5FU (31). In the present study, we found that depletion of TS by RNA interference enhanced the induction of apoptosis by 5FU in gastric cancer cells with *HER2* amplification, suggesting that the proapoptotic effect of the combination of 5FU and HER2-targeting agents is attributable to TS inhibition. The abundance of TS in neoplastic cells has been found to increase after exposure to 5FU, resulting in maintenance of the amount of the free enzyme in excess of that of enzyme bound to 5FU (32–34). Such an increase in TS expression and activity has been viewed as a mechanistic driver of 5FU resistance in cancer cells (22, 35–39). Downregulation of TS by HER2-targeting agents might thus contribute to reversal of the 5FU-induced increase in TS expression, resulting in enhancement of 5FU-induced apoptosis. In addition, prolonged inhibition of TS has been shown to trigger apoptosis by inducing an imbalance in the deoxyribonucleoside pool and consequent disruption of DNA synthesis and repair (40–42). Given that the TS siRNA itself induced apoptosis in gastric cancer cells positive for *HER2* amplification in the present study, the depletion of TS by HER2-targeting agents might also contribute directly to the combined proapoptotic action with 5FU.

The *HER2* amplification–positive gastric cancer cell line MKN-7 has been found to be insensitive to trastuzumab. In contrast to their insensitivity to trastuzumab, we found that MKN-7 cells retain sensitivity to lapatinib (IC_{50} values of >200 μg/mL and 0.99 ± 0.055 μmol/L for trastuzumab and lapatinib, respectively; data not shown). Most HER2-positive breast cancer patients who initially respond to trastuzumab ultimately develop resistance to this drug (25). Preclinical studies have indicated several molecular mechanisms that might contribute to the development of trastuzumab resistance, including

signaling by a HER2-HER3-PI3K-PTEN pathway (43, 44). One possible explanation for trastuzumab resistance in MKN-7 cells is activation of the EGFR signaling pathway (45, 46). MKN-7 cells might prove to be a good model for the study of trastuzumab-resistant cells positive for *HER2* amplification. We found that lapatinib and trastuzumab each inhibit TS expression and activity in MKN-7 cells, likely accounting for the synergistic antiproliferative effect observed with 5FU. These data suggest that the synergistic antitumor effect of the combination of 5FU and HER2-targeting agents is conserved in trastuzumab-resistant cells with *HER2* amplification.

In conclusion, we have shown that the combination of S-1 and HER2-targeting agents exerts a synergistic antitumor effect mediated by TS inhibition in gastric cancer cells with *HER2* amplification, but not in those negative for *HER2* amplification. Our observations provide a rationale for clinical evaluation of combination chemotherapy with S-1 and HER2-targeting agents according to *HER2* amplification status.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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Molecular
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Identification of c-Src as a Potential Therapeutic Target for Gastric Cancer and of MET Activation as a Cause of Resistance to c-Src Inhibition

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Abstract

Therapeutic strategies that target c-Src hold promise for a wide variety of cancers. We have now investigated both the effects of dasatinib, which inhibits the activity of c-Src and several other kinases, on cell growth as well as the mechanism of dasatinib resistance in human gastric cancer cell lines. Immunoblot analysis revealed the activation of c-Src at various levels in most gastric cancer cell lines examined. Dasatinib inhibited the phosphorylation of extracellular signal-regulated kinase (ERK) and induced G_1 arrest, as revealed by flow cytometry, in a subset of responsive cell lines. In other responsive cell lines, dasatinib inhibited both ERK and AKT phosphorylation and induced apoptosis, as revealed by an increase in caspase-3 activity and cleavage of poly(ADP-ribose) polymerase. Depletion of c-Src by RNA interference also induced G_1 arrest or apoptosis in dasatinib-responsive cell lines, indicating that the antiproliferative effect of dasatinib is attributable to c-Src inhibition. Gastric cancer cell lines positive for the activation of MET were resistant to dasatinib. Dasatinib had no effect on ERK or AKT signaling, whereas the MET inhibitor PHA-665752 induced apoptosis in these cells. The subsets of gastric cancer cells defined by a response to c-Src or MET inhibitors were distinct and nonoverlapping. Our results suggest that c-Src is a promising target for the treatment of gastric cancer and that analysis of MET amplification might optimize patient selection for treatment with c-Src inhibitors. Mol Cancer Ther; 9(5); 1188–97. ©2010 AACR.

Introduction

Gastric cancer is the second most frequent cause of cancer deaths worldwide (1). Chemotherapy has a beneficial effect on survival in individuals with advanced-stage gastric cancer, but overall survival is still usually <1 year (1, 2). Advanced gastric cancer is treated predominantly with the combination of fluoropyrimidine derivatives and platinum compounds, although a globally accepted standard regimen remains to be established. Improved therapy for affected individuals is thus urgently needed.

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c-Src is a nonreceptor tyrosine kinase that plays key roles in intracellular signaling by interacting with and phosphorylating multiple proteins and protein complexes (3). Activation of c-Src has been found to contribute to the transformation, proliferation, survival, and motility of malignant cells as well as to tumor angiogenesis (3, 4). c-Src is highly activated in a wide variety of human cancers and clinical studies have shown that such aberrant activation is correlated with malignant progression (5). These properties have rendered c-Src a potential target for the treatment of solid tumors.

Dasatinib is an oral, multitargeted inhibitor of tyrosine kinases that inhibits the activities of c-Src, Bcr-Abl, and other kinases (6). It has been approved for clinical use in patients with chronic myelogenous leukemia or Philadelphia chromosome–positive acute lymphoblastic leukemia and it is currently under investigation as a potential therapy for solid tumors. Recent studies have shown that c-Src inhibitors induce apoptosis or arrest cell cycle progression in various cancer cell types (7–14). The activation of c-Src has pleiotropic effects that depend on cell type and context. Although c-Src activity has been found to be increased in most gastric cancers (15–18), the responses of gastric cancer cells to c-Src inhibition have not previously been characterized. We have therefore now examined the effects of c-Src inhibition by

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dasatinib on cell growth and signal transduction in human gastric cancer cell lines. Furthermore, we have investigated the mechanism of resistance to dasatinib in such cells. Our results provide a rationale for the clinical investigation of c-Src inhibition in individuals with gastric cancer.

Materials and Methods

Cell culture and reagents. The human gastric cancer cell lines SNU1, SNU5, Hs746T, and AGS were obtained from the American Type Culture Collection; MKN1, MKN7, MKN45, NUGC3, and AZ521 were from the Health Science Research Resources Bank; OKAJIMA, MKN28, and HSC39 were from Immuno-Biological Laboratories; and SNU216 was from the Korean Cell Line Bank. HSC58, 58As1, and 58As9 are established cell lines derived from human scirrhous gastric carcinoma as previously described (19). All cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma,) supplemented with 10% fetal bovine serum and were passaged for ≤3 mo before the renewal from frozen, early-passage stocks obtained from the indicated sources. Cells were regularly screened for *Mycoplasma* with the use of a MycoAlert Mycoplasma Detection kit (Lonza). Dasatinib was kindly provided by Bristol-Myers Squibb and PHA-665752 was obtained from Tocris Bioscience.

Immunoblot analysis. Cells were washed twice with ice-cold PBS and then lysed with 1× Cell Lysis Buffer (Cell Signaling Technology) containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA (disodium salt), 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, leupeptin (1 μg/mL), and 1 mmol/L phenylmethylsulfonyl fluoride. The protein concentration of cell lysates was determined with a BCA protein assay kit (Thermo Fisher Scientific) and equal amounts of protein were subjected to SDS-PAGE on 7.5 or 12% gels (Bio-Rad). The separated proteins were transferred to a nitrocellulose membrane, which was then incubated with Blocking One solution (Nacalai Tesque) for 20 minutes at room temperature before incubation overnight at 4°C with primary antibodies. Antibodies to phosphorylated c-Src (Y416), total c-Src, phosphorylated MET (Y1234/1235, Y1349), phosphorylated AKT, total AKT, phosphorylated extracellular signal-regulated kinase (ERK), p27, or poly (ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology; those to total ERK were from Santa Cruz Biotechnology; those to total MET were from Zymed/Invitrogen; and those to β-actin were from Sigma. The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase–conjugated antibodies to rabbit or mouse IgG (GE Healthcare). Immune complexes were finally detected with enhanced chemiluminescence (Amersham) Western Blotting Detection Reagents (GE Healthcare).

