR-D7F by mass analysis of immunoprecipitates; however, no clear mediator molecules were identified. As a possible indirect mechanism, Sasaoka *et al.* postulated that ErbB2–Shc signals from EGFR lacking C-terminal autophosphorylation sites.[34] However, we consider this unlikely from the results of our experiments because no significant expression of Erb2 was detected in the HER293 cells.

The results of the crosslinking assay demonstrated that a complex of lower molecular weight was present in the HEK293/D-Tr cells compared with the HER293/Wt cells, indicating that truncated EGFR forms homodimers in the HEK293/D-Tr cells. Thus, it can be speculated that homodimerized truncated EGFR directly transduces signals downstream.

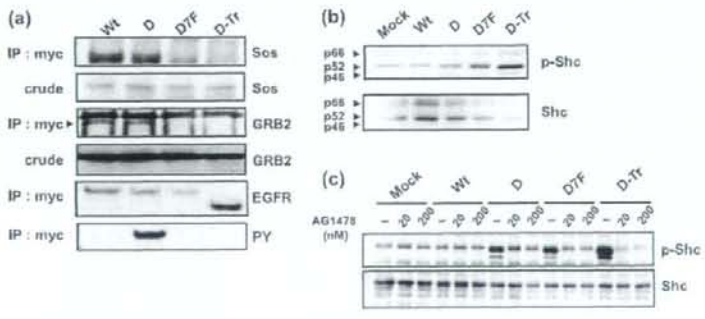

Fig. 6. Interaction between mutant epidermal growth factor receptor (EGFR) and adaptor proteins. The cells were cultured under normal conditions. (a) The lysates of HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were immunoprecipitated with anti-myc tag antibody; the precipitates were immunoblotted with anti-son of sevenless homolog (Sos) and anti-growth factor receptor-bound protein (Grb) 2 antibodies. (b) Whole-cell lysates containing equal amounts of protein were immunoblotted with anti-phospho-Src homology and collagen homology (Shc) and anti-Shc antibodies. (c) The cells incubated in 1% serum starve medium for 12 h were treated with 20 or 200 nM AG1478 for 3 h and then the lysates were immunoblotted with anti-phospho-Shc or anti-Shc antibodies. Mock, HEK293/Mock; Wt, HEK293/Wt; D, HEK293/D; D7F, HEK293/D7F; D-Tr, HEK293/D-Tr. PY, anti-phospho-tyrosine.

In conclusion, our results indicate that an as-yet-unknown signaling pathway of EGFR exists that is independent of the C-terminal region of EGFR, and these regions are not required for cellular sensitivity to EGFR TKI.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer set for epidermal growth factor receptor cDNA with substitution of seven tyrosine residues to phenylalanine in the C-terminal region

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Mitogen-activated protein kinase phosphatase-1 modulated JNK activation is critical for apoptosis induced by inhibitor of epidermal growth factor receptor-tyrosine kinase

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Keywords
AG1478; c-Jun N-terminal kinase; epidermal growth factor receptor; mitogen-activated protein kinase phosphatase-1; non-small-cell lung cancer

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Alterations resulting in enhanced epidermal growth factor receptor (EGFR) expression or function have been documented in a variety of tumors. Therefore, EGFR-tyrosine kinase is a promising therapeutic target. Although *in vitro* and *in vivo* studies have shown the anti-tumor activity of EGFR-tyrosine kinase inhibitors against various tumor types, little is known about the mechanism by which such inhibitors effect their anti-tumor action. AG1478 is known to selectively inhibit EGFR-tyrosine kinase. In this study, we showed that AG1478 caused apoptosis and apoptosis-related reactions such as the activation of caspase 3 in human non-small cell lung cancer cell line PC-9. To investigate the signaling route by which AG1478 induced apoptosis, we examined the activation of c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase p38 in AG1478-treated PC-9 cells. JNK, but not p38, was significantly activated by AG1478 as determined by both immunoblot analysis for levels of phosphorylated JNK and an *in vitro* activity assay. Various types of stimuli activated JNK through phosphorylation by the dual-specificity JNK kinases, but the dual-specificity JNK kinases MKK4 and MKK7 were not activated by AG1478 treatment. However, JNK phosphatase, i.e. mitogen-activated protein kinase phosphatase-1 (MKP-1), was constitutively expressed in the PC-9 cells, and its expression level was reduced by AG1478. The inhibition of JNK activation by ectopic expression of MKP-1 or a dominant-negative form of JNK strongly suppressed AG1478-induced apoptosis. These results reveal that JNK, which is activated through the decrease in the MKP-1 level, is critical for EGFR-tyrosine kinase inhibitor-induced apoptosis.

Epidermal growth factor receptor (EGFR), a member of the ErbB family, is important in the regulation of growth, differentiation and survival of various cell types. Ligand binding to EGFR results in receptor dimerization, activation of its tyrosine kinase and phosphorylation of its C-terminal tyrosine residues. The tyrosine-phosphorylated motifs of EGFR recruit various adaptors or signaling molecules [1,2]. EGFR is able to activate a variety of signaling pathways through its association with these molecules. The mitogen-activated protein kinase (MAPK) pathway leading to phosphorylation of extracellular signal-regulated

Abbreviations
EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKP-1, mitogen-activated protein kinase phosphatase-1; NSCLC, non-small-cell lung cancer; PI, propidium iodide; PtdIns3-K, phosphatidylinositol 3-kinase; SAPK, stress-activated MAPK.

kinase (ERK) 1/2 plays an essential role in EGF-induced cell growth; and the phosphatidylinositol 3-kinase (PtdIns3K) pathway is also important for cell growth and cell survival. One way by which PtdIns3K signals cells to survive is by activating protein kinase PDK1 which in turn phosphorylates Akt.

