

Hepatocyte Growth Factor Induces Gefitinib Resistance of Lung Adenocarcinoma with Epidermal Growth Factor Receptor–Activating Mutations

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Abstract

Lung cancer with epidermal growth factor receptor (EGFR)–activating mutations responds favorably to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib. However, 25% to 30% of patients with EGFR-activating mutations show intrinsic resistance, and the responders invariably acquire resistance to gefitinib. Here, we showed that hepatocyte growth factor (HGF), a ligand of MET oncoprotein, induces gefitinib resistance of lung adenocarcinoma cells with EGFR-activating mutations by restoring the phosphatidylinositol 3-kinase/Akt signaling pathway via phosphorylation of MET, but not EGFR or ErbB3. Strong immunoreactivity for HGF in cancer cells was detected in lung adenocarcinoma patients harboring EGFR-activating mutations, but no T790M mutation or MET amplification, who showed intrinsic or acquired resistance to gefitinib. The findings indicate that HGF-mediated MET activation is a novel mechanism of gefitinib resistance in lung adenocarcinoma with EGFR-activating mutations. Therefore, inhibition of HGF-MET signaling may be a considerable strategy for more successful treatment with gefitinib. [Cancer Res 2008;68(22):9479–87]

Introduction

Lung cancer is one of the most prevalent malignancies and the leading cause of cancer-related death worldwide. Non–small-cell lung cancer (NSCLC) accounts for nearly 80% of lung cancer cases. The median survival of metastatic NSCLC is 8 to 10 months when treated with the most active combination of conventional chemotherapeutic agents (1, 2). Recent therapeutic strategies for NSCLC have focused on the development of molecular targeted therapies.

Epidermal growth factor receptor (EGFR) is a member of a family of closely related growth factor receptor tyrosine kinases, which includes EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3),

and HER4 (ErbB4; ref. 3). As EGFR is expressed in the majority of cases of NSCLC, it is an attractive target for the development of therapeutic agents. The small-molecule EGFR tyrosine kinase inhibitors, including gefitinib and erlotinib, have been evaluated in clinical trials for patients with NSCLC. Both agents cause partial responses in 10% to 20% of all NSCLC patients (4, 5).

Activating mutations in *EGFR* have been found in a proportion of NSCLC (6, 7). Almost 90% of the activating mutations in *EGFR* consist of in-frame deletions in exon 19 and L858R point mutation in exon 21. These mutations are predominantly found in female, nonsmoking, adenocarcinoma patients and in patients of East Asian origin, and are associated with favorable response to the EGFR tyrosine kinase inhibitors, gefitinib and erlotinib (8). Multiple prospective trials have shown a response rate of 70% to 75% for patients with tumors harboring these mutations (8, 9). This indicates, however, that another 25% to 30% of the patients are intrinsically resistant to EGFR tyrosine kinase inhibitors although their tumors harbor activating mutations in *EGFR*. In addition, even patients who show a dramatic response to initial treatment also develop acquired resistance to the EGFR tyrosine kinase inhibitors almost without exception after varying periods of time (8).

To overcome the intrinsic and acquired resistance to EGFR tyrosine kinase inhibitors, it is necessary to clarify the molecular mechanisms of the resistance. Recently, two mechanisms, second-site point mutation that substitutes methionine for threonine at position 790 (T790M) in *EGFR* (10, 11) and amplification of *MET* proto-oncogene (12, 13), which contribute to acquired resistance to EGFR tyrosine kinase inhibitors, have been reported. T790 is located in the ATP-binding pocket of the catalytic region of EGFR and seems to be critical for the binding of erlotinib and gefitinib (10). On acquisition of a secondary T790M mutation, the bulkier methionine residue prevents EGFR tyrosine kinase inhibitor from binding with EGFR tyrosine kinases. On the other hand, *MET* amplification has been shown to restore the phosphatidylinositol 3-kinase (PI3K)/Akt pathway mediated by ErbB3 transactivation and thereby induces acquired resistance to gefitinib. T790M secondary mutation and *MET* amplification are found in ~50% and 20%, respectively, of patients acquiring resistance to EGFR tyrosine kinase inhibitors (8, 10–13). However, the mechanisms of intrinsic resistance and the other 30% of cases of acquired resistance are still unknown.

The *MET* gene encodes a high-affinity receptor for hepatocyte growth factor (HGF; refs. 14, 15). HGF, originally cloned as a mitogenic protein for hepatocytes (16), specifically activates MET receptor tyrosine kinase and induces pleiotropic biological effects

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in a wide variety of cells, including mitogenic, motogenic, morphogenic, and antiapoptotic activities (17, 18). As HGF is expressed in lung cancer cells and stromal cells (17–19), we investigated whether HGF is involved in gefitinib resistance of lung adenocarcinoma cells with *EGFR*-activating mutations. In contrast with *MET* amplification, we found that HGF induces resistance to gefitinib by restoring Akt phosphorylation independently of ErbB3 and that HGF can be involved in intrinsic resistance as well as acquired resistance to gefitinib in lung adenocarcinoma patients with *EGFR*-activating mutations.

Materials and Methods

Cell culture and reagents. The *EGFR*-mutant human lung adenocarcinoma cell lines PC-9 and HCC827 were purchased from Immuno-

Biological Laboratories Co. and American Type Culture Collection, respectively. These cell lines were extensively characterized (12, 20, 21). Human lung squamous cell carcinoma, EBC-1 (22), was purchased from the Health Science Research Resources Bank (Japan Health Sciences Foundation). PC-9, HCC827, and EBC-1 cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mmol/L glutamine.

Gefitinib was obtained from AstraZeneca. Recombinant HGF and anti-HGF antibody for immunohistochemistry were prepared as reported previously (23, 24). Recombinant epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) were obtained from Invitrogen. Transforming growth factor α (TGF- α) was from Biosource. Goat anti-human HGF neutralizing antibody and control goat IgG were purchased from R&D Systems.