Cell growth inhibition assay. Cells were transferred to 96-well flat-bottomed plates and cultured for 24 hours before exposure to various concentrations of dasatinib or PHA-665752 for 72 hours. Tetra Color One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku Kogyo) was then added to each well and the cells were incubated for 3 hours at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems). Absorbance values were expressed as a percentage of that for nontreated cells and the concentration of dasatinib resulting in 50% growth inhibition (IC_{50}) was calculated.

Fluorescence in situ hybridization analysis. MET gene copy number per cell was determined by fluorescence in situ hybridization with the use of the LSI D7S522 (7q31) Spectrum Orange and chromosome 7 centromere (CEP7) Spectrum Green probes (Vysis; Abbott). Cells were centrifuged onto glass slides with a Shandon cytocentrifuge (Thermo Electron) and were fixed by consecutive incubations with ice-cold 70% ethanol for 10 minutes, 85% ethanol for 5 minutes, and 100% ethanol for 5 minutes. Slides were stored at −20°C until analysis. Cells were subsequently subjected to digestion with pepsin for 10 minutes at 37°C, washed with water, dehydrated with a graded series of ethanol solutions, denatured with 70% formamide in 2× SSC for 5 minutes at 72°C, and dehydrated again with a graded series of ethanol solutions before incubation with a hybridization mixture consisting of 50% formamide, 2× SSC, Cot-1 DNA, and labeled DNA. The slides were washed for 5 minutes at 73°C with 3× SSC, for 5 minutes at 37°C with 4× SSC containing 0.1% Triton X-100, and for 5 minutes at room temperature with 2× SSC before counterstaining with an antifade solution containing 4′,6-diamidino-2-phenylindole. Hybridization signals were scored in 40 nuclei with the use of a ×100 immersion objective lens. Nuclei with a disrupted boundary were excluded from the analysis. Gene amplification was defined by a mean MET/chromosome 7 copy number ratio of >2.2 or by a mean MET copy number of >6 per cell, corresponding to the previous definition for HER2 amplification (20).

Cell cycle analysis. Cells were harvested, washed with PBS, fixed with ice-cold 70% methanol, washed again with PBS, and stained with propidium iodide–RNase staining buffer (BD Biosciences) for 15 minutes at room temperature. The stained cells were then analyzed for DNA content with a flow cytometer (FACSCalibur, BD Biosciences) and the Modfit software (Verity Software House).

Assay of caspase-3 activity. The activity of caspase-3 in cell lysates was measured with a CCP32/Caspase-3 Fluorometric Protease Assay kit (Medical Biological Laboratories). Fluorescence attributable to the cleavage of the Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) substrate was measured at excitation and emission wavelengths of 390 and 460 nm, respectively.

Gene silencing. Cells were plated at 50% to 60% confluence in six-well plates or 25-cm^2 flasks and were incubated for 24 hours before transient transfection with small interfering RNAs (siRNA) for 48 or 72 hours with the use of the Lipofectamine RNAiMAX reagent (Invitrogen). siRNAs specific for human c-Src mRNA (5'-CCAC-CUUUGU-GGCCCUCUATT-3') or human MET mRNA (5'-ACAAGAUCGUCAACAAAAATT-3') as well as a nonspecific siRNA (control) were obtained from Nippon EGT. The cells were then subjected to flow cytometry, immunoblot analysis, or assay of cell growth inhibition.

Statistical analysis. Data were analyzed by Student's two-tailed *t* test. A *P* value of <0.05 was considered statistically significant.

Results

Effects of dasatinib on the growth of gastric cancer cell lines. The baseline levels of total c-Src and activated c-Src

(phospho-Y416) in 16 human gastric cancer cell lines were measured by immunoblot analysis. All the cell lines expressed detectable levels of total c-Src, whereas all lines with the exception of SNU1 and HSC39 manifested detectable (albeit different) levels of c-Src phosphorylation (Fig. 1A).

To assess the effects of dasatinib on cell growth, we exposed the gastric cancer cell lines to various concentrations of the drug and then measured cell viability. Seven cell lines were responsive to dasatinib with IC$_{50}$ values ranging from approximately 40 to 540 nmol/L, whereas nine cell lines remained resistant to dasatinib at concentrations up to 5 μmol/L (Table 1; Fig. 1B). SNU1 and HSC39 cells, both of which lack activated c-Src, were resistant to dasatinib. For the remaining cell lines positive for phosphorylated c-Src, there was no correlation between the antiproliferative effect of dasatinib and the baseline phosphorylation level of c-Src (Fig. 1A).
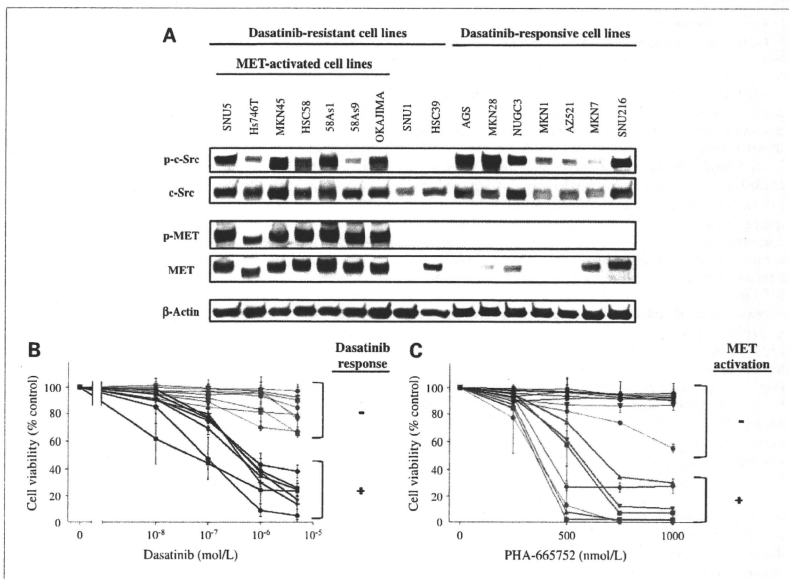

Figure 1. Phosphorylation of c-Src or MET and growth-inhibitory effects of c-Src or MET inhibitors in gastric cancer cell lines. A, the indicated gastric cancer cell lines maintained in a medium containing 10% serum were lysed and subjected to immunoblot analysis with antibodies to phosphorylated (p-) or total forms of c-Src or MET or to β-actin (loading control). B and C, gastric cancer cell lines were cultured in medium containing 10% serum for 72 hours in the presence of various concentrations of dasatinib (B) or PHA-665752 (C), after which cell viability was assessed as described in Materials and Methods. The number of viable cells is expressed as a percentage of the value for nontreated cells. Black and red lines, dasatinib-responsive and dasatinib-resistant cells, respectively. Points, mean of values from three independent experiments; bars, SD.

Table 1. IC_{50} values of dasatinib for inhibition of the growth of gastric cancer cells *in vitro*

Dasatinib response		Cell line	Dasatinib IC_{50} (µmol/L)	MET activation
Resistant		SNU5	>5	+
		Hs746T	>5	+
		MKN45	>5	+
		HSC58	>5	+
		58As1	>5	+
		58As9	>5	+
		OKAJIMA	>5	+
		SNU1	>5	
		HSC39	>5	
Responsive				
	Moderately responsive	AGS	0.54 ± 0.12	
		MKN28	0.50 ± 0.11	
		NUGC3	0.45 ± 0.16	
		MKN7	0.42 ± 0.26	
		MKN1	0.28 ± 0.20	
	Highly responsive	AZ521	0.06 ± 0.03	
		SNU216	0.04 ± 0.03	

NOTE: Data are means ± SD of triplicates from experiments that were repeated a total of three times with similar results.

