Hepatocyte Growth Factor Induces Resistance to Anti-Epidermal Growth Factor Receptor Antibody in Lung Cancer

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Introduction: Epidermal growth factor receptor (EGFR) is an attractive drug target in lung cancer, with several anti-EGFR antibodies and small-molecule inhibitors showing efficacy in lung cancer patients. Patients, however, may develop resistance to EGFR inhibitors. We demonstrated previously that hepatocyte growth factor (HGF) induced resistance to EGFR tyrosine kinase inhibitors in lung cancers harboring EGFR mutations. We therefore determined whether HGF could induce resistance to the anti-EGFR antibody (EGFR Ab) cetuximab in lung cancer cells, regardless of EGFR gene status.

Methods: Cetuximab sensitivity and signal transduction in lung cancer cells were examined in the presence or absence of HGF, HGF-producing fibroblasts, and cells transfected with the HGF gene in vitro and in vivo.

Results: HGF induced resistance to cetuximab in H292 (EGFR wild) and Ma-1(EGFR mutant) cells. Western blotting showed that HGF-induced resistance was mediated by the Met/Gab1/Akt signaling pathway. Resistance of H292 and Ma-1 cells to cetuximab was also induced by coculture with lung fibroblasts producing high levels of HGF and by cells stably transfected with the HGF gene. This resistance was abrogated by treatment with anti-HGF neutralizing antibody.

Conclusions: HGF-mediated resistance is a novel mechanism of resistance to EGFR Ab in lung cancers, with fibroblast-derived HGF inducing cetuximab resistance in H292 tumors in vivo. The involvement of HGF-Met-mediated signaling should be assessed in acquired resistance to EGFR Ab in lung cancer, regardless of EGFR gene status.

Key Words: Hepatocyte growth factor, Lung cancer, Anti-EGFR antibody, Drug resistance.

Lung cancer is one of the most prevalent malignancies and the leading cause of malignancy-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for ~80% of cases of lung cancers. The median survival of patients with metastatic NSCLC is 8 to 10 months, even if treated with the most active combination of conventional chemotherapeutic agents.1,2

Epidermal growth factor receptor (EGFR) is a cell surface receptor tyrosine kinase that transduces growth signals through dimerization with the HER of family receptors. As EGFR is highly expressed in a variety of human tumors,3,4 it is regarded as an attractive target for the development of therapeutic agents. EGFR overexpression has been observed in 40 to 80% of NSCLCs and is associated with tumor development.5,6 Recent clinical trials have demonstrated that NSCLC patients with EGFR mutants had good outcomes with the EGFR tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib.7–9 Overall, however, EGFR-TKIs, with or without standard chemotherapy, have not yielded good outcomes in patients with NSCLC.10,11

Cetuximab, a chimeric human-mouse anti-EGFR IgG1 monoclonal antibody (EGFR Ab), has shown clinical success in patients with colorectal and head and neck cancers.12–15 Because EGFR mutations in NSCLCs were associated with sensitivity to gefitinib but not to cetuximab,16 cetuximab was hypothesized to be effective in NSCLC patients overall, regardless of EGFR mutations. Clinical trials showed that the addition of cetuximab to platinum-based chemotherapy resulted in significantly longer survival time in patients with EGFR-positive advanced NSCLC than chemotherapy alone and that first-cycle skin rash may be a surrogate clinical marker for the efficacy of cetuximab.17,18 Nevertheless, other phase III clinical trials demonstrated that the addition of cetuximab to chemotherapy did not significantly improve clinical outcomes in patients with advanced NSCLC and that efficacy parameters was not correlated with K-Ras mutation status or with any EGFR-related biomarker, mutation, protein expression, or gene copy number.19,20 Thus, treatment with cetuximab may have only limited effects in patients with EGFR-positive NSCLC, and the identification of resistance biomarkers to cetuximab may improve the efficacy of cetuximab in these patients.

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We previously showed that hepatocyte growth factor (HGF) induces resistance to reversible or irreversible EGFR-TKIs by activating Met, restoring the phosphorylation of downstream MAPK-extracellular signal-regulated kinase1/2 (ERK1/2) and PI3K-Akt pathways in lung cancers with mutant EGFR.\textsuperscript{21,22} We sought to determine whether HGF could induce resistance to EGFR Ab in lung cancer cells, regardless of the presence or absence of EGFR mutations.