EGFR gene mutations or EGFR gene amplification is detected in various types of malignancy [1,2]; therefore, EGFR-tyrosine kinase is a promising therapeutic target. Orally active small molecules against EGFR (e.g. gefitinib and erlotinib) show evident anti-tumor effects in patients with various cancers, particularly non-small cell lung cancer (NSCLC) [3–5]. Beneficial responsiveness to EGFR-targeting chemicals in NSCLC patients is closely associated with EGFR mutations in the kinase domain [6–8].

The induction of apoptosis has been considered as a major mechanism for gefitinib-mediated anti-cancer effects [9,10]. Lung cancer cells harboring mutant EGFRs become dependent on them for their survival and, consequently, undergo apoptosis following inhibition of EGFR tyrosine kinase by gefitinib. Gefitinib has been shown to inhibit cell survival and growth signaling pathways such as the Ras-MAPK pathway and PtdIns3K/Akt pathway, as a consequence of inactivation of EGFR [10–13]. The PtdIns3K/Akt pathway is downregulated in response to gefitinib only in NSCLC cell lines that are growth-inhibited by gefitinib [14]. So, it is thought that the PtdIns3K/Akt pathway plays a critical role in the gefitinib-induced anti-tumor action. Furthermore, some reports have demonstrated that blockage of the EGFR activity with gefitinib is able to cause suppression of a downstream signaling pathway through Ras-MAPK and/or PtdIns3K/Akt, and induce apoptosis through activation of the pro-apoptotic Bcl-2 family protein Bad or Bax [9,15].

In mammals, three major groups of MAPK have been identified [16–18]. The c-Jun N-terminal kinase (JNK), also known as stress-activated MAPK (SAPK), represents a group of MAPKs that are activated by treatment of cells with cytokines or by exposure of cells to a variety of stresses [19–21]. JNK activity has been implicated in both apoptosis and survival signaling and is tightly controlled by both protein kinases and protein phosphatases [22–24]. Various types of stimuli activate JNK through phosphorylation by the dual-specificity kinase MKK4 or MKK7 [18,25]. By contrast, any types of stimuli can inactivate JNK through induction of the expression of JNK phosphatases, which include dual-specificity (threonine/tyrosine) phosphatases [26–28].

PC-9 cells are gefitinib-sensitive human NSCLC cell lines with a mutation (delE746-A750) in their EGFR, which allows the receptor to be autophosphorylated independent of EGF. In this study, we investigated the signaling route by which the EGFR tyrosine kinase inhibitor AG1478 induces apoptosis in PC-9 cells. There is a general agreement on the hypothesis that the inhibition of ERK1/2 MAPK and/or PtdIns3K/Akt growth/survival signaling cascades leads to apoptosis of cancer cells. However, there are no studies addressing the role of JNK in apoptosis induced by EGFR tyrosine kinase inhibitors. Here, we demonstrate that JNK-phosphatase MKP-1 expression is controlled by a signal downstream of EGFR and that if this signal is abolished by an inhibitor of EGFR tyrosine kinase, the decreased MKP-1 activity can result in JNK activation, leading to the induction of apoptosis.

Results

We first examined the effect of AG1478 on the viability of human NSCLC cell line PC-9. Treatment of the cells with AG1478 markedly suppressed the cell viability, as determined by the results of a colorimetric assay (Fig. 1A). Photographic observation of AG1478-treated PC-9 cells revealed that AG1478 decreased the percentage of adherent cells in a time-dependent manner (Fig. 1B). When AG1478-treated PC-9 cells were stained with Hoechst–propidium iodide (PI), cells with condensed chromatin and fragmented nuclei, which are characteristic of the nuclear changes in apoptotic cells, were seen in both adherent and non-adherent cell populations (data not shown). To confirm whether this AG1478-induced cell death resulted from apoptosis, we examined caspase 3 activity after exposing the cells to 500 nM AG1478. As shown in Fig. 1C, caspase 3 activity was increased in a time-dependent manner. It thus appears that AG1478 reduced the survival rate of PC-9 cells by activating the apoptotic pathway.

It is important to know how AG1478 affected the survival rate of PC-9 cells. Many studies have shown that enhanced JNK activity may be required for initiation of stress-induced apoptosis [29,30]. To examine whether JNK might be activated by AG1478, we treated PC-9 cells with AG1478 for various periods (Fig. 2A). Activation of JNK was measured by performing an immune complex kinase assay using bacterially expressed GST–c-Jun as a substrate. Phosphorylation of c-Jun appeared 1 h after AG1478 addition, with a maximum level at 24 h. We next determined the phosphorylation of JNK in the presence of AG1478. PC-9 cells were incubated with AG1478 for several periods, and cell lysates were prepared from these cells to determine the phosphorylation of JNK by immunoblotting (Fig. 2B). AG1478