MET activation is associated with dasatinib resistance in gastric cancer cell lines. The level of c-Src phosphorylation was thus not sufficient to distinguish dasatinib-responsive from dasatinib-resistant cells. Redundancy of tyrosine kinases has been shown to contribute to *de novo* resistance to tyrosine kinase inhibitors (21, 22). Given that amplification of *MET* is frequent in gastric cancer (23–25), we examined whether resistance to dasatinib in gastric cancer cell lines positive for c-Src activation might be due to MET activation. We first determined the abundance and activation status of MET in the gastric cancer cell lines. Immunoblot analysis revealed that all dasatinib-resistant cells positive for phosphorylated c-Src manifested high levels of both MET expression and baseline activation, as reflected by phosphorylation of tyrosine residues 1234/1235 (Fig. 1A) and tyrosine-1349 (data not shown). In contrast, cells categorized as responsive to dasatinib had undetectable levels of phosphorylated MET. We next examined the gastric cancer cell lines for *MET* amplification by fluorescence *in situ* hybridization analysis. Six of the seven cell lines positive for MET activation, all of which were resistant to dasatinib, were found to be positive for *MET* amplification, whereas the one remaining cell line (OKAJIMA) showed no evidence of *MET* amplification. In contrast, all dasatinib-responsive cell lines as well as SNU1 and HSC39 were found to be negative for *MET* amplification (data not shown). These results thus suggested that MET activation is associated with dasatinib resistance in gastric cancer cells.

A MET inhibitor suppresses the growth of dasatinib-resistant gastric cancer cell lines with activated MET but not that of dasatinib-responsive cells. The specific MET inhibitor PHA-665752 was previously shown to inhibit the proliferation of cancer cells in which MET is constitutively activated (26, 27). Given that MET was found to be activated in all dasatinib-resistant gastric cancer cell lines with activated c-Src (Fig. 1A), we examined the effect of PHA-665752 on the growth of gastric cancer cell lines. Consistent with previous observations (27), PHA-665752 inhibited the growth of the dasatinib-resistant cell lines with activated MET (Fig. 1C). In contrast, all dasatinib-responsive cell lines as well as SNU1 and HSC39 were resistant to PHA-665752 (Fig. 1C). These results thus suggested that the subsets of gastric cancer cells defined by the response to c-Src or MET inhibitors are distinct and nonoverlapping.

Dasatinib inhibits ERK or AKT signaling in dasatinib-responsive gastric cancer cell lines but not in dasatinib-resistant cells. The effects of dasatinib on cell signaling were evaluated in the gastric cancer cell lines with activated c-Src. Cells were exposed to various concentrations of dasatinib and then subjected to immunoblot analysis of phosphorylated and total forms of c-Src, ERK, and AKT (Fig. 2). Dasatinib induced marked inhibition of c-Src phosphorylation in all cell lines tested. In dasatinib-responsive cells, dasatinib also inhibited ERK phosphorylation in a concentration-dependent manner. It also inhibited AKT phosphorylation in SNU216, AGS, and MKN1 cells. In contrast, dasatinib exhibited no substantial inhibitory effect on the phosphorylation of ERK or AKT even at a concentration of 300 nmol/L in dasatinib-resistant cells. These findings indicated that the antiproliferative effect of dasatinib in gastric cancer cells correlates with the inhibition of ERK or AKT signaling.

Dasatinib induces G1 arrest or apoptosis in dasatinib-responsive gastric cancer cell lines. To investigate the mechanism by which dasatinib inhibits gastric cancer cell growth, we first analyzed the cell cycle profile by flow cytometry after exposure of cells to the drug for 0, 24, or 48 hours. We chose a dasatinib concentration of 300 nmol/L for these experiments because it approximated the IC_{50} values for dasatinib-responsive cell lines. Dasatinib increased the percentage of cells in G_0-G_1 phase of the cell cycle and decreased the percentage of those in S phase in a subset of dasatinib-responsive cell lines, including AZ521, MKN28, NUGC3, and MKN7 (Fig. 3A). The other dasatinib-responsive cell lines, including SNU216, MKN1, and AGS, in which dasatinib inhibited both ERK and AKT phosphorylation, showed an increase in the sub-G_1 cell population on exposure to dasatinib, indicative of the induction of apoptosis (Fig. 3A). In dasatinib-resistant cells with MET activation, dasatinib had minimal effects on cell cycle distribution (Supplementary Fig. S1A). We also examined the effect of dasatinib on the abundance of the cyclin-dependent kinase inhibitor p27, which contributes to the regulation of G_1-S progression. Dasatinib induced the upregulation of

p27 in the four dasatinib-responsive cell lines in which it induced G_1 arrest (Fig. 3B), but not in cell lines in which it did not trigger such arrest (Supplementary Fig. S1B). As a further test for apoptosis in SNU216, MKN1, and AGS cells, we measured the activity of caspase-3 and probed for cleavage of PARP. Dasatinib increased caspase-3 activity (Fig. 3C) and induced PARP cleavage (Fig. 3D) in these three cell lines. These findings thus indicated that induction of G_1 arrest or apoptosis underlies the antiproliferative effect of dasatinib in responsive cells. On the other hand, PHA-665752 was previously shown to induce apoptosis in gastric cancer cells with *MET* amplification (27). Consistent with these previous results, we showed that PHA-665752 induced a substantial increase in the frequency of apoptosis, as revealed by an increase in caspase-3 activity and PARP cleavage, in dasatinib-resistant cells with MET activation, whereas PHA-665752 had minimal effects on apoptosis in dasatinib-responsive cells (Fig. 3C and D).

Effects of c-Src depletion in dasatinib-responsive gastric cancer cell lines. To verify that the inhibitory effect of dasatinib on cell growth is indeed mediated by c-Src inhibition rather than by nonspecific inhibition of
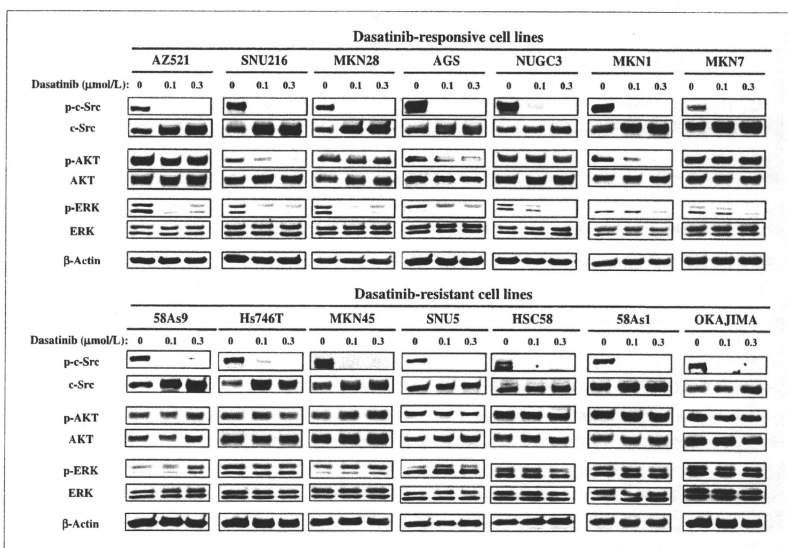