**MATERIALS AND METHODS**

**Cell Lines and Reagents**

The H292 human lung adenocarcinoma cell line, which expresses wild type EGFR, was purchased from the American Type Culture Collection (Manassas, VA). The Ma-1 human lung adenocarcinoma cell line with an EGFR-activating mutation (deletion in exon 19) was kindly provided by Dr. Eiji Shimizu (Tottori University, Yonago, Japan). The MRC-5 lung embryonic fibroblast cell line was obtained from RIKEN Cell Bank. H292 and Ma-1 cells were cultured in RPMI 1640 medium and MRC-5 (P 25–30) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM), each supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (50 µg/mL), in a humidified CO2 incubator at 37°C.

Cetuximab was obtained from Merck Serono (Darmstadt, Germany) and erlotinib hydrochloride from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). BIBW2992, BEZ235, and AZD6244 were purchased from Selleck Chemicals (Houston, TX). SU11274 was purchased from Calbiochem (San Diego, CA). Recombinant EGF, Amphiregulin, PDGF-AA, and IGF-1 were obtained from R&D Systems (Minneapolis, MN). Recombinant HGF was prepared as described.\textsuperscript{23–25} Its purity, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein staining, was 96.4% and >98%, respectively. Goat anti-human HGF neutralizing antibody and control goat IgG were purchased from R&D Systems.

**Cell Proliferation Assay**

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method.\textsuperscript{26} Tumor cells at 80% confluence were harvested, seeded at 2 × 10^5 cells per well in 96-well plates and incubated in RPMI 1640 for 24 hours. Several concentrations of cetuximab, erlotinib, BIBW2992, SU11274, BEZ235, AZD6244, goat anti-human HGF neutralizing antibody, control goat IgG, and/or HGF, EGF, Amphiregulin, PDGF-AA, and IGF-1 were added to each well, and incubation was continued for a further 72 hours. A 50 µL aliquot of MTT solution (2 mg/mL; Sigma, St. Louis, MO) was added to each well, followed by incubation for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 µL of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage growth is shown relative to untreated controls. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

**Antibodies and Western Blotting**

Tumor cells were incubated in 10 mL of RPMI 1640 with 10% FBS in the presence or absence of cetuximab (1 µg/mL) for 1 hour, and then in the presence or absence of cetuximab (1 µg/mL) and/or HGF (20 ng/mL) for 15 minutes. The cells were washed twice with phosphate-buffered saline (PBS), harvested in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and flash-frozen on dry ice. After allowing the cells to thaw, the lysates were collected with a rubber scraper, sonicated, and centrifuged at 14000 × g for 20 minutes at 4°C. The total protein concentration of each lysate was measured using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL).

For Western blotting assays, cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) and the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature and incubated at 4°C overnight with antibodies to Met (25H2), phospho-Met (Y1234/Y1235) (3D7), Gab1, phospho-Gab1 (Tyr627), PTEN (138G6), ErbB3, phospho-ErbB3 Akt, phospho-Akt (Ser473), or β-actin (13E5; each at 1:1000 dilution; Cell Signaling Technology, Danvers, MA), or with antibodies to human EGFR (1 µg/mL), human/mouse/rat ERK1/ERK2 (0.2 µg/mL), or phospho-ERK1/ERK2 (T202/Y204) (0.1 µg/mL) (R&D Systems). After washing three times, the membranes were incubated for 1 hour at room temperature with secondary Ab (horseradish peroxidase-conjugated species-specific Ab). Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce). Each experiment was performed at least three times independently.

**RNA Interference**

Duplexed Stealth RNAi (Invitrogen), targeted against nucleotides 1014-1038 of the EGF receptor (5’-CGGATAGTGTATTGGTGATTTAAA-3’), and Stealth RNAi Negative Control Low GC Duplex 3 (Invitrogen) were used for RNA interference (RNAi) assay. Briefly, aliquots of 1 × 10^5 cells in 2 mL of antibiotic-free medium were plated on six-well plates and incubated at 37°C for 24 hours. The cells were then transfected with siRNA (250 pmol) or scramble RNA using Lipofectamine 2000 (5 µL) in accordance with the manufacturer’s instructions (Invitrogen). After 24 hours, the cells were washed twice with PBS and incubated with or without recombinant human HGF (20 ng/mL) for an additional 15 minutes in antibiotic-containing medium. These cells were then used for Western blotting assay as described above. EGF receptor knockdown was confirmed by Western blotting. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