Sequence analyses. We searched for mutations in the *EGFR* gene in lung adenocarcinoma cell lines by sequencing the cDNAs synthesized from

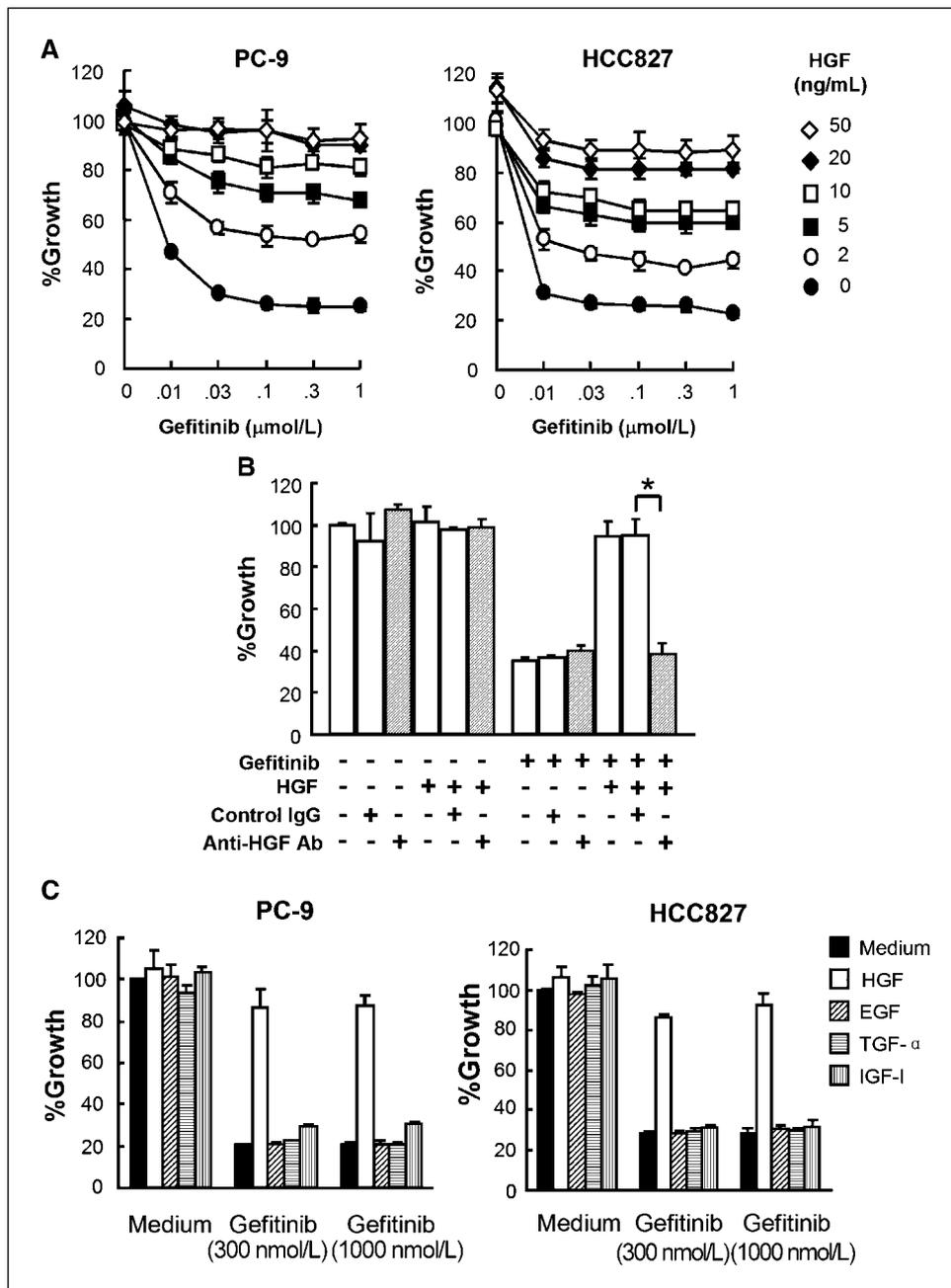


Figure 1. HGF induces gefitinib resistance of lung adenocarcinoma cells with *EGFR*-activating mutations. **A**, HGF induced gefitinib resistance of PC-9 and HCC827 cells with *EGFR*-activating mutation. Tumor cells were incubated with increasing concentrations of gefitinib and/or HGF, and cell growth was determined after 72 h of treatment by MTT assay. **B**, pretreatment of HGF with anti-HGF antibody abrogated HGF-induced resistance of HCC827 cells to gefitinib. HGF (20 ng/mL) was pretreated with control IgG (2 μ g/mL) or anti-HGF antibody (2 μ g/mL) at 37°C for 1 h. The resultant solutions were added to the cultures of tumor cells with or without gefitinib (300 nmol/L). Cell growth was determined in the same way as in **A**. *, $P < 0.01$ (one-way ANOVA). **C**, HGF was most potent in induction of gefitinib resistance of PC-9 and HCC827 cells. PC-9 and HCC827 cells were incubated with or without gefitinib and/or 50 ng/mL of HGF, EGF, TGF- α , or IGF-I. Cell growth was determined after 72 h of treatment. The percentage of growth is shown relative to untreated controls.

mRNAs. Gene-specific primer pairs were designed to cover the region from exons 18 to 22 of *EGFR*, which included all mutations reported to date. Primers for PCR were 5'-CCTCTTGTGCTGGTGGT-3' and 5'-GATTCGGT-CATATGGCTTGG-3'. PCR was done in reaction mixtures containing cDNA, 1× Ex Taq buffer, 0.2 mmol/L deoxynucleotide triphosphate mixture, 1.25 units of Ex Taq HS Enzyme (TaKaRa), and 0.5 μmol/L of forward and reverse primers in a volume of 50 μL. PCR cycling parameters were one cycle of 94°C for 7 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; followed by one cycle of 72°C for 7 min. The resulting PCR products were purified using a QIAquick PCR purification kit (Qiagen), followed by bidirectional dye-terminator fluorescent sequencing. All sequencing reactions were done in both forward and reverse directions with two primers for PCR and sequencing primer designed within the amplicon. The sequencing primer was 5'-ATGTCCGGGAACACAAAGAC-3'. Sequencing fragments were detected by capillary electrophoresis using an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All mutations were confirmed by examination of at least two independently derived PCR products.

Quantitative PCR for analysis of *MET* genomic amplification. Quantitative PCR was done on genomic DNA purified with a QIAamp DNA Mini kit (Qiagen). Primers and probe used for *MET* were (5' to 3') F-TGTTGCCAAGCTGTATTCTGTTTAC, R-TCTCTGAATTAGACGGATGTTGACA, and FAM-TGGATAATTGTGTCTTCTCTAG-MGBNFQ (22). Primers and probe for the single-copy reference gene *RNase P*, which encodes the RNA moiety of the enzyme RNase P, were obtained from Applied Biosystems, as well as TaqMan Gene Expression Master Mix. Reactions were done in quadruplicate with 20 ng of genomic DNA, primers at 900 nmol/L, and probes at 250 nmol/L under standard thermocycling conditions (2 min at 50°C; 10 min at 95°C; 40 cycles of 15 s at 92°C and 1 min at 60°C). Data were normalized to RNase P.