Figure 2. Effects of dasatinib on cell signaling in gastric cancer cell lines. The indicated cell lines were incubated in a medium containing 10% serum for 24 hours in the absence or presence of dasatinib at 100 or 300 nmol/L. Cell lysates were then subjected to immunoblot analysis with antibodies to the indicated proteins.
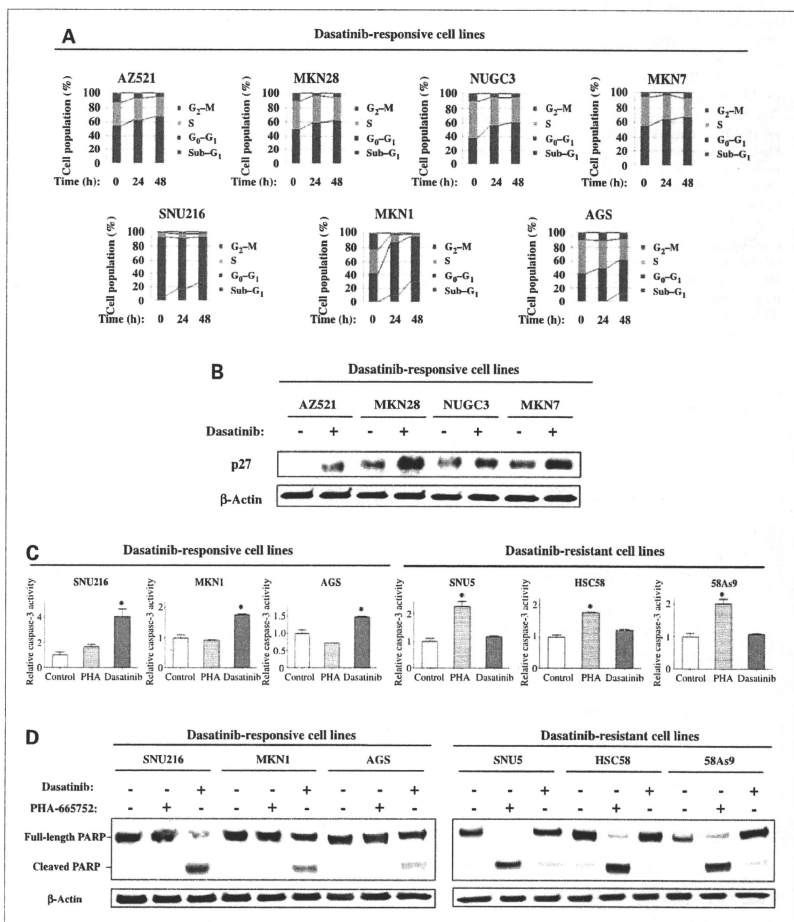
Figure 3. Effects of dasatinib on cell cycle distribution and apoptosis in gastric cancer cell lines. A, cells were incubated in medium containing 10% serum for 0, 24, or 48 hours in the presence of 300 nmol/L dasatinib, after which they were fixed, stained with propidium iodide, and analyzed for cell cycle distribution by flow cytometry. All data are means of triplicates from experiments that were repeated a total of three times with similar results. B, cells were incubated in a medium containing 10% serum for 24 hours in the absence or presence of dasatinib (300 nmol/L). Cell lysates were then subjected to immunoblot analysis with antibodies to p27. C, cells were incubated in medium containing 10% serum for 48 hours in the absence or presence of either dasatinib (300 nmol/L) or PHA-665752 (500 nmol/L). Cell lysates were then assayed for caspase-3 activity. Columns, mean of values from three independent experiments; bars, SD. *, $P < 0.05$ versus the corresponding value for control cells. D, cells were incubated in medium containing 10% serum for 72 hours in the absence or presence of either dasatinib (300 nmol/L) or PHA-665752 (500 nmol/L). Cell lysates were then subjected to immunoblot analysis with antibodies to PARP.

other kinases such as the platelet-derived growth factor receptor, c-Kit, or Bcr-Abl (6), we transfected dasatinib-responsive cells with an siRNA that targets c-Src mRNA. Similar to the effects of dasatinib, depletion of c-Src resulted in G_1 arrest (Fig. 4A), accompanied by accumulation of p27 (Fig. 4B), in AZ521, MKN28, NUGC3, or MKN7 cells. Moreover, also similar to the effects of dasatinib, depletion of c-Src in SNU216, MKN1, or AGS cells triggered apoptosis as revealed by an increase in the sub-G_1 cell population (Fig. 4A) and PARP cleavage (Fig. 4C). These results thus indicated that the effects of dasatinib on cell growth or survival in gastric cancer cell lines are mediated by inhibition of c-Src.

Mechanism of dasatinib resistance in gastric cancer cells with MET activation. Given the association of activated MET with resistance to dasatinib, we examined whether the depletion of MET might affect dasatinib cytotoxicity in dasatinib-resistant cells with MET activation. Immunoblot analysis revealed that transfection of 58As9 or OKAJIMA cells with an siRNA spe-

cific for MET mRNA resulted in the marked depletion of the corresponding protein (Fig. 5A). Such depletion of MET restored the sensitivity of these dasatinib-resistant cells to the inhibition of cell growth by dasatinib (Fig. 5B). These results thus indicated that activated MET indeed contributes to dasatinib resistance in gastric cancer cells.

To examine the mechanism by which MET activation gives rise to dasatinib resistance, we determined the effects of PHA-665752 or dasatinib on the phosphorylation of MET, c-Src, AKT, and ERK in gastric cancer cells with MET activation. PHA-665752 inhibited the phosphorylation of MET, c-Src, AKT, and ERK in such cells (Fig. 5C). In contrast, dasatinib had no effect on either MET phosphorylation or downstream signaling by AKT or ERK in these cells (Fig. 5D). These data thus suggested that MET activation results in AKT and ERK phosphorylation in a c-Src–independent manner, although c-Src is activated at least in part by increased MET signaling in dasatinib-resistant cells with MET activation.
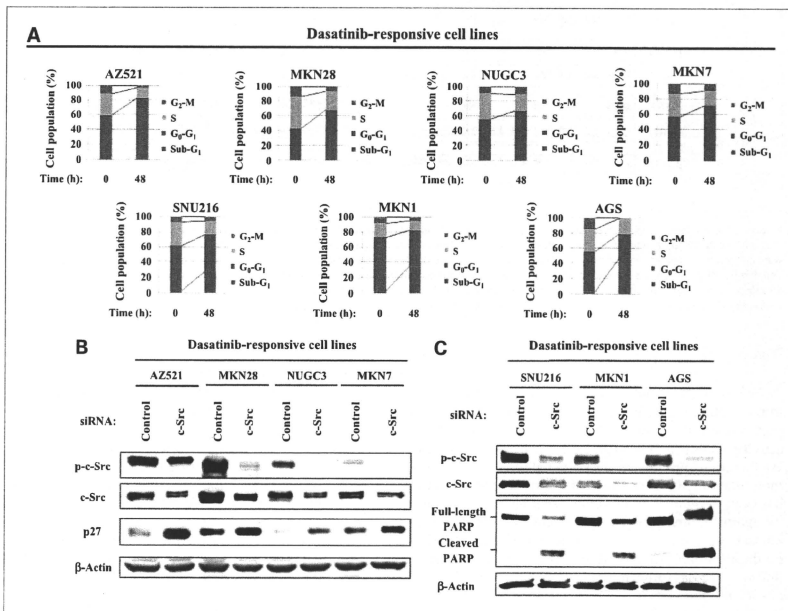