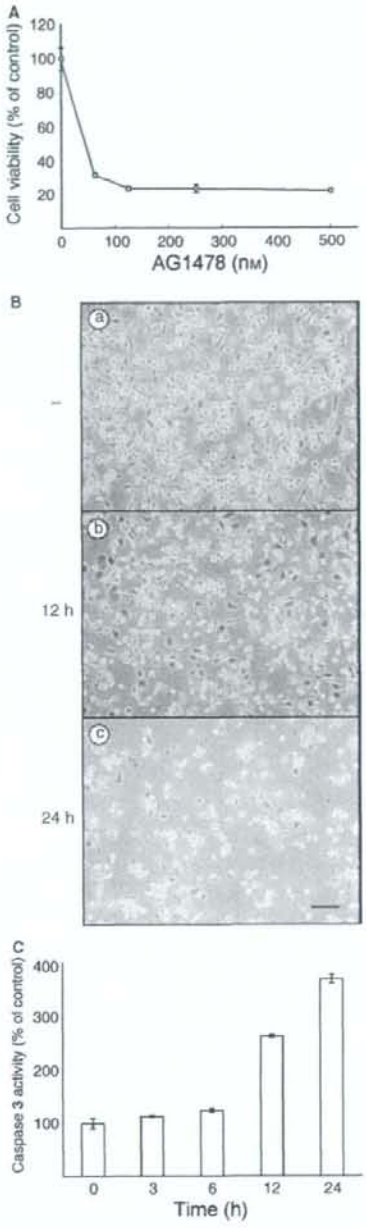

Fig. 1. Induction of apoptosis by AG1478. (A) PC-9 cells were seeded into a 96-well microplate, and treated with AG1478 at various concentrations for 48 h. The viability of cells was determined by conducting WST-8 assays. The value of untreated cells was considered as 100% viability. The data presented are the mean ± SD ($n = 6$). (B) PC-9 cells were seeded at a density 3×10^5 cells per 60 mm dish and then treated with 500 nM AG1478. The phase-contrast photomicrographs were taken 0 (a), 12 (b) or 24 h (c) after incubation with AG1478. Scale bar, 100 μm. (C) PC-9 cells were treated with 500 nM AG1478. Lysates were prepared at the indicated time points after the AG1478 addition and analyzed for caspase **3** activity by using a fluorometric substrate-based assay. Each point is the mean of triplicate samples, and the bar represents the standard deviation. Similar results were obtained from three separate experiments.

Fig. 2. JNK activation by AG1478. PC-9 cells were treated with 500 nM AG1478 and lysed on ice at the indicated time points. (A) JNK–c-Jun complexes were collected by glutathione *S*-transferase–c-Jun agarose beads and then assayed *in vitro* for kinase activity by using c-Jun as a substrate. The phospho-c-Jun product was detected by immunoblotting. (B) The cell lysates were normalized for protein content and analyzed for phospho-JNK content (upper), as well as for JNK content (lower). (C) The cell lysates were analyzed for phospho-p38 content (upper panel), as well as for p38 (lower). Similar results were obtained from three separate experiments.

intensively stimulated phosphorylation of JNK on its threonine 183 and tyrosine 185, and their phosphorylation levels continued to increase for at least 24 h. However, the activation of p38, another MAP kinase sub-family member, was not evident up to 12 h after AG1478 treatment; although an increase in the phosphorylation of p38 was detected at 24 h (Fig. 2C). Phosphorylation of ERK1/2, prototypical MAPK, was decreased by the treatment with AG1478 at the same time as activation of JNK (data not shown).

Neither SB203580 nor PD98059, inhibitors of p38 and ERK1/2, respectively, affected AG1478-induced apoptosis in PC-9 cells (data not shown), suggesting that neither p38 nor ERK1/2 mainly transmit the apoptotic signal of AG1478 in the PC-9 cells. If JNK plays an important role in AG1478-induced apoptosis,

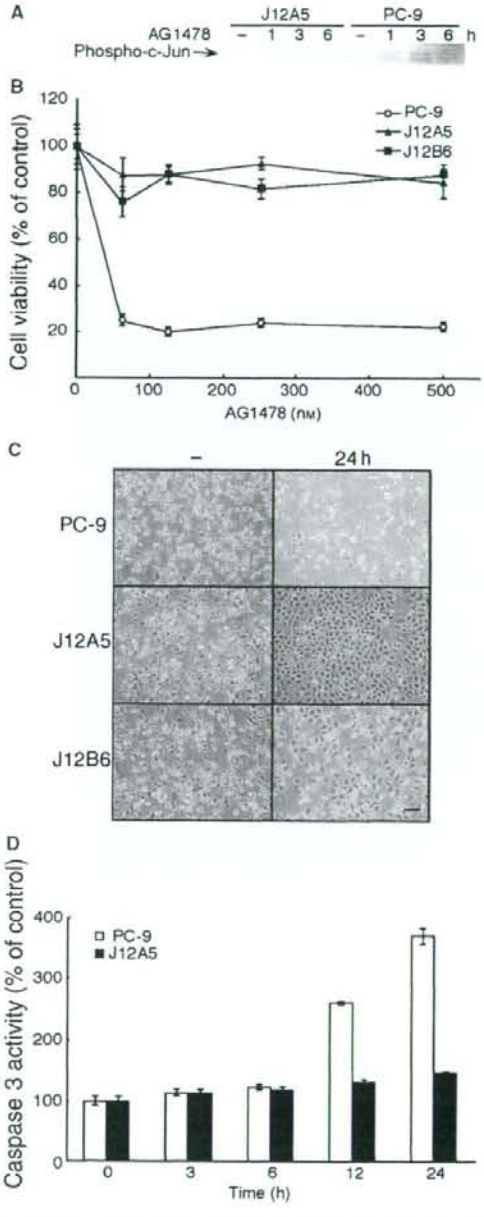

Fig. 3. Expression of dominant-negative JNK prevents AG1478-induced apoptosis. (A) Subconfluent PC-9 and J12A5 cells were incubated with 500 nM AG1478 for the indicated times. JNK activity was determined as described in Experimental Procedures. (B) PC-9, J12A5 and J12B6 cells were incubated with the indicated concentrations of AG1478 for 48 h. The viability of cells was determined by conducting WST-8 assays. The reading obtained for untreated cells was considered as 100% viability. The data presented are the mean ± SD ($n = 6$). (C) Phase-contrast photomicrographs were taken 24 h after incubation with 500 nM AG1478. Scale bar, 100 μm. (D) PC-9 and J12A5 cells were treated with 500 nM AG1478. Lysates were prepared at the indicated time points after the AG1478 addition and analyzed for caspase **3** activity by using a fluorometric substrate-based assay. Each point is the mean of the triplicate samples, and the bar represents the standard deviation. Similar results were obtained from three separate experiments.

inactivation of JNK should suppress this AG1478-induced apoptosis. To test this scenario, we stably transfected PC-9 cells with a mammalian expression vector encoding a dominant-negative form of JNK, and isolated two clones, J12A5 and J12B6. The results of a JNK kinase assay confirmed that J12A5 cells had no detectable activity (Fig. 3A). A colorimetric assay for cell viability, microscopic observation of cells, and an assay for caspase **3** activity revealed that this dominant-negative kinase efficiently blocked AG1478-induced apoptosis (Fig. 3B–D), indicating that activation of JNK mediated the AG1478-induced apoptosis.