**HGF Production in Cell Culture Supernatant**

Cells (2 × 10^5) were cultured in 2 mL of RPMI 1640 or DMEM with 10% FBS for 24 hours, washed with PBS and incubated for 48 hours with or without cetuximab (1 µg/mL) in
RPMI 1640 or DMEM with 10% FBS. The culture media were harvested and centrifuged, and the supernatants were stored at −70°C until analysis. HGF was measured by enzyme-linked immunosorbent assay (Immunis HGF EIA; B-Bridge International, Mountain View, CA; limit of detection, 0.1 ng/mL), according to the manufacturer’s recommendations. All samples were assayed in triplicate. Color intensity was measured at 450 nm with a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

Coculture of Lung Cancer Cells with Fibroblasts

Cells were cocultured in Transwell chambers separated by filters of 8 μm pore size. Tumor cells (8 × 10^3 cells/700 μL) with or without cetuximab (1 μg/mL) were placed in the lower chambers, and the human fibroblast MRC-5 (1 × 10^4 cells/300 μL) cell lines, with or without 2 hours of pretreatment with control IgG (2 μg/mL) or anti-HGF neutralizing antibody (2 μg/mL), were placed in the upper chambers. After coculture for 72 hours, the upper chambers were removed, 200 μL of MTT solution (2 mg/mL; Sigma) was added, and cell viability was determined as in A. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

* $p < 0.05$ (Mann-Whitney U test).

FIGURE 1. HGF induces resistance to anti-EGFR antibody in lung adenocarcinoma cells regardless of EGFR gene status. A, H292 cells, containing wild-type EGFR, and Ma-1 cells, containing EGFR with an exon19 deletion, were incubated with the anti-EGFR antibody cetuximab, at various concentrations and/or with 0, 2, and 20 ng/mL HGF for 72 hours, with cell growth determined by the MTT assay. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. B, HGF (20 ng/mL) was pretreated with control IgG (2 μg/mL) or anti-HGF antibody (2 μg/mL) at 37°C for 1 hour. The resultant solutions were added to cultures of H292 and Ma-1 cells in the presence or absence of cetuximab (1 μg/mL), and cell growth was determined as in A. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. *$p < 0.05$ (Mann-Whitney U test). C, HGF (20 ng/mL) was pretreated with SU11274 (1 μM), BEZ235 (0.001 μM), or AZD6244 (1 μM) at 37°C for 1 hour. The resultant solutions were added to cultures of H292 and Ma-1 cells in the presence or absence of cetuximab (1 μg/mL), and cell growth was determined as in A. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.
added to each well, and the cells were incubated for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 400 μL of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage growth is shown relative to untreated controls. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

**HGF Gene Transfection**

One day before transfection, aliquots of 1 × 10^5 Ma-1 cells in 1 mL of antibiotic-free medium were plated on six-well plates. The full-length HGF cDNA cloned into a BCMGSneo expression vector (25-A) was transfected using Lipofectamine 2000 in accordance with the manufacturer’s instructions. After 24 hours, the cells were washed with PBS and incubated for an additional 72 hours in antibiotic-containing medium. The cells were selected in G418 sulfate (Calbiochem, La Jolla, CA). After the limiting dilution, HGF-producing cells, Ma-1/HGF, were established. HGF production by Ma-1/HGF was confirmed by enzyme-linked immunosorbent assay.

**Xenograft Studies in SCID Mice**

Suspensions of H292 cells (5 × 10^6) with or without MRC-5 cells (5 × 10^6) were injected subcutaneously into the backs of 5-week-old female SCID mice (Japan Clea). After 4 days (tumors diameter > 4 mm), mice were randomly allocated into groups of six animals to receive intraperitoneal cetuximab (4 mg/kg, twice/wk) or vehicle. Tumor size was measured with digital calipers, and tumor volume was calculated as 0.5 × length × (width)^2. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval no. AP-081088).