Figure 4. Effects of c-Src depletion on cell cycle distribution and apoptosis in gastric cancer cell lines. A, cells were transfected with nonspecific (control) or c-Src siRNAs for 48 hours, fixed, stained with propidium iodide, and analyzed for cell cycle distribution by flow cytometry. All data are means of triplicates from experiments that were repeated a total of three times with similar results. B and C, cells were transfected as in A for 48 hours (B) or 72 hours (C), after which cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins.
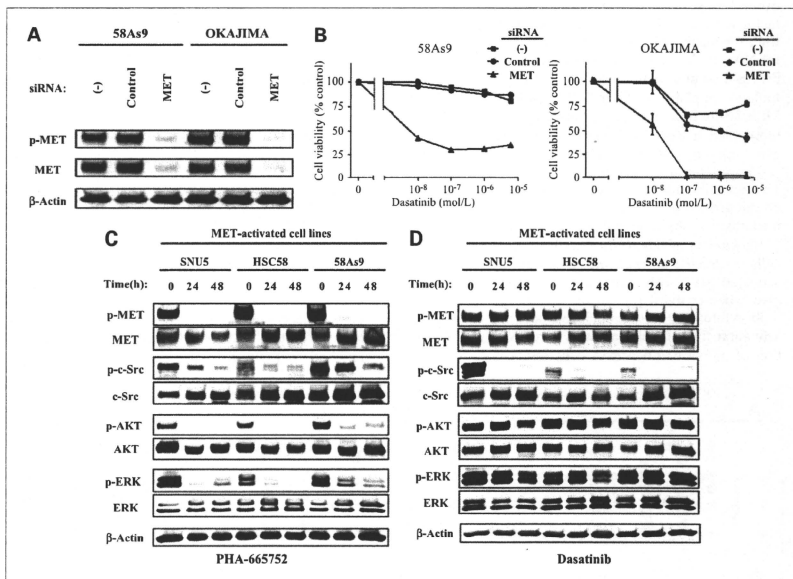
Figure 5. Mechanism of dasatinib resistance in gastric cancer cells with MET activation. A, cells were transfected or not with nonspecific (control) or MET siRNAs for 48 hours, after which cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins. B, cells transfected as in A were cultured in a medium containing 10% serum and various concentrations of dasatinib for an additional 48 hours, after which cell viability was assessed. The number of viable cells is expressed as a percentage of the corresponding value for cells not exposed to dasatinib. Points, mean of values from three independent experiments; bars, SD. C and D, cells were incubated in medium containing 10% serum for 0, 24, or 48 hours in the presence of 500 nmol/L PHA-665752 (C) or 300 nmol/L dasatinib (D). Cell lysates were then subjected to immunoblot analysis with antibodies to the indicated proteins.

Discussion

We have shown that c-Src is activated at various levels in most human gastric cancer cell lines, consistent with previous studies showing the upregulation of the kinase activity of c-Src in most gastric cancers examined (15–18). We therefore investigated the potential utility of c-Src as a molecular target for treatment of gastric cancer. Dasatinib has been developed as a multikinase inhibitor with activity against c-Src, Bcr-Abl, and several receptor tyrosine kinases (6). We examined the effects of c-Src inhibition by dasatinib on cell growth in gastric cancer cell lines, finding that dasatinib inhibited the growth of a subset of such cell lines exhibiting c-Src phosphorylation. No relation was apparent between the response to dasatinib and the level of c-Src phosphorylation in cell lines with activated c-Src, whereas SNU1 and HSC39, both of which did not manifest detectable c-Src phosphorylation, were resistant to dasatinib, consistent with previous observations

(7, 8, 14, 28, 29). These findings suggest that c-Src promotes cell proliferation and survival in a subset of gastric cancer cell lines positive for c-Src activation but not in those without c-Src activation.

We found that the level of ERK phosphorylation was reduced by dasatinib in all responsive cell lines but not in resistant cell lines, suggesting that the inhibition of ERK might correlate with the antiproliferative effects of c-Src inhibitors in gastric cancer cells. In addition, inhibition of both ERK and AKT phosphorylation by dasatinib was associated with the induction of apoptosis in SNU216, MKN1, and AGS cells. To confirm that the effects of dasatinib on cell cycle progression and apoptosis are indeed attributable to c-Src inhibition in dasatinib-responsive gastric cancer cells, we specifically depleted the cells of c-Src by RNA interference. In cells in which dasatinib induced G_1 arrest, depletion of c-Src also triggered G_1 arrest accompanied by the upregulation of p27. Similarly, in cells in which dasatinib induced apoptosis, c-Src depletion also

elicited apoptosis, as revealed by the detection of PARP cleavage. These data thus provide more definitive support for the notion that c-Src signaling promotes the proliferation and survival of dasatinib-responsive gastric cancer cell lines. A c-Src inhibitor was previously shown to induce G_1 arrest in prostate cancer cells (11). On the other hand, dasatinib was found to induce apoptosis in non–small cell lung cancer cells harboring an epidermal growth factor receptor gene mutation (8). Sensitive cells thus exhibit different responses to c-Src inhibitors. The mechanisms underlying the cellular decision to undergo G_1 arrest or apoptosis in response to such inhibitors remain unclear but may be related to differences in cell type.

Gastric cancer cell lines positive for MET activation were resistant to dasatinib, despite the activation of c-Src apparent in these cells. We showed that dasatinib sensitivity was restored in such cells by the depletion of MET, suggesting that MET activation contributes to resistance to c-Src inhibitors. The MET inhibitor PHA-665752 suppressed c-Src, ERK, and AKT phosphorylation in cells with activated MET, whereas dasatinib had minimal effects on either ERK or AKT phosphorylation. These findings suggested that MET-c-Src and MET-ERK/AKT pathways operate independently of each other in such cells. We found that PHA-665752 inhibited the growth of cells with MET activation, with this effect being accompanied by the induction of apoptosis. Consistent with our findings, MET amplification was previously shown to identify a subset of gastric cancers likely to respond to MET inhibitors (27). These results suggest that cells with MET activation may have switched their cell growth dependence from the c-Src-ERK/AKT pathway to the MET-ERK/AKT pathway, although the precise mechanism of the altered signal transduction remains unknown. We further found that the combination of dasatinib and PHA-665752 manifested an additive to synergistic inhibitory effect on the growth of gastric cancer cells with MET activation but not on that of cells without MET activation (Supplementary Table S1 and Fig. S2). These data suggest that the survival of cells with MET activation depends at least in part on activated c-Src in the presence of a MET inhibitor. Further studies are required to determine the mechanism of dasatinib resistance in gastric cancer with MET activation. Amplification of MET is often responsible for the activation of MET signaling, with such amplification occurring most frequently (10-20%) in gastric cancer (23–25). Our data therefore suggest that the analysis of MET amplification might optimize patient selection for gastric cancer treatment with c-Src or MET inhibitors.

The recent success of molecularly targeted agents seems to depend on the identification of drug targets and patients who are likely to benefit from these agents. In the present study, we show that c-Src is a promising target for the treatment of gastric cancer. Moreover, our results indicate that testing to exclude the possibility of MET amplification should be done in consideration of c-Src inhibitors for the treatment of gastric cancer. Dasatinib inhibited the growth of SNU216 and AZ521 cells with IC_{50} values in the low nanomolar range (40 and 60 nmol/L, respectively); however, the IC_{50} values for most of the responsive cell lines were greater than the maximum achievable plasma concentration (100 nmol/L) of dasatinib (30, 31). Novel c-Src inhibitors with increased potency are currently under development (32) and might prove beneficial for the treatment of gastric cancer. Our results provide a rationale for future clinical investigation of the therapeutic efficacy of c-Src inhibitors in individuals with gastric cancer as well as for the selection of patients likely to benefit from such treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Successful Treatment with Erlotinib after Gefitinib-Induced Severe Interstitial Lung Disease

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Masahiro Fukuoka, MD, Phd,† and Kazuhiko Nakagawa, MD, Phd*