Cell proliferation assay. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method (25). Tumor cells (2×10^3 /100 μL/well) were plated into each well of 96-well plates in RPMI 1640 with 10% FBS. After 24-h incubation, several concentrations of gefitinib, cytokines, and/or culture supernatants were added to each well, and incubation was continued for a further 72 h. Then, 50 μL of MTT solution (2 mg/mL; Sigma) were added to all wells, and incubation was continued for a further 2 h. The media containing MTT solution were removed, and the dark blue crystals were dissolved by adding 100 μL of DMSO. The absorbance was measured with a microplate reader at test and reference wavelengths of 550 and 630 nm, respectively. The percentage of growth is shown relative to untreated controls. Each experiment was done at least in triplicate, and thrice independently.

Cell apoptosis assay. Cell apoptosis induced by gefitinib was detected with an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen) in accordance with the manufacturer's protocols. Briefly, aliquots of 5×10^5 cells were seeded into 100-mm dishes and incubated for 24 h at 37°C. On the following day, various concentrations of gefitinib and/or HGF (20 ng/mL), diluted in culture media, were added to the dishes and incubated for an additional 36 h. After trypsinization and gently washing cells once with medium, the cells were washed twice with PBS and then resuspended, and aliquots of 1×10^5 cells were transferred into new 5-mL culture tubes in 100 μL of 1× binding buffer. Then 5 μL of Annexin V-FITC and 5 μL of propidium iodide were added to the resuspended cells. After incubation at room temperature for 15 min in the dark, 400 μL of binding buffer were added to the resuspended cells. The analysis was done on a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson).

Antibodies and Western blotting. Tumor cells were incubated in 10 mL RPMI 1640 with 10% FBS in the presence or absence of HGF and/or gefitinib. Then, cells were lysed in cell lysis buffer containing phosphatase inhibitor cocktail and proteinase inhibitor cocktail (Sigma), and the protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce Biotechnology). Aliquots of 500 μg of total proteins were immunoprecipitated with the appropriate antibodies; immune complexes were recovered with Protein G-Sepharose beads (Zymed Laboratories). For Western blotting analysis, immunoprecipitates or 40 μg of total protein were resolved by SDS-polyacrylamide gel (Bio-Rad) electrophoresis and the proteins were then transferred onto polyvinylidene difluoride membranes

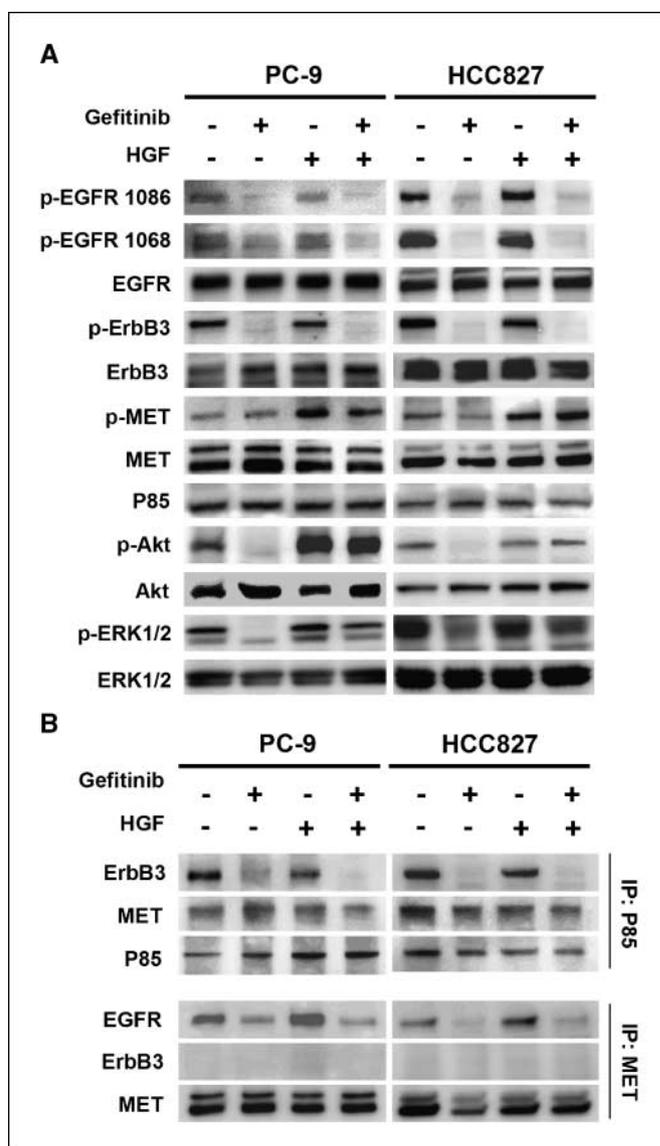


Figure 2. HGF induces gefitinib resistance of lung adenocarcinoma cells with *EGFR*-activating mutations by restoring phosphorylation of Akt, but not EGFR or ErbB3. **A**, the phosphorylation of Akt, but not EGFR or ErbB3, was restored by HGF. Tumor cells were treated with or without gefitinib (300 nmol/L) and/or HGF (20 ng/mL) for 1 h. Cells were lysed, and the indicated proteins were detected by immunoblotting. **B**, cell extracts were immunoprecipitated with an antibody to MET or p85. The precipitated proteins were determined by immunoblotting with the indicated antibodies.

(Bio-Rad). After washing four times, membranes were incubated with Blocking One (Nacalai Tesque, Inc.) for 1 h at room temperature and then incubated overnight at 4°C with the following primary antibodies: anti-Met (25H2), anti-phospho-Met (Y1234/Y1235) (3D7), anti-phospho EGFR (Y1068), anti-ErbB3 (1B2), anti-phospho-ErbB3 (Tyr1289) (21D3), PI3K p85 (19H8), phospho-PI3K p85(Tyr458)/p55(Tyr199), anti-Akt, or phospho-Akt (Ser473) antibodies (1:1,000 dilution, Cell Signaling Technology), and anti-human EGFR (1 μg/mL), anti-human/mouse/rat extracellular signal-regulated kinase (ERK)-1/ERK2 (0.2 μg/mL), or anti-phospho ERK1/ERK2 (T202/Y204) (0.1 μg/mL) antibodies (R&D Systems). After washing thrice, membranes were incubated for 1 h at room temperature with species-specific horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology). Each experiment was done at least thrice independently.