A multitude of stimuli including osmotic stress activate JNK through phosphorylation of the JNK kinases MKK4 and MKK7 [18,31]. To examine the mechanism by which AG1478 induced JNK activation, we incubated PC-9 cells in the presence of AG1478 for several periods, and then prepared cell lysates from these cells to determine the phosphorylation of MKK4 and MKK7 by immunoblotting (Fig. 4A). No phosphorylated MKK4 or MKK7 was observed in the presence of AG1478, although phosphorylation of both JNK kinases in response to osmotic stress could be detected. Next, we determined the effect of AG1478 on the levels of MAPK phosphatases MKP-1 and MKP-2. As shown in Fig. 4B, AG1478 decreased the expression of the MKP-1 protein. As for the MKP-2 protein, however, AG1478 did not affect its expression level.

To check the role of MKP-1 as an anti-apoptotic signal molecule, we constitutively expressed MKP-1 in PC-9 cells. The cells were transfected with a vector directing the expression of MKP-1; and two clones, M1A4 and M1B2, were isolated as cell lines over-expressing MKP-1 (Fig. 5A). Using PC-9 and M1A4 cells, we examined the effect of AG1478 on the amounts of dually phosphorylated JNK (Fig. 5B). In PC-9 cells, AG1478 treatment decreased the expression of the MKP-1 protein and concomitantly stimulated the phosphorylation of JNK. However, the expression

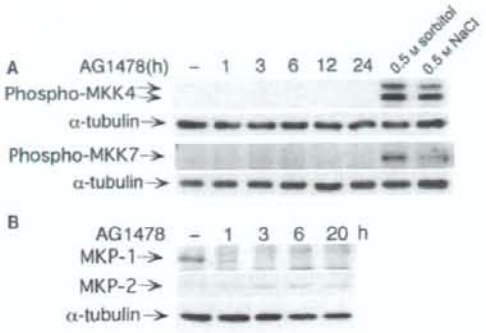

Fig. 4. Effect of AG1478 on phosphorylation of MKK4 and MKK7, and expression of MKP-1 and MKP-2. A, PC-9 cells were treated with 500 nM AG1478 for the indicated periods, and cellular lysates were analyzed by SDS/PAGE and immunoblotting with anti-[phospho SEK1/MKK4 (Ser254/Thr261)] Ig and anti-[phospho MKK7 (Ser271/Thr275)] Ig, respectively (upper). α-Tubulin levels were examined as a control for equal loading (lower). As a control for MKK4 and MKK7 activation, parallel cultures were treated with 0.5 M sorbitol for 30 min or with 0.5 M sodium chloride for 15 min. (B) The cellular lysates were prepared at the indicated time points after AG1478 treatment. Total protein (40 μg) was subjected to immunoblotting, and the membranes were hybridized with antibodies against MKP-1 (upper) or MKP-2 (middle). The equal loading of the samples was checked by using an antibody against α-tubulin (lower). The experiments corresponding to (A) and (B) were repeated three times with similar results.

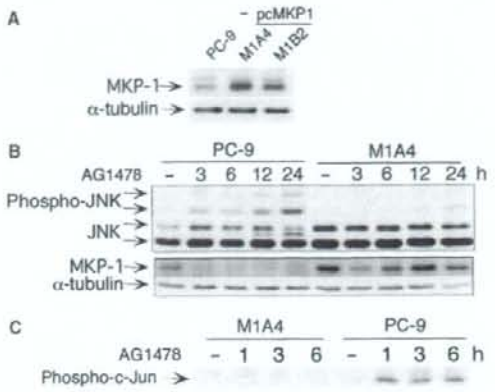

Fig. 5. Expression of MKP-1 prevents JNK activation. (A) Cellular lysates were prepared from parent PC-9 cells and pcMKP1- transfected PC-9 cells (M1A4 and M1B2). The lysates were analyzed by SDS/PAGE and immunoblotting with specific antibody against MKP-1 (upper) or α-tubulin (lower). (B) Subconfluent PC-9 and M1A4 cells were incubated with 500 nM AG1478 for the indicated times. The cells were then harvested, and equal aliquots of protein extracts (40 μg per lane) were analyzed for phospho-JNK (upper) and MKP-1 (lower) by immunoblotting. Each membrane was reprobed with JNK (upper) or an α-tubulin antibody (lower). Similar results were obtained from three separate experiments. (C) Cell lysates were prepared from PC-9 and M1A4 cells at the indicated time points after treatment with 500 nM AG1478. JNK activity was determined as described in Experimental procedures. The experiments were repeated three times with similar results.

level of MKP-1 in M1A4 cells remained high, in contrast to that in PC-9 cells; although MKP-1 expression was lowered once at 3 h after AG1478 treatment. JNK phosphorylation was extremely low in M1A4 cells. The expression patterns of MKP-1 and phospho-JNK seen in M1A4 were also observed in M1B2 cells (data not shown). The results of the JNK kinase assay indicated that JNK was not activated in M1A4 cells, where the MKP-1 expression level remained high even after exposure to AG1478 (Fig. 5C).

We next tested whether the expression level of MKP-1 correlated with sensitivity to AG1478. As shown in Fig. 6A,B, overexpression of MKP-1 resulted in resistance to AG1478. We also examined whether AG1478 could activate the effector caspase 3 in M1A4 cells (Fig. 6C). In PC-9 cells, activation of caspase 3 was observed with a maximal increase (480%) at 24 h after AG1478 treatment; however, in M1A4 cells, only a slight increase in caspase 3 enzyme activity (28% and 39% at 12 and 24 h, respectively) was detected. These results show that the MKP-1 expression level correlated with the susceptibility to AG1478-induced apoptosis.