A 62-year-old woman with an 18-pack-year history of smoking was found to have a solitary spiculated lesion in the right upper lung lobe and multiple pulmonary nodules on a computed tomography (CT) scan of the chest. A transcutaneous CT-guided needle biopsy specimen of the lung revealed adenocarcinoma. She received four cycles of chemotherapy with docetaxel and cisplatin, after which CT revealed marked tumor shrinkage. During follow-up for 3 months, progressive disease was diagnosed on the basis of the presence of an enhancement of cortical sulci on a CT scan of the brain. Mutation analysis of the lung cancer specimen showed the presence of an exon-19 deletion in the *epidermal growth factor receptor (EGFR)* gene, and gefitinib was administered orally at a dose of 250 mg once daily (Figure 1A). After 24 days of gefitinib, the patient manifested acutely deteriorating dyspnea with fever and cough. A chest CT scan revealed extensive bilateral ground-glass opacities throughout both lungs (Figure 1B). The results of sputum culture of bacteria or fungi and general blood tests, including those for fungal antigen and cytomegalovirus antigen, were negative. The patient had no prior interstitial lung disease (ILD) or preexisting collagen-vascular disease. Therefore, we concluded that the clinical course and imaging results were consistent with gefitinib-induced ILD. Gefitinib therapy was discontinued, and the treatment with high-dose methylprednisolone was initiated. Given that her symptoms and radiologic findings of gefitinib-induced ILD improved, the patient was discharged 14 days after discontinuation of gefitinib. As a result of progression of brain metastasis soon after discontinuation of gefitinib, whole-brain radiation therapy was delivered. Three months after discontinuation of gefitinib, the patient again experienced neuro-

logic progression because of the brain metastasis, and the development of symptomatic brain metastasis was considered to be a contraindication for treatment with cytotoxic agents. After receiving full informed consent for the risk of recurrent ILD and high mortality rate after the onset of ILD, we administered erlotinib at a dose of 150 mg/d (Figure 1C). Nine weeks after the initiation of erlotinib treatment, a chest CT scan revealed no evidence of ILD and a reduction in the size of multiple pulmonary metastatic nodules (Figure 1D). After 11 weeks of erlotinib treatment, she developed meningeal carcinomatosis with progressive neurologic symptoms and discontinued erlotinib treatment. ILD has not been identified during the course of treatment with erlotinib.

DISCUSSION

Gefitinib-induced ILD is the most problematic toxicity, with an incidence reported to be approximately 4% in Japan and with about one third of the cases being fatal.[1] The predictive risk factors for ILD development include male sex, smoking, and the existence of pulmonary fibrosis. The discovery of somatic mutations in the tyrosine kinase domain of *EGFR* and of the association of such mutations with a high response rate to EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib and erlotinib has had a profound impact on the treatment of advanced non-small cell lung cancer. Gefitinib-induced severe ILD has been reported only rarely in *EGFR* mutation-positive patients, although the prevalence of EGFR mutations is estimated to be low in patients with risk factors for ILD.[2]

The mechanism of gefitinib-induced ILD has not been fully elucidated. The previous findings suggest that inhibition of EGFR-mediated signaling by gefitinib impairs the repair of and thereby exacerbates lung injury.[3–5] In the present case, the patient received oral erlotinib (150 mg) daily, which results in a steady-state plasma trough concentration, that is approximately 3.5 times that for gefitinib (250 mg), and tumor shrinkage was observed with no evidence of ILD. These observations suggest that blockade of the EGFR signaling pathway by EGFR-TKIs is not necessarily associated with the incidence of drug-related ILD. Erlotinib and gefitinib share a 4-anilinoquinazoline base structure but differ in the substituents attached to the quinazoline and anilino rings. Minor differences in

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A   Before gefitinib   B   After gefitinib (day 24)

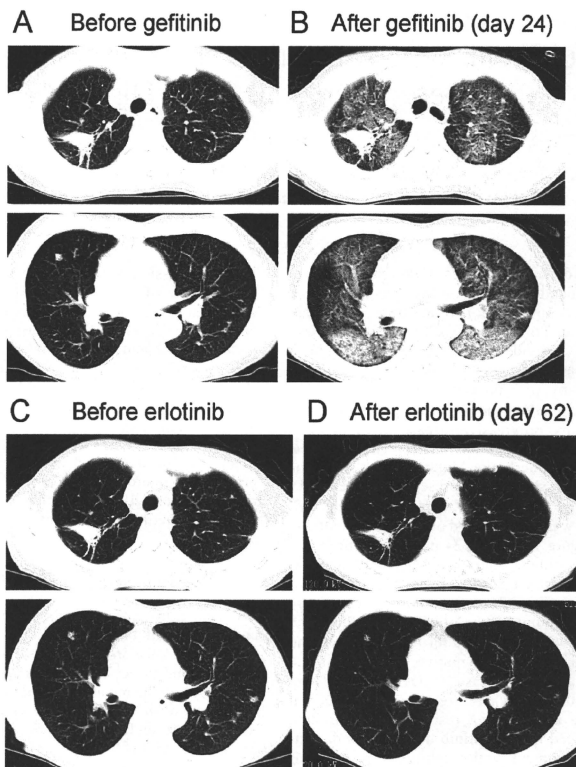## C   Before erlotinib   D   After erlotinib (day 62)

FIGURE 1. Chest computed tomography (CT) scans of the patient before (*A*) and 24 days after initiation of treatment with gefitinib (*B*). Bilateral pulmonary infiltrates on the chest CT were apparent on day 24 of gefitinib administration (*B*). After improvement of gefitinib-induced ILD, chest CT scans demonstrated that no ILD was observed in the patients receiving erlotinib (*C* and *D*).

the chemical structure of these compounds may, thus, influence lung toxicity.

We could successfully manage the patient who had once developed gefitinib-induced ILD with a full dose of erlotinib. The present case demonstrated that erlotinib may be one of the treatment options in such patients when alternative therapeutic options are limited. Because EGFR TKI-induced ILD has a high associated mortality, a careful assessment of clinical symptoms and radiographic findings and informed consent are needed in this setting.

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Successful Treatment With Erlotinib After Gefitinib-Related Severe Hepatotoxicity

A 66-year-old nonsmoking woman presented with enlarged left supraclavicular lymph nodes. She had no history of liver disease, alcohol intake, or hepatitis. Baseline blood tests showed cell counts, electrolytes, as well as renal and liver function to be normal. She had not previously received medication for her condition. A chest x-ray revealed a nodular shadow in the right upper lung field. A computed tomography scan of the chest confirmed a solitary spiculated lesion in the right upper lung lobe, disseminated nodules in the interlobar fissures, and multiple pulmonary nodules. Core biopsy of left supraclavicular lymph nodes revealed adenocarcinoma, consistent with metastasis from the primary non–small-cell lung carcinoma. Mutation analysis of lung cancer specimens obtained before first-line chemotherapy showed the presence of an exon 19 deletion of the epidermal growth factor receptor gene, and gefitinib was administered orally at a dose of 250 mg once daily. Eight weeks after the initiation of treatment, computed tomography revealed marked tumor shrinkage, which was categorized as a partial response. After 13 weeks of gefitinib treatment, laboratory investigations showed a substantial increase in serum transaminase levels (AST of 84 U/L, compared with a normal range of lower than 40; ALT of 181 U/L, compared with a normal range of lower than 35; Fig 1). Initiation of treatment with ursodeoxycholic acid and ammonium glycyrrhizate resulted in a gradual decrease in transaminase levels (to values of 31 U/L and 35 U/L for AST and ALT, respectively; Fig 1). Thirty-six weeks after the initiation of daily gefitinib administration, the transaminase levels of the proband had begun to increase again, reaching a pronounced high of 599 U/L for AST and 1,011 U/L for ALT at 37 weeks (Fig 1). Gefitinib treatment was discontinued at 36 weeks. The patient had taken no other medications or supplements, and an abdominal ultrasound revealed a normal liver with no other substantial abnormalities. A drug lymphocyte stimulation test yielded a strong positive result for gefitinib, sug-

gesting that the hepatitis of the proband was attributable to drug allergy rather than to dose-dependent toxicity. We therefore concluded that gefitinib should not be administered further at any schedule in this patient. In the 7 weeks after gefitinib withdrawal, the patient's liver function normalized but her lung cancer progressed slightly. We initiated treatment with erlotinib accompanied by careful monitoring of liver function, and the patient has continued daily oral erlotinib (150 mg) for 15 weeks with no evidence of increased hepatic toxicity or disease progression.