Assay for RNA interference and HGF gene transfection. Duplexed Stealth RNAi (Invitrogen) against *MET* and *ErbB3* and Stealth RNAi Negative Control Low GC Duplex #3 (Invitrogen) were used for RNA interference (RNAi) assay. The full-length *HGF* cDNA cloned into an expression vector (26) was used for *HGF* gene transfection assay. One day before transfection, aliquots of 2×10^4 tumor cells in 400 μ L of antibiotic-free medium were plated on 24-well plates. After incubation for 24 h, the cells were transfected with small interfering RNA (siRNA; 50 pmol) or scramble RNA using Lipofectamine 2000 (1 μ L) in accordance with the manufacturer's instructions. After 24-h incubation, the cells were washed with PBS and incubated with or without gefitinib (300 nmol/L) and/or recombinant human HGF (20 ng/nL) for an additional 72 h in antibiotic-containing medium. Cell proliferation was measured with a Cell Counting Kit-8 (Dojin) in accordance with the manufacturer's instructions. Knock-down of *MET* and *ErbB3* was confirmed by reverse transcription-PCR (RT-PCR) analysis. The sequences of siRNAs were as follows: *MET*, 5'-UCCAGAAGAUCAGUUUCCUAAUUA-3' and 5'-UGAAUUAGGAAACUGAUCUUCUGGA-5'; *ErbB3*, 5'-GGCCAUGAAUGAAUUCUCUACUCUA-3'

and 5'-UAGAGUAGAGAAUUAUCAUGGCC-3'. Each experiment was done at least in triplicate, and thrice independently.

HGF production. Cells (1×10^6) were cultured in RPMI 1640 with 10% FBS for 24 h. The cells were washed with PBS and incubated for 48 h in RPMI 1640 with 10% FBS. Then, culture medium was harvested and centrifuged, and the supernatant was stored at -70°C until analysis. For determination of HGF, ELISA was done in accordance with the manufacturer's recommended procedures (Immunis HGF EIA, Institute of Immunology, Tokyo, Japan). All samples were run in triplicate. Color intensity was measured at 450 nm with a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves. The detection limit was 0.1 ng/mL.

Patients. In total, 20 tumor specimens with *EGFR*-activating mutations were obtained from 16 lung adenocarcinoma patients, who provided written informed consent, at the Tokushima University Hospital (Tokushima, Japan) and Aichi Cancer Center Hospital (Nagoya, Japan) in Institutional Review Board-approved studies. Of the 16 patients, 3 patients showed intrinsic resistance and other 13 patients showed partial response

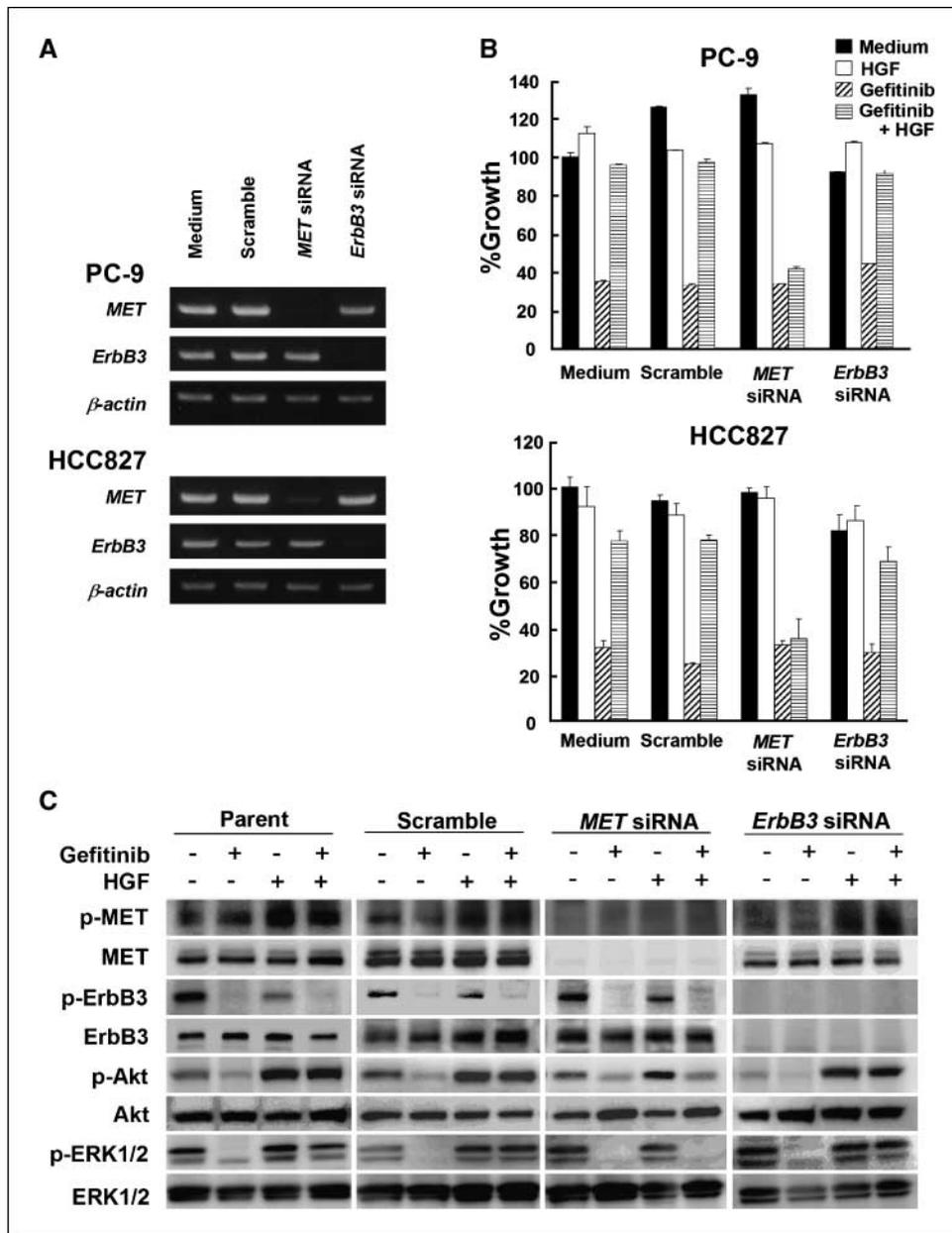


Figure 3. Specific down-regulation of MET, but not ErbB3, reversed gefitinib resistance and phosphorylation of Akt induced by HGF. *A*, siRNA specific for *MET* and *ErbB3* down-regulated mRNA expression of *MET* and *ErbB3*, respectively. Control, *MET*-specific, or *ErbB3*-specific siRNAs were introduced into PC-9 and HCC827 cells. mRNA was extracted 24 h later and RT-PCR was done. *B*, siRNA specific for *MET*, but not for *ErbB3*, reversed gefitinib resistance by HGF. The growth of cells from *A* with or without gefitinib (300 nmol/L) and/or HGF (20 ng/mL) was measured with a Cell Counting Kit-8. *, $P < 0.05$ (one-way ANOVA). *C*, down-regulation of MET, but not ErbB3, by specific-siRNA inhibited restored Akt-phosphorylation by HGF in cells treated with gefitinib. Control, *MET*-specific, or *ErbB3*-specific siRNAs were introduced into PC-9 cells. After 48 h, the cells were treated with or without gefitinib (300 nmol/L) and/or HGF (20 ng/mL) for 1 h, and then cell extracts were prepared and immunoblotted with the indicated antibodies.