Discussion

Gefitinib, an EGFR-tyrosine kinase inhibitor, has been reported to inhibit cell survival and proliferation signaling pathways such as MAPK and PtdIns3K/Akt pathways [10–13]. Furthermore, some reports have shown that gefitinib reduces Akt activity only in NSCLC cell lines, in which it inhibits growth [14,32]. The ErbB family of receptor tyrosine kinases includes four members, namely, the EGFR (ErbB1), ErbB2, ErbB3 and ErbB4. Among these members, ErbB3 effectively couples to the PtdIns3K/Akt pathway. Therefore, it is likely that ErbB3 serves to couple EGFR to the PtdIns3K/Akt pathway and that ErbB3 expression serves as an effective predictor of sensitivity to gefitinib in NSCLC cell lines [14]. In this study, we used PC-9 cells, which are gefitinib-sensitive human NSCLC cells with a mutation (delE746-A750) in their EGFR. In these PC-9 cells, autophosphorylation of EGFR took place independent of EGF, and it was suppressed by AG1478. Because AG1478 inhibited the phosphorylation of multiple down-stream targets including ERK1/2 in the PC-9 cells, but its effect on Akt phosphorylation was not so

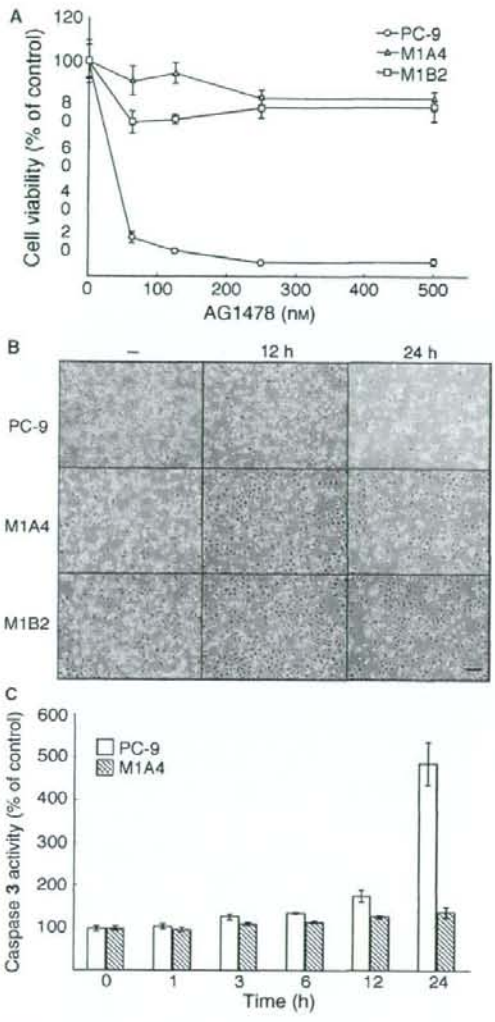

Fig. 6. Expression of MKP-1 prevents AG1478-induced apoptosis. A, PC-9, M1A4, and M1B2 cells were incubated with the indicated concentrations of AG1478 for 48 h. The viability of cells was determined by conducting WST-8 assays. The reading obtained for untreated cells was considered as 100% viability. The data presented are the mean ± SD ($n = 6$). (B) Phase-contrast photomicrographs were taken 12 and 24 h after incubation with 500 nM AG1478. Scale bar, 100 μm. (C) PC-9 and M1A4 cells were treated with 500 nM AG1478. Lysates were prepared at the indicated time points after the AG1478 addition and analyzed for caspase **3** activity by using a fluorometric substrate-based assay. Each point is the mean of the triplicate samples, and the bar represents the standard deviation. Similar results were obtained from three separate experiments.

significant (K. Takeuchi & F. Ito, unpublished data), intracellular signaling pathways other than PtdIns3K/Akt could be responsible for the AG1478-induced apoptosis in PC-9 cells.

Stress stimuli that induce apoptosis, including UV- and γ-irradiation, heat shock, protein synthesis inhibitors, DNA-damaging agents and the proinflammatory cytokines, are potent activators of JNK. Several anti-neoplastic agents such as cisplatin, etoposide, camptothecin and taxol, which are also strong inducers of apoptosis, also activate the JNK pathway [33]. In this study, we found that AG1478 induced the activation of JNK in PC-9 cells. Furthermore, a dominant-negative form of JNK efficiently blocked AG1478-induced apoptosis. It thus appears that EGFR-tyrosine kinase inhibitors induce apoptosis in PC-9 cells via activation of JNK.

ERK1 and ERK2, also known as p44 and p42 MAPK, respectively, represent the prototypical MAPK in mammalian cells. ERK MAP kinase catalytic activation was observed in PC-9 cells, and it was inhibited by AG1478. Increased phosphorylation of the other MAPK family member, p38, was also observed at 24 h after AG1478 treatment; but it was not observed at 12 h when apoptosis could be detected (Figs 1A and 2C). Our experiment indicated that neither SB203580 nor PD98059, inhibitors of p38 and ERK1/2, respectively, affected AG1478-induced apoptosis in PC-9 cells. Taken together, our data indicate that JNK, but not other MAPK family members such as p38 and ERK1/2, mainly transmits the apoptotic signal of AG1478 in the PC-9 cells.

JNK signaling can regulate apoptosis both positively and negatively, depending on the cell type, cellular context and the nature and dose of treatment [22,23]. Strong and sustained JNK activation is predominantly associated with induction or enhancement of apoptosis, whereas transient JNK activation can result in cell survival [23,24]. AG1478 induced strong and sustained JNK activation in PC-9 cells (Fig. 2A,B). This finding strengthens the possibility that JNK is a mediator of the apoptotic action of AG1478.

JNK activity in cells is tightly controlled by both protein kinases such as MKK4 or MKK7 and protein phosphatases such as MKPs. MKP-1, the first member of the MKP family to be identified as an ERK-specific phosphatase, is also able to inactivate JNK and p38 [34–38]. MKP-1 is an immediate-early gene whose expression is regulated by mitogenic, inflammatory and DNA-damaging stimuli [39–41]. In this study, we observed no activation of MKK4 or MKK7 in AG1478-treated PC-9 cells (Fig. 4A). However, the expression level of MKP-1, but not that of MKP-2,

was significantly decreased by the AG1478 treatment (Fig. 4B), indicating that JNK activity in the PC-9 cells may be regulated by MKP-1. Another member of the dual-phosphatase family of proteins, MKP-2 shows a 60% sequence homology to MKP-1, and also similar substrate specificity [42]. However, the expression level of MKP-2 was not affected by AG1478 treatment, indicating that the expression of MKP-1, but not that of MKP-2, is controlled by signals via EGFRs.