Gefitinib-induced hepatitis has received little attention to date, even though phase I trials revealed hepatotoxicity as a dose-limiting toxicity of the drug and the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL 1) trial showed that 2% of patients receiving gefitinib alone at a dose of 250 mg per day developed elevations of liver enzymes of grade 3 or 4 that necessitated cessation of treatment.[1] Exploration of new strategies for management of gefitinib-induced severe hepatotoxicity is thus warranted. Resumption of gefitinib treatment after its discontinuation as a result of the development of drug-induced hepatitis has been reported in three cases. However, gefitinib was again discontinued because of repeated elevation of serum transaminase levels in two of three cases[2,3]; the other case showed that an intermittent schedule of gefitinib administration (250 mg/d every 5 days) reduced hepatotoxicity, although the response had been maintained for only 8 weeks at the time of report submission.[4] These findings prompted us not to recommend resumption of gefitinib treatment after the development of severe hepatotoxicity in this patient. Erlotinib acts in a manner similar to that of gefitinib and has been shown to provide clinical benefit in patients with tumors positive for epidermal growth factor receptor gene mutations. We thus treated the proband of this study with erlotinib (150 mg once daily) as an alternative to gefitinib after discontinuation of the latter drug.

With regard to the toxicity profiles of gefitinib and erlotinib, it is important to clarify the mechanism responsible for drug-induced hepatotoxicity. Gefitinib and erlotinib share a common chemical backbone structure and exhibit similar disposition characteristics in humans after oral administration. They manifest similar bioavailabilities and both undergo extensive metabolism primarily by cytochrome P450 3A4, with more than 80% of the administered dose being found in feces.[5,6] Administration of erlotinib at the maximum-tolerated dose and approved dose of 150 mg once daily resulted in a steady-state plasma trough concentration that was approximately 3.5 times that for gefitinib administered at the recommended dose (approximately one third of the maximum tolerated dose) of 250 mg once daily.[7,8] This patient received no medications that influence the pharmacokinetics of gefitinib or erlotinib, suggesting that the plasma concentration of gefitinib per se did not give rise to the drug-induced hepatotoxicity, although the toxicity of gefitinib has not been directly compared with that of erlotinib alone. Instead, the positive result of the drug lymphocyte stimulation test supports a diagnosis of gefitinib-induced allergic hepatitis. Erlotinib and gefitinib share a
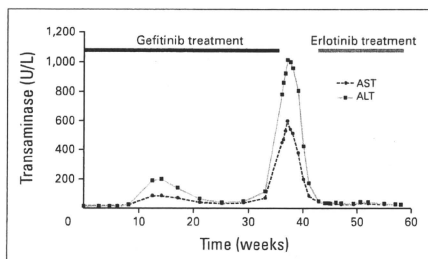

Fig 1.

4-anilinoquinazoline base structure, but differ in the substituents attached to the quinazoline and anilino rings. Minor differences in the chemical structures of these compounds may thus influence hepatotoxicity. In conclusion, erlotinib is an effective and well-tolerated treatment option for patients for whom gefitinib has been discontinued because of severe hepatotoxicity. Clinical trials to evaluate the administration of erlotinib after severe hepatotoxicity induced by daily administration of gefitinib are warranted.

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The author(s) indicated no potential conflicts of interest.

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CASE REPORT

Retreatment of recurrent malignant pleural mesothelioma with cisplatin and pemetrexed

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Abstract Combination chemotherapy with cisplatin and pemetrexed is the most active first-line regimen for malignant pleural mesothelioma (MPM). However, no drugs have been approved for second-line treatment of MPM, with effective regimens remaining to be identified for patients in relapse. We have now evaluated the combination of cisplatin and pemetrexed for retreatment of patients with recurrent MPM. Four men with MPM, all of whom received initial treatment with cisplatin and pemetrexed, underwent retreatment with this drug combination. Two of the patients achieved an objective response to the first-line chemotherapy with no evidence of disease progression for 6.4 or 11.4 months, respectively. The other two patients had stable disease with a duration of 7.8 or 5.0 months, respectively. The two patients who showed an objective response to first-line chemotherapy showed a partial response to retreatment, with a time to progression of 5.0 or 8.2 months, whereas the other two patients had progressive disease with a time to progression of 1.0 or 1.4 months, respectively. Retreatment with cisplatin plus pemetrexed was generally well tolerated. Retreatment with cisplatin and pemetrexed is a potential therapeutic option for certain patients with recurrent epithelioid MPM, possibly including those who show tumor regression with a time to progression of 6 months or more

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after the initial chemotherapy. Further studies are warranted to evaluate the efficacy of such retreatment and to clarify the criteria for patient selection.

Keywords Malignant pleural mesothelioma ·
Retreatment · Pemetrexed · Cisplatin

Introduction

Malignant pleural mesothelioma (MPM) is a highly aggressive neoplasm. Treatment of MPM patients with the combination of cisplatin and pemetrexed has been associated with an increased survival time (12.1 vs. 9.3 months), longer time to progression (5.7 vs. 3.9 months), and greater response rate (41.3% vs. 16.7%), as well as with improved pulmonary function and symptom control compared with treatment with cisplatin alone [1, 2]. However, most patients eventually manifest disease progression after the initial response to such combination chemotherapy. Although a previous study has suggested that second-line chemotherapy after initial treatment with cisplatin and pemetrexed has a positive impact on the survival of MPM patients [3], no drugs have been approved for second-line treatment of MPM. Effective chemotherapy is thus needed for the treatment of patients with MPM who relapse after first-line chemotherapy. We now present a report of four patients with recurrent MPM who received an initial course of treatment with pemetrexed and cisplatin and who subsequently underwent retreatment with this drug combination.

Case report

Four patients with MPM underwent retreatment with cisplatin and pemetrexed. Treatment response was evaluated

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according to the modified RECIST criteria for MPM proposed by Byrne and Nowak [4]. Time to progression was defined as the period from the start of treatment to the date of disease progression or death, whichever occurred first. Overall survival was defined as the time from the initial visit until death from any cause. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria (version 3).

Patient characteristics are summarized in Table 1. The patients were men aged 65, 60, 53, or 69 years. Tumor histology was epithelial in three patients and biphasic in one patient. All patients received four cycles of treatment with cisplatin and pemetrexed as the first-line chemotherapy. Patient 1 achieved a partial response (Fig. 1) and patient 2 achieved a complete response, with no evidence of disease progression for 6.4 and 11.4 months, respectively. Patients 3 and 4 had stable disease for a duration of 7.8 and 5.0 months, respectively.

At the time of this analysis, all four patients were no longer undergoing retreatment with cisplatin and pemetrexed. Patients 1 and 2 showed a partial response to retreatment, with a time to progression of 5.0 and 8.2 months, respectively (Fig. 1, Table 1). Patients 3 and 4 manifested progressive disease, with a time to progression of only 1.0 and 1.4 months, respectively. With the exception of hyponatremia of grade 3 observed in one patient (patient 1), no toxicities of grade 3 or 4 were apparent during retreatment.

Discussion

We have presented four patients who were retreated with the same chemotherapy regimen on progression of their MPM after initial treatment with cisplatin plus pemetrexed and durable tumor control. Two of the four patients achieved an objective response after four cycles of retreatment with acceptable toxicity.