Table 1. Summary of tissue samples from patients with lung adenocarcinoma with *EGFR*-activating mutations

Patient	Specimen	Type	BOR	<i>EGFR</i> mutation	T790M	<i>MET</i> amplification	HGF	MET
Intrinsic resistance								
Pretreatment specimens								
1	Pre-gefitinib	Primary lung cancer	PD	L858R	N	N	High	2+
2	Pre-gefitinib	Primary lung cancer	SD	L858R	N	N	High	3+
3	Pre-gefitinib	Lymph node	PD	L858R	N	N	High	3+
Acquired resistance								
Paired specimens								
4	Pre-gefitinib	Primary lung cancer	PR	Ex19Del		N	Low	3+
	Post-gefitinib			Y	N	Low	1+	
5	Pre-gefitinib	Primary lung cancer	PR	Ex19Del		N	Low	3+
	Post-gefitinib			Y	N	Low	3+	
6	Pre-gefitinib	Primary lung cancer	PR	Ex19Del		N	Low	3+
	Post-gefitinib			Y	N	Low	2+	
Posttreatment specimens								
7	Post-gefitinib	Lymph node	PR	L858R	N	N	High	3+
8	Post-gefitinib	Lung metastasis	PR	L858R	N	N	Low	2+
	Post-gefitinib	Lung metastasis	PR	L858R	Y	N	Low	2+
Responder								
Pretreatment specimens								
9	Pre-gefitinib	Primary lung cancer	PR	Ex19Del	N	NE	Low	3+
10	Pre-gefitinib	Lymph node	PR	L858R	N	N	Low	1+
11	Pre-gefitinib	Primary lung cancer	PR	Ex19Del	N	NE	Low	2+
12	Pre-gefitinib	Primary lung cancer	PR	Ex19Del	N	NE	Low	1+
13	Pre-gefitinib	Lymph node	PR	L858R	N	N	Low	3+
14	Pre-gefitinib	Primary lung cancer	PR	L858R	N	NE	Low	3+
15	Pre-gefitinib	Primary lung cancer	PR	L858R	N	NE	Low	3+
16	Pre-gefitinib	Lymph node	PR	Ex19Del	N	N	High	3+

Abbreviations: BOR, best overall response to gefitinib treatment; PR, partial response; SD, stable disease; PD, progressive disease; L858R, L858R point mutation in exon 21; Ex19Del, in-frame deletion in exon 19; N, no; Y, yes; NE, not evaluated.

to initial gefitinib treatment. For the 3 patients who showed intrinsic resistance and 8 of the 13 patients who had shown partial response, tumor specimens were obtained only before gefitinib treatment. The other 5 had shown partial response to gefitinib during initial treatment but showed signs of tumor regrowth (i.e., acquired resistance) while still receiving gefitinib. For 3 of those 5 patients, we were able to obtain pairs of tumor specimens before treatment and after the development of acquired resistance to gefitinib. In the other 2 patients, tumor specimens were available only after the development of acquired resistance to gefitinib. Of 6 tumors from 5 patients who showed acquired resistance, 4 tumors had T790M second mutation. No *MET* amplification was detected in any of these tumors.

Histology and immunohistochemistry. Immunohistochemical staining was carried out on formalin-fixed, paraffin-embedded tissue sections of lung adenocarcinoma specimens. Sections of 5- μ m thickness were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. For detection of *MET*, sections were retrieved by microwave treatment for 10 min, with immersion in antigen retrieval medium (Immunosaver, Nissin EM Ltd.). After blocking the endogenous peroxidase activity with 3% aqueous H₂O₂ solution for 10 min, the sections were treated with 5% normal horse serum. The sections were then reacted with primary antibodies (anti-HGF antibody, 5 μ g/mL; anti-total *MET* antibody, 1:100 dilution) at 4°C overnight. The primary antibodies used were rabbit polyclonal anti-HGF antibody (24) and rabbit polyclonal anti-*MET* antibody (IBL Ltd.). After washing with PBS, the sections were treated with biotin-conjugated anti-rabbit IgG (1:200 dilution) for 30 min at room temperature and allowed to react for 30 min with avidin-biotin-peroxidase complex (ABC) using a Vectastain ABC kit (Vector Laboratories). The DAB (3,3'-diaminobenzidine tetrahydrochloride) Liquid System (DakoCytomation) was used to detect

immunostaining. Omission of primary antibodies served as negative control.

Evaluation of immunohistochemical results. Immunoreactivities were evaluated as follows, in accordance with the method of Nakamura and colleagues (27). Immunoreactivities for HGF were evaluated as either high or low, with a cutoff value of 50% of positively stained cancer cells. Immunoreactivities for *MET* protein were evaluated as follows: -, complete absence of staining or only focal weak staining; 1+, weak to moderate staining in <40% of cancer cells; 2+, weak to moderate staining in at least 40% of cancer cells; 3+, strong staining in at least 10% of cancer cells, among the specimens with weak to moderate staining in at least 40% of cancer cells. Weak to moderate staining was defined as staining similar to or weaker than the staining of normal bronchial epithelium; strong staining was defined as staining clearly more than intense than that of normal bronchial epithelium. Immunoreactivity was evaluated independently by two investigators (S.Y. and W.W.). Cases with discrepancies were jointly reevaluated and a consensus was reached.

Statistical analysis. The statistical significance of differences was analyzed by one-way ANOVA. In cases in which the *P* values for the overall comparisons were <0.05, post hoc pairwise comparisons were done with the Newman-Keuls Multiple comparison test. Statistical analyses were done using GraphPad Prism ver. 4.01 (GraphPad Software, Inc.).