Brondello *et al.* reported that activation of the ERK cascade is sufficient to promote the expression of MKP-1 and MKP-2 [43]. It has also been suggested that MKP-1 expression is regulated by ERK-dependent and -independent signals [44]. Because the ERK inhibitor PD98059 did not affect MKP-1 expression or activation of JNK in PC-9 cells (K. Takeuchi & F. Ito, unpublished data), MPK-1 expression in PC-9 cells may be controlled in an ERK-independent manner. Recently, Ryser *et al.* reported that MKP-1 transcription is regulated in the transcriptional elongation step: under basal conditions, a strong block to elongation in the first exon regulates MKP-1 gene transcription [45]. Thus, EGFR-mediated signals may overcome this block to stimulate MKP-1 gene transcription in PC-9 cells. Another possible mechanism responsible for EGFR-mediated enhancement of MKP-1 expression is that MKP-1 degradation via the ubiquitin–proteasome pathway is suppressed by EGFR activation. In fact, some research groups have reported that the expression level of MKP-1 is controlled via the ubiquitin–proteasome pathway [46,47]. Our preliminary experiment also indicated that AG1478-induced MKP-1 degradation was suppressed in the presence of proteasome inhibitors such as MG-132 and ALLN (K. Takeuchi & F. Ito, unpublished data).

Gene disruption studies demonstrate that JNK is required for the release of mitochondrial proapoptotic molecules (including cytochrome *c*) and apoptosis in response to UV radiation [48]. Bax and Bak (members of the proapoptotic group of multidomain Bcl-2-related proteins) are essential for the JNK-stimulated release of cytochrome *c* and apoptosis [49]. Other studies have shown that 14-3-3 proteins are direct targets of JNK and that phosphorylation of 14-3-3 proteins by JNK results in dissociation of Bax from 14-3-3 proteins, leading to apoptosis [50]. Because translocation of Bax to mitochondria was observed in AG1478-treated PC-9 cells (K. Takeuchi & F. Ito, unpublished data), AG1478 may exert its apoptotic actions, at least in part, by promoting the translocation of Bax to mitochondria.

Some reports have shown that the activation of the Fas/FasL system may be one of the mechanisms responsible for drug-induced apoptosis in a variety of cancer cells of different histotype [51]. Chang *et al.* recently reported that an increase in Fas protein expression might be the molecular mechanism by which gefitinib induces apoptosis in lung cancer cell lines [52]. Furthermore, it has been reported that c-Jun-dependent FasL expression plays a critical role in the induction of apoptosis by genotoxic agents [53]. To understand the causal relationship between JNK activation and AG1478-induced apoptosis, we need to study whether AG1478 induces the expression of Fas or FasL in PC-9 cells.

Overexpression of MKP-1 inhibited the AG1478-induced JNK activation and also AG1478-induced apoptosis. These results indicate that there is a link between the decreased MKP-1 activity and AG1478-induced apoptosis: MKP-1 expression is controlled by signals downstream of EGFR, and it is downregulated in the presence of an inhibitor of EGFR tyrosine kinase. This downregulation could be followed by JNK activation, triggering the apoptosis pathway.

Understanding the molecular basis of responsiveness to gefitinib is important to identify patients who will have a positive response to this drug. The EGFR gene in tumors from patients with gefitinib-responsive lung cancer was recently examined for mutations, and clustering of mutations was detected in the part of the gene encoding the ATP-binding pocket. Screening for such mutations may identify patients who will have a positive response to the drug. However, this study showed that NSCLC cell line PC-9 was dependent on the MKP-1/JNK pathway for its growth and survival. Thus, sensitivity to gefitinib may be predicted from the detailed analysis of the MKP-1/JNK pathway as described in this study. Although the MKP-1 level in normal cells is low, an increased level of MKP-1 has been found in human ovarian, breast, and prostate cancer [54–56]. Our results suggest that MKP-1 may be a candidate drug target in order to optimize gefitinib-based therapeutic protocols.

Experimental procedures

Materials

EGF (ultra-pure) from mouse submaxillary glands was purchased from Toyobo Co., Ltd (Osaka, Japan). Fetal calf serum came from Gibco (Grand Island, NY, USA). Phenylmethanesulfonyl fluoride, pepstatin A, aprotinin and leupeptin were obtained from Sigma (St Louis, MO, USA). RPMI-1640 medium was from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). Antibodies used and their sources were: ERK1/2 (pT202/pY204) phospho-specific antibody (clone 20A), JNK(pT183/pY185) phospho-specific antibody

(clone 41), p38 MAPK (pT180/pY182) phospho-specific antibody (clone 36), p38α antibody (clone 27), MKP2 antibody (clone 48) and pan-JNK/SAPK1 antibody (clone 37), from BD Transduction Laboratories (San Jose, CA, USA); MKP-1 antibody (C-19), from Santa Cruz Biotechnology (Santa Cruz, CA, USA); α-tubulin antibody (clone B-5-1-2) and MAP kinase antibody, from Sigma; phospho-SEK1/MKK4 (Ser254/Thr261) antibody and phospho-MKK7 (Ser271/Thr275) antibody, from Cell Signaling Technology (Danvers, MA, USA); swine horseradish peroxidase (HRP)-linked anti-rabbit Ig, from DAKO (Glostrup, Denmark); and sheep HRP-linked anti-mouse Ig, from GE Healthcare UK Ltd (Amersham, UK). Plasmid pcMKP1 was generated from *Homo sapiens* dual-specificity phosphatase 1 cDNA, MGC clone (ID 4794895) purchased from Invitrogen (Carlsbad, CA, USA). The MGC clone had been cloned into pBluscriptR. This clone was digested with *Aval*, treated with T4 DNA polymerase, ligated to the pcDNA 3.1 mammalian expression vector (Invitrogen) prepared by digestion with *Eco*RV and treated with calf intestinal phosphatase to produce pcMKP1. Plasmid DNA was prepared by standard techniques (Qiagen Plasmid Midi Kit). pBabePuro, a puromycin-resistant vector, was kindly provided by K. Shuai (UCLA, USA). pcDL-SRα296JNK2(VPF), a dominant-negative JNK expression vector, was kindly donated by E. Nishida (Kyoto University, Japan).