**Statistical Analysis**

The statistical significance of differences was analyzed by one-way analysis of variance. All statistical analyses were performed using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA). p < 0.05 was considered statistically significant.

**RESULTS**

**HGF Induces Resistance to Anti-EGFR Antibody in Lung Cancer Cells Regardless of EGFR Gene Status**

We first tested the effects of the anti-EGFR Ab cetuximab against H292 cells, an NSCLC cell line with wild type in EGFR, and Ma-1 cells, an NSCLC cell line harboring the exon 19 deletion in the EGFR gene, resulting in its constitutive activa-

**FIGURE 2.** HGF was most potent in induction of cetuximab resistance of lung adenocarcinoma cells. H292 and Ma-1 cells were incubated with or without cetuximab (1 μg/mL) and/or 20 ng/mL of HGF, EGF, Amphiregulin, PDGF-AA, or IGF-I. Cell growth was determined after 72 hours of treatment. The percentage of growth is shown relative to untreated controls. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

**FIGURE 3.** HGF induces resistance of lung adenocarcinoma cells to cetuximab by restoring phosphorylation of Akt and ERK1/2. Tumor cells were treated with or without cetuximab (1 μg/mL) for 1 hour and incubated in the presence or absence of cetuximab (1 μg/mL) and/or HGF (20 ng/mL) for 15 minutes. The cells were lysed, and the indicated proteins were detected by immunoblotting. The results shown are representative of three independent experiments.
tion. We found that both cell lines were sensitive to cetuximab (Figure 1A). Although HGF (20 ng/mL) alone had no effect on the growth of these lung cancer cells, HGF (at 2 and 20 ng/mL) markedly and dose-dependently induced the resistance of both H292 and Ma-1 cells to cetuximab (Figure 1A). Moreover, HGF (20 ng/mL) also significantly enhanced the resistance of H292 cells to the reversible EGFR-TKI erlotinib and the irreversible EGF-TKI BIBW2992 (Supplemental Figure 1A, B, http://links.lww.com/JTO/A195). These effects of HGF were abrogated by an anti-HGF neutralizing antibody (2 μg/mL) but not by control IgG (2 μg/mL) (Figure 1B). In addition, these effects of HGF were abrogated by Met-TKI SU11274 (1 μM) and phosphatidylinositol 3-kinase (PI3K) inhibitor BEZ235 (0.001 μM) but not by MEK inhibitor AZD6244 (1 μM) (Figure 1C). We also examined the effects of other receptor’s ligands, including EGF, Amphiregulin, PDGF-AA, and IGF-I. Although EGF and IGF-1 tend to induce resistance of H292 and Ma-1 cells to cetuximab, HGF showed the strongest effect in induction of resistance to cetuximab in H292 and Ma-1 cells (Figure 2).

HGF-Induced Cetuximab Resistance Is Mediated by the Restoration of Gab1, Akt, and ERK1/2 Phosphorylation

To investigate the molecular mechanism by which HGF induces resistance to cetuximab in lung cancer cells, we assessed the level of expression and phosphorylation of Met protein and downstream molecules by Western blotting. We found that both H292 and Ma-1 cells expressed Met, PTEN, Gab1, and ErbB3 proteins, with Met, Gab1, and ErbB3 being phosphorylated to at least some extent. We also found that Akt and ERK1/2, molecules downstream to these receptors, were phosphorylated. Cetuximab inhibited the phosphorylation of Akt and ERK1/2 but not of ErbB3, Met, and Gab1. HGF alone did not affect the phosphorylation of ErbB3, but it stimulated the phosphorylation of Met and Gab1. Even in the presence of cetuximab, HGF restored the phosphorylation of Met, Gab1, Akt, and ERK1/2 (Figure 3), indicating that HGF-induced resistance to cetuximab is mediated by Met/Gab1/Akt signaling.

Specific Down-Regulation of EGFR Also Induces Phosphorylation of Gab1, Akt, and ERK1/2 Induced by HGF

Using EGFR-specific siRNA to knockdown the expression of EGFR in H292 cells, we found that the phosphorylation of Akt and ERK1/2 was also inhibited. However, down-regulation of EGFR expression by EGFR-specific siRNA had no effect on the HGF-induced phosphorylation of Met, Gab1, Akt, and ERK1/2 (Supplemental Figure 1C), indicating that HGF also induces resistance to