Results

HGF induces gefitinib resistance of lung adenocarcinoma cells with *EGFR*-activating mutations. We first confirmed the presence of activating mutations in *EGFR* in human lung adenocarcinoma cell lines by direct sequencing. PC-9 and HCC827 had

deletions in exon 19 (Supplementary Fig. S1). Consistent with a previous report (12), neither PC-9 nor HCC827 cells had *MET* amplification or T790M mutation in exon 20 of *EGFR* (Supplementary Fig. S2 and data not shown). Both PC-9 and HCC827 cells were highly sensitive to gefitinib. HGF alone did not affect proliferation of PC-9 cells, but it slightly stimulated the proliferation of HCC827 cells. Under these experimental conditions, HGF induced resistance of PC-9 and HCC827 cells to gefitinib in a dose-dependent manner (Fig. 1A). The effect of HGF was abrogated by pretreatment of HGF with anti-HGF neutralizing antibody but not control IgG (Fig. 1B). In parallel experiments, HGF did not induce resistance of PC-9 or HCC827 cells to conventional chemotherapeutic drugs such as carboplatin, gemcitabine, docetaxel, or pemetrexed (Supplementary Fig. S3). We also explored the effects of other cytokines reported to be related to gefitinib sensitivity. EGF (28), TGF- α (29), and IGF-I (30) tended to promote resistance of PC-9 cells to gefitinib, but their effects were marginal (Fig. 1C). Gefitinib has been shown to induce apoptosis of PC-9 and HCC827 cells (12, 20). Whereas HGF alone had no effect on induction of apoptosis of PC-9 or HCC827 cells, it prevented gefitinib-induced apoptosis of these cells (Supplementary Fig. S4).

HGF-induced gefitinib resistance is mediated by restoring phosphorylation of Akt, but not EGFR or ErbB3. *MET* amplification has been shown to restore the PI3K/Akt pathway mediated by ErbB3 transactivation and thereby induces acquired resistance to gefitinib (12). To investigate the molecular mechanism by which HGF induces gefitinib resistance, we examined the phosphorylation of MET, EGFR, ErbB3, and the PI3K/Akt pathway by Western blotting. PC-9 and HCC827 cells expressed EGFR, ErbB3, and MET proteins, and these molecules were phosphorylated at various levels. These receptors and downstream Akt and ERK1/2 were also phosphorylated. Gefitinib inhibited the phosphorylation of EGFR, ErbB3, Akt, and ERK1/2, but not MET. HGF alone did not affect phosphorylation of EGFR or ErbB3, whereas it stimulated phosphorylation of MET, Akt, and ERK1/2. Importantly, even in the presence of gefitinib, HGF restored phosphorylation of Akt and ERK1/2, but not EGFR or ErbB3 (Fig. 2A).

To investigate the mechanism by which PI3K/Akt phosphorylation is restored by HGF, we immunoprecipitated the p85 regulatory subunit of PI3K and examined the association with MET. In PC-9 and HCC827 cells, ErbB3 was associated with p85, and this association was inhibited by gefitinib. HGF did not affect the association of ErbB3 with p85 irrespective of the presence of gefitinib. MET was constitutively associated with p85, and this association was unaffected by gefitinib and/or HGF. MET was not associated with ErbB3, and neither gefitinib nor HGF induced the association of these two molecules. On the other hand, MET was constitutively associated with EGFR, and this association was disrupted by gefitinib, suggesting that the association between EGFR and MET may be regulated by EGFR phosphorylation status. However, HGF failed to restore the association inhibited by gefitinib (Fig. 2B). These results suggest that HGF-induced resistance is mediated by the MET/PI3K signaling axis but not by the ErbB3/PI3K axis.

Specific down-regulation of MET, but not ErbB3, reversed gefitinib resistance and phosphorylation of Akt induced by HGF. We used RNAi technique to confirm this possibility. Down-regulation of ErbB3 expression by an *ErbB3*-specific siRNA did not affect either HGF-induced resistance to gefitinib or phosphorylation of Akt and ERK1/2 restored by HGF in PC-9 or HCC827 cells. In contrast, down-regulation of MET expression by *MET*-specific siRNA canceled HGF-induced resistance to gefitinib, as well as phosphorylation of Akt and ERK1/2 restored by HGF (Fig. 3; Supplementary Fig. S5). These results indicate that HGF induces gefitinib resistance by restoring the Akt signaling pathway via MET phosphorylation independently of ErbB3.

High immunoreactivity for HGF in cancer cells of intrinsic resistant tumors and those with acquired resistance without T790M second mutation or MET amplification. To assess the clinical relevance of this resistance mechanism, we examined whether HGF as well as MET could be detected in tumors with *EGFR*-activating mutations obtained from lung adenocarcinoma patients who showed intrinsic resistance (3 cases) or acquired resistance (5 cases) to gefitinib, and a comparison was also made

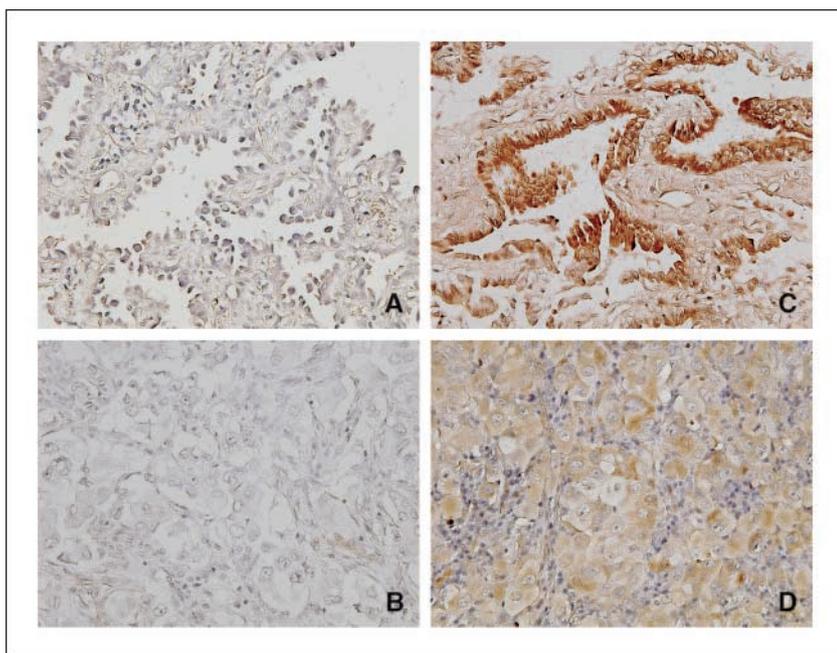


Figure 4. High immunoreactivity for HGF in cancer cells of intrinsic resistant tumors and those with acquired resistance without T790M second mutation or *MET* amplification. Immunohistochemical HGF staining of tumor obtained before gefitinib treatment from patient 4 who showed partial response (A), tumor obtained from patient 4 after acquired resistance by T790M second mutation (B), tumor obtained before gefitinib treatment from patient 1 who showed intrinsic resistance (C), and tumor obtained from patient 7 after acquired resistance without T790M second mutation or *MET* amplification (D). Magnification, $\times 200$.