Cell culture and transfection

Human non-small cell lung cancer cell line PC-9 was cultured to subconfluence in RPMI-1640 medium supplemented with 5% fetal calf serum and used for all of the experiments. PC-9 cells were plated 24 h before transfection and co-transfected with 8.5 μg of pcDL-SRα 296JNK2(VPF) or pcMKP-1 and 1.5 μg of pBabePuro by using the Lipofectamine reagent, and the transfected cells were selected by exposure to 2.5 mg of puromycin (Sigma) per mL of medium for 3 weeks. Empty vector and pBabePuro were used for co-transfection as a negative control. The expression of JNK protein and MKP-1 protein were verified by immunoblot analysis using anti-(pan-JNK/SAPK1 aa264–415) and anti-(MKP-1) (Santa Cruz Biotechnology), respectively.

Determination of cell viability

The anti-proliferative effect of AG1478 on PC-9 cells was assessed by using a Cell Counting Kit-8 (DOJIN, Kumamoto, Japan) according to the manufacturer's instructions. The Cell Counting Kit-8 is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. Briefly, 200 μL of a suspension of PC-9 cells was seeded into each well of a 96-well plate at a density of 2000 cells·well^{-1}. After 48 h, the culture medium was replaced with 100 μL of AG1478 solution at various concentrations. After incubation for 48 h at 37 °C, 10 μL of WST-8 solution was added to each well, and the cells were incubated for a further 40 min at 37 °C. A_{450} was measured using a Bio-Rad microplate reader model 550. Each experiment was performed by using six replicate wells for each drug concentration and was carried out independently three times.

Preparation of cellular lysates and immunoblotting

Preparation of cellular lysates and immunoblotting were performed as described previously [57]. Briefly, cells were lysed with buffer A (20 mM Tris/HCl, pH 7.4, containing 137 mM NaCl, 2 mM EGTA, 5 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 100 μg·mL^{-1} phenylmethanesulfonyl fluoride, 1 μg·mL^{-1} pepstatin A, 1 μg·mL^{-1} *p*-toluenesulfonyl-L-arginine methyl ester, 2 μg·mL^{-1} leupeptin, 1 mM sodium orthovanadate, 50 mM sodium fluoride and 30 mM $Na_4P_2O_7$). Lysates were then incubated on ice for 30 min, and the insoluble material was cleared by centrifugation. Samples were normalized for protein content and separated by SDS/PAGE, after which they were transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) for immunoblotting with antibodies.

Caspase 3 activity assay

Caspase activity was assayed as described previously [57]. Briefly, cells were lysed with buffer A, and the protein concentration in each sample was adjusted to 100 μg·50 μL^{-1} of buffer A. Fifty microliters of 2× Reaction Buffer (0.2 M Hepes/NaOH, pH 7.4, containing 20% sucrose, 0.2% Chaps and 1 mM dithiothreitol) was added to each sample, which was then incubated with Z-DEVD-AFC substrate (50 μM final concentration) at 37 °C for 1 h. The samples were read in a fluorometer (VersaFluor, Bio-Rad) equipped with a 340–380 nm excitation filter (EX 360/40) and 505–515 nm emission filter (EM 510/10).

JNK assay

PC-9 cells were cultured in RPMI-1640 supplemented with 5% fetal calf serum at a density of 6.0×10^5 per 100 mm dish for 2 days and then assayed for JNK activity. JNK assays were performed by using a SAPK/JNK Assay kit (Cell Signaling Technology) according to the manufacturer's specifications. In brief, after various times of treatment with AG1478, adherent cells and floating cells were harvested by centrifugation and washed once in NaCl/P_i. Subsequently, the cells were lysed with lysis buffer (consisting of 20 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM

deltamethrin, 180 nM nodularin, 100 $\mu g \cdot mL^{-1}$ phenylmethanesulfonyl fluoride, 25 $\mu g \cdot mL^{-1}$ aprotinin, 25 $\mu g \cdot mL^{-1}$ leupeptin and 25 $\mu g \cdot mL^{-1}$ pepstatin), and scraped into microcentrifuge tubes. Extracts were prepared by sonicating each sample on ice (BRANSON SONIFIER 250, Danbury, CT, USA), and insoluble material was removed by microcentrifugation. Soluble fractions were mixed with 2 μg glutathione *S*-transferase-c-Jun (1–89) agarose beads (Cell Signaling Technology) and rotated overnight at 4 °C. JNK–c-Jun complexes were collected and washed with lysis buffer followed by kinase buffer, consisting of 25 mM Tris/HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM Cleland's reagent, 0.1 mM Na_3VO_4 and 10 mM $MgCl_2$. The *in vitro* kinase reaction was initiated by the addition of kinase buffer containing 100 μM ATP, samples were incubated at 30 °C for 45 min, and reactions were terminated by the addition of SDS sample buffer and heating to 95 °C for 5 min. Phosphorylated c-Jun was detected by western blotting using a phospho-specific c-Jun antibody (Cell Signaling Technology).

Hoechst- PI staining

For the study of nuclear morphologic changes induced by AG1478, PC-9 cells were seeded on coverslips, grown to sub-confluence, and treated with AG1478 for the desired times. After fixation with formalin solution, the cells were stained with 10 μM Hoechst33342 and 10 μM PI in 5% fetal calf serum/RPMI. Coverslips were mounted on slides by using Dakocytomation Fluorescent Mounting Medium (DAKO) and observed under a fluorescence microscope (Axioskop: Carl Zeiss, Jena, Germany).