For many types of malignant neoplasm, the standard treatment options for disease progression after first-line chemotherapy are chemotherapeutic regimens that differ from the initial treatment. However, in the case of MPM, no drugs have been approved for second-line treatment. We elected to retreat the present patients after disease recurrence with the same regimen as that used for the initial chemotherapy, given that all four individuals manifested disease control (one a partial response, one a complete response, and two stable disease) after the first-line treatment. Retreatment of ovarian cancer patients with the combination of carboplatin and paclitaxel is established for individuals who show sensitive relapse, defined as disease that responds to first-line chemotherapy but which relapses more than 6 months after the last dose of the first-line treatment [5]. A previous report presented four patients with relapsed MPM who achieved long-lasting tumor control with the combination of platinum and pemetrexed for retreatment [6]. The time to progression after initial platinum–pemetrexed chemotherapy was unusually long in these patients, ranging from 23 to 73 months. In the present report, the time to progression after the initial chemotherapy was 6.4 or 11.4 months for the two patients who achieved a second response, times that are substantially shorter than those in the previous study [6]. Our findings suggest that patients who show a time to progression of 6 months or more after initial chemotherapy with cisplatin plus pemetrexed may show a response on retreatment. The histological subtype of the two patients who responded to the retreatment was epithelioid histology, consistent with

Table 1 Patient characteristics and response to first-line chemotherapy and retreatment

	Patient 1	Patient 2	Patient 3	Patient 4
Age, years	65	60	53	69
Sex	Male	Male	Male	Male
Histology	Epithelial	Epithelial	Epithelial	Biphasic
Dose of first-line chemotherapy[a]	P500 + C60	P500 + C75	P500 + C75	P500 + C75
No. of cycles of first-line chemotherapy	4	4	4	4
Time to progression (months) after first-line chemotherapy	6.4	11.4	7.8	5.0
Response to first-line chemotherapy)	Partial response	Complete response	Stable disease	Stable disease
Dose of retreatment[a]	P500 + C60	P500 + C75	P500 + C75	P500 + C60
No. of cycles of retreatment	4	4	1	1
Time to progression (months) after retreatment	5.0	8.2	1.0	1.4
Response to retreatment	Partial response	Partial response	Progression disease	Progression disease
Overall survival[b] (months)	19.0	26+	10.0	9.3

[a] Doses for pemetrexed (P) and cisplatin (C) are given in milligrams per square meter (mg/m^2)

[b] Overall survival was defined as the time from the initial visit until death from any cause
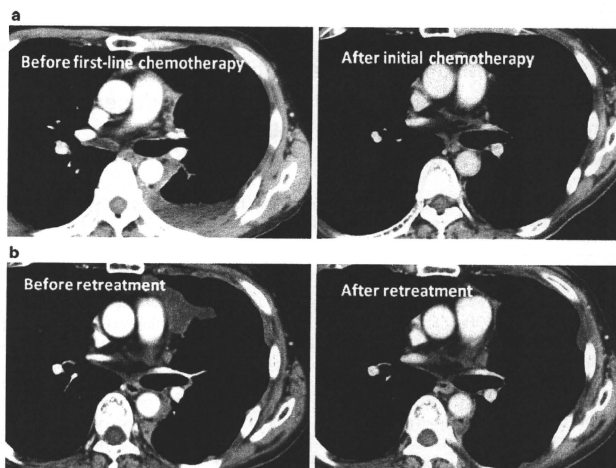
Fig. 1 Responses of patient 1 to first- and second-line chemotherapy with cisplatin plus pemetrexed. Computed tomography scans revealed that pleural nodules of patient 1 showed a partial response to first-line chemotherapy (**a**) and that a second response was obtained after retreatment with cisplatin plus pemetrexed (**b**)

the previous report [6]. These findings suggest that epithelioid histological subtype may also define a response on retreatment.

Our cohort included two patients who developed progressive disease with retreatment. These two patients did not develop an objective response to initial chemotherapy with cisplatin–pemetrexed, instead manifesting stable disease, whereas the two patients who achieved a response to retreatment showed a partial or complete response to first-line treatment. This observation suggests that failure to respond to initial chemotherapy may be a negative predictive factor for the effectiveness of retreatment.

The overall survival of the two patients who achieved a second response was 19 and more than 26 months, respectively, suggesting that successful retreatment with cisplatin plus pemetrexed can prolong survival time. Our observations thus suggest that retreatment with cisplatin plus pemetrexed may yield clinical benefits in patients who show a partial or complete response of long duration (>6 months) to the initial combination chemotherapy. Further studies are warranted to evaluate the efficacy of such second-line treatment and to clarify the criteria for selection of patients likely to respond to retreatment with cisplatin plus pemetrexed.

Conflict of interest statement I. Okamoto and K. Nakagawa received honoraria from Boehringer Ingelheim. The other authors have no conflict of interest.

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TAK-701, a Humanized Monoclonal Antibody to Hepatocyte Growth Factor, Reverses Gefitinib Resistance Induced by Tumor-Derived HGF in Non–Small Cell Lung Cancer with an EGFR Mutation

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Abstract

Most non–small cell lung cancer (NSCLC) tumors with an activating mutation of the epidermal growth factor receptor (EGFR) are initially responsive to EGFR tyrosine kinase inhibitors (TKI) such as gefitinib but ultimately develop resistance to these drugs. Hepatocyte growth factor (HGF) induces EGFR-TKI resistance in NSCLC cells with such a mutation. We investigated strategies to overcome gefitinib resistance induced by HGF. Human NSCLC cells with an activating *EGFR* mutation (HCC827 cells) were engineered to stably express HGF (HCC827-HGF cells). HCC827-HGF cells secreted large amounts of HGF and exhibited resistance to gefitinib *in vitro* to an extent similar to that of HCC827 GR cells, in which the gene for the HGF receptor MET is amplified. A MET-TKI reversed gefitinib resistance in HCC827-HGF cells as well as in HCC827 GR cells, suggesting that MET activation induces gefitinib resistance in both cell lines. TAK-701, a humanized monoclonal antibody to HGF, in combination with gefitinib inhibited the phosphorylation of MET, EGFR, extracellular signal-regulated kinase, and AKT in HCC827-HGF cells, resulting in suppression of cell growth and indicating that autocrine HGF-MET signaling contributes to gefitinib resistance in these cells. Combination therapy with TAK-701 and gefitinib also markedly inhibited the growth of HCC827-HGF tumors *in vivo*. The addition of TAK-701 to gefitinib is a promising strategy to overcome EGFR-TKI resistance induced by HGF in NSCLC with an activating *EGFR* mutation. *Mol Cancer Ther; 9(10); 2785–92. ©2010 AACR.*

Introduction

Somatic mutations in the kinase domain of the epidermal growth factor receptor (EGFR) are associated with a high rate of response to EGFR tyrosine kinase inhibitors (TKI) such as gefitinib (Fig. 1) and erlotinib in advanced non–small cell lung cancer (NSCLC; refs. 1–3). Despite the therapeutic benefit of EGFR-TKIs in NSCLC, however, most patients ultimately develop resistance to these drugs. A secondary T790M mutation of *EGFR* and amplification of the *MET* gene are major causes of acquired resistance to EGFR-TKIs (4–7). In addition, hepatocyte growth factor (HGF), a ligand of the MET oncoprotein (8, 9), induces gefitinib resistance in *EGFR*

mutation–positive NSCLC by activating MET and downstream signaling (10).

HGF was originally identified as a mitogenic protein for hepatocytes (11). Both HGF and its MET receptor are expressed, and often overexpressed, in a broad spectrum of human solid tumors including lung, mesothelioma, breast, and brain cancer (12–16). HGF thus acts as an autocrine or paracrine growth factor for these tumor cells (17, 18). TAK-701 is a potent humanized monoclonal antibody to HGF that blocks various HGF-induced biological activities as well as inhibits tumor growth in an autocrine HGF-MET–driven xenograft model.[6] To identify strategies or agents capable of overcoming resistance to EGFR-TKIs induced by HGF, we have now established sublines of the *EGFR* mutation–positive human NSCLC cell line HCC827 that stably express transfected HGF cDNA. With the use of these cells, we investigated the effects of TAK-701 on HGF-MET signaling and gefitinib resistance induced by cell-derived HGF both *in vitro* and *in vivo*.

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