with tumors from responders to gefitinib treatment (8 cases). We analyzed 20 tumors from 16 patients (Table 1). For 3 of 5 patients who developed acquired resistance (i.e., who showed partial response to gefitinib treatment but showed signs of tumor regrowth while still receiving gefitinib), we were able to obtain pairs of tumor specimens before treatment and after the development of acquired resistance to gefitinib. Of 6 acquired resistance tumors from 5 patients, 4 tumors had T790M second mutation. No *MET* amplification was detected in any of these tumors. HGF and MET immunoreactivity were detected predominantly in cancer cells, as reported by other groups (27). Immunoreactivity for MET protein was detected diffusely in all of 20 tumors (Table 1). Importantly, high levels of immunoreactivity for HGF were detected in cancer cells of 3 of 3 intrinsic resistant tumors and in 1 (patient 7) of 2 tumors with acquired resistance without T790M second mutation or *MET* amplification, whereas only a low level of HGF immunoreactivity was detected in cancer cells of responders' tumors (except patient 16) or tumors with acquired resistance with T790M second mutation (Fig. 4; Table 1). These results strongly suggest that HGF produced by cancer cells is involved in resistance to gefitinib in lung adenocarcinomas harboring *EGFR*-activating mutations.

HGF derived from tumor cells induces gefitinib resistance of lung adenocarcinoma cells with *EGFR*-activating mutation. To further confirm this, we sought to transfect *HGF* gene into lung adenocarcinoma cells. PC-9 and HCC827 cells, which are sensitive to gefitinib, secreted undetectable levels of HGF (Fig. 5A). PC-9 cells transfected with human *HGF* gene (PC-9/HGF), but not vector alone (PC-9/mock), secreted a high level of HGF, and such cells became highly resistant to gefitinib (Fig. 5A and B), but the resistance was abrogated by treatment with anti-HGF neutralizing antibody (Fig. 5C). These results indicate that tumor cell-derived HGF could induce gefitinib resistance of lung adenocarcinoma cells with *EGFR*-activating mutations.

Discussion

Both intrinsic and acquired resistance to *EGFR* tyrosine kinase inhibitors are a serious problem in the treatment of lung cancer harboring *EGFR*-activating mutations. In the present study, we found that HGF induces gefitinib resistance in lung adenocarcinoma cells with *EGFR*-activating mutations. Despite the limited number of clinical specimens examined, high levels of HGF immunoreactivity were detected in tumors from patients who showed intrinsic resistance (3 of 3 cases) or acquired resistance to gefitinib with no relation to T790M second mutation or *MET* amplification (1 of 2 cases). These findings indicate that HGF-mediated MET activation is one of the novel mechanisms contributing to intrinsic or acquired gefitinib resistance of lung adenocarcinomas harboring *EGFR*-activating mutations.

Engelman and colleagues reported that *EGFR* is linked to PI3K/Akt signaling via ErbB3 in NSCLC cell lines that are sensitive to *EGFR* tyrosine kinase inhibitors (31) and that *MET* amplification leads to gefitinib resistance by binding and activation of ErbB3 (12). In the present study, inhibition of ErbB3 with *ErbB3* siRNA tended to reduce the phospho-Akt level and proliferation of both PC-9 and HCC827 cells (Fig. 3C), confirming the importance of ErbB3 in lung adenocarcinoma cells with *EGFR*-activating mutations. However, HGF treatment completely restored PI3K/Akt signaling independently of ErbB3. The results of the present study clearly indicated that HGF induced gefitinib resistance without the involvement of ErbB3.