Acknowledgements

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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.

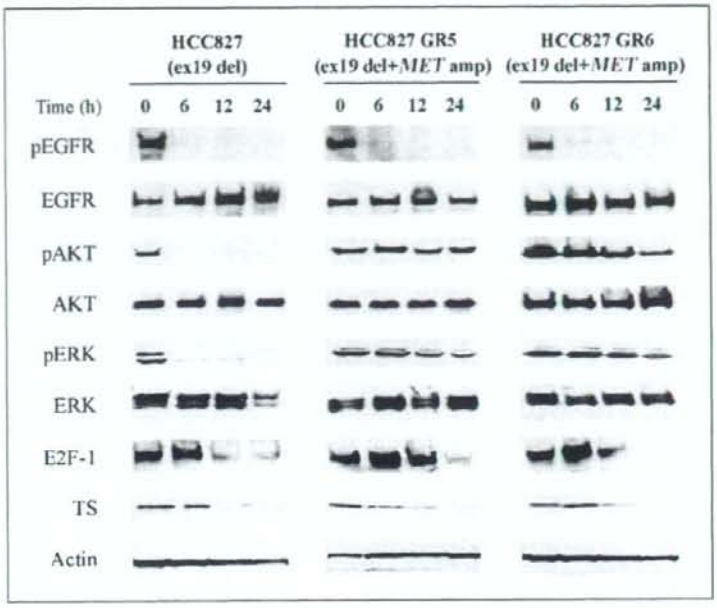

Fig. 1. Effects of gefitinib on EGFR, AKT, and ERK phosphorylation as well as on E2F-1 and TS expression in gefitinib-resistant NSCLC cells with *MET* amplification. Parental HCC827 cells and gefitinib-resistant clones with *MET* amplification (HCC827 GR5 and HCC827 GR6) were incubated with gefitinib (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

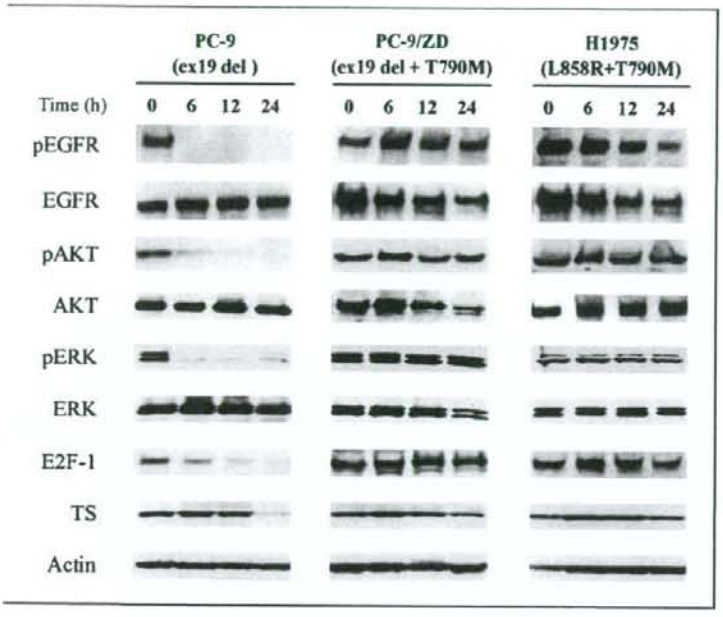

Fig. 2. Effects of gefitinib on EGFR, AKT, and ERK phosphorylation as well as on E2F-1 and TS expression in gefitinib-resistant NSCLC cells with the T790M mutation of *EGFR*. PC-9 cells as well as the gefitinib-resistant lines PC-9/ZD and H1975 harboring the T790M mutation of *EGFR* were incubated with 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.

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

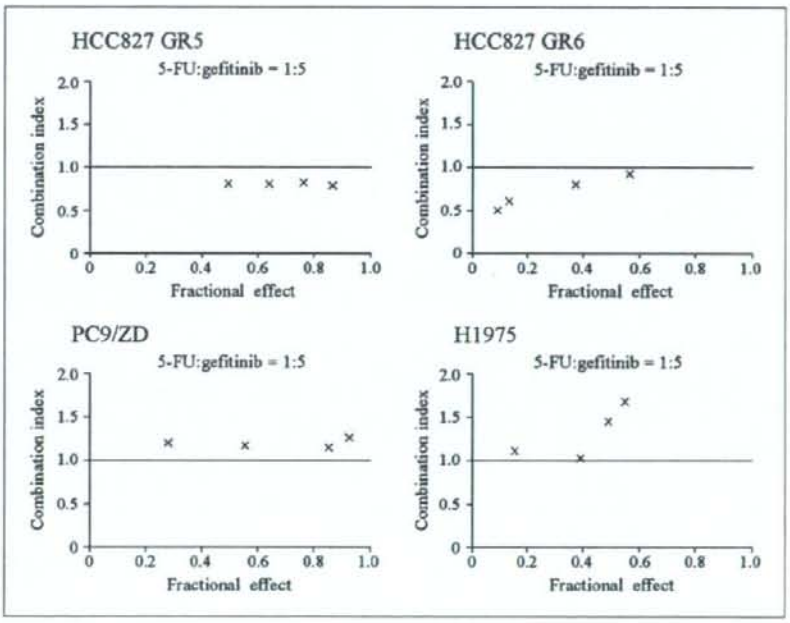

Fig. 3. Effects of the combination of 5-FU and gefitinib on the growth of gefitinib-resistant NSCLC cells *in vitro*. Cells with *MET* amplification (HCC827 GR5 and HCC827 GR6) or those harboring the T790M mutation of *EGFR* (PC-9/ZD and H1975) were incubated for 72 h with 5-FU or gefitinib alone or 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.