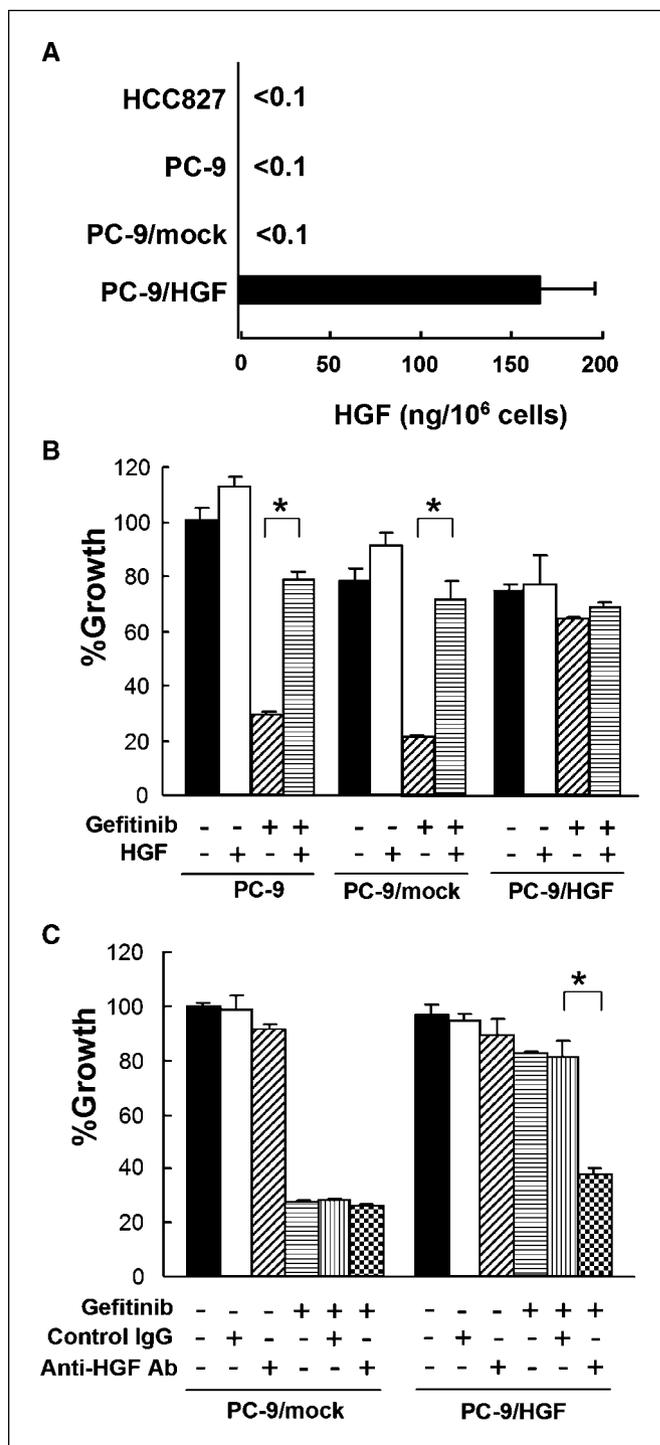


Figure 5. HGF derived from tumor cells induces gefitinib resistance of lung adenocarcinoma cells with *EGFR*-activating mutation. **A**, HGF production by PC-9, HCC827, mock-transfected PC-9 (PC-9/mock), and HGF gene-transfected PC-9 (PC-9/HGF) cells. The cells were incubated in medium for 48 h and culture supernatants were harvested. The level of HGF in the supernatants was determined by ELISA. **B**, HGF gene transfection into PC-9 cells resulted in resistance to gefitinib. Human HGF cDNA was introduced into PC-9 cells (PC-9/HGF). Vector alone served as a mock control (PC-9/mock). The cells were incubated with or without gefitinib (300 nmol/L) in the presence or absence of HGF (20 ng/mL). Cell growth was measured with a Cell Counting Kit-8. **C**, anti-HGF antibody abrogated the resistance of HGF gene-transfected PC-9 cells to gefitinib. PC-9/mock and PC-9/HGF cells were cultured in the presence or absence of control IgG (2 μ g/mL) or anti-HGF antibody (2 μ g/mL) with or without gefitinib (300 nmol/L). The growth of cells was measured with an MTT assay. *, $P < 0.001$ (one-way ANOVA).

MET can be activated by various mechanisms, including binding by its ligand, HGF (18), amplification of *MET* gene itself (12), and transactivation by other factors such as EGFR (21). We observed that although human lung adenocarcinoma PC-9 and HCC827 cells did not show *MET* amplification or produce detectable levels of HGF, MET was phosphorylated at an appreciable level, suggesting transactivation of MET in these cells. Because PC-9 and HCC827 are highly sensitive to gefitinib, MET phosphorylation induced by transactivation may be insufficient to protect cells from apoptosis by gefitinib, and thus HGF-induced activation may be necessary in the presence of gefitinib for survival of lung adenocarcinoma cells harboring *EGFR*-activating mutations.

HGF is known to be produced by various stromal component cells as well as tumor cells. Histologic analyses of clinical specimens indicated that HGF is expressed by a significant population of cancer cells, including NSCLC, although HGF is not expressed in a large population of cancer cell lines of many different types *in vitro* (18). In accordance with these reports, we found that whereas the human lung adenocarcinoma cell lines PC-9 and HCC827 did not secrete detectable levels of HGF *in vitro*, a high level of HGF was detected predominantly in cancer cells in clinical specimens (32). Therefore, it will be important to clarify the regulatory mechanism of HGF expression in lung cancer cells. Recently, Chen and colleagues (33) reported that cigarette smoking induced overexpression of HGF in lung cancer cells. Nicotine was shown to stimulate HGF production in several NSCLC cell lines. In addition, overexpression of HGF in the tumors was correlated with history of cigarette smoking in lung cancer patients. It is well known that nonsmokers with lung cancer show a more favorable response to gefitinib than smokers (34). Therefore, HGF induced by cigarette smoking may be involved in gefitinib resistance, at least in a specific population of lung cancer patients.

Gene alternation of the target itself or its associated molecules, including secondary mutations (*EGFR*, *KIT*, *PDGFR*, or *BCR/ABL*) and gene amplification (*MET*, *KIT*, or *BCR/ABL*), were identified as mechanisms of acquired resistance to kinase inhibitors in lung cancer, chronic myeloid leukemia, and gastrointestinal stromal tumors (10–13, 35–38). Our findings provide the new concept that receptor (MET) activation by natural ligand (HGF) without gene mutation or amplification could mediate survival signaling independently of the target or associated molecules (EGFR and ErbB3) against tyrosine kinase inhibitor (gefitinib). Moreover, HGF-MET signaling is a common pathway that has been reported in a number of cancers, including lung, breast, colorectal, prostate, pancreatic, head and neck, gastric, hepatocellular, ovarian, renal, glioma, melanoma, and a number of sarcomas. Therefore, it will be valuable to investigate the involvement of HGF-MET-mediated signaling in acquired resistance to kinase inhibitors in other types of tumors.

The mechanisms responsible for intrinsic resistance to gefitinib in lung cancer harboring *EGFR*-activating mutations are poorly understood, although several mechanisms of acquired resistance have been reported (8). To our knowledge, this is the first study to clarify the mechanism of intrinsic resistance to gefitinib in lung cancer harboring *EGFR*-activating mutations. Interestingly, all of three intrinsically resistant lung cancers examined showed high levels of HGF immunoreactivity, indicating that HGF plays an important role in intrinsic resistance to gefitinib. In clinical trials, 25% to 30% of lung cancer patients with *EGFR*-activating mutations show intrinsic resistance. Thus, our findings suggest the usefulness of combined therapy with EGFR tyrosine kinase inhibitor and HGF-MET antagonists in these patients. Moreover, because HGF also contributes to acquired resistance to gefitinib, combined therapy may also be applicable for lung cancer showing acquired resistance without T790M second mutation or *MET* amplification.

There are several possible strategies to inhibit HGF-MET signaling, including anti-HGF neutralizing antibody, HGF antagonist (NK4), and MET tyrosine kinase inhibitors (SU11274, PHA-665752; refs. 18, 39). We found that anti-HGF antibody as well as NK4 (data not shown) efficiently abrogated gefitinib resistance induced by HGF under our *in vitro* experimental conditions. With regard to EGF-EGFR targeting, both receptor tyrosine kinase inhibitors and monoclonal antibodies have been developed for clinical use. On the other hand, in the case of vascular endothelial growth factor (VEGF)-VEGF receptor targeting, treatment with monoclonal antibody (bevacizumab) has been more successful than with receptor tyrosine kinase inhibitors. Thus, further studies are required to determine which strategy is the most successful for HGF-MET targeting and *in vivo* experiments are currently ongoing in our laboratory.

In conclusion, we show that HGF-mediated MET activation is a novel mechanism of gefitinib resistance in lung adenocarcinoma with *EGFR*-activating mutations. Therefore, inhibition of HGF-MET signaling may be a considerable strategy for more successful treatment with gefitinib.

Disclosure of Potential Conflicts of Interest

T. Mitsudomi: speakers bureau/honoraria and expert testimony, AstraZeneca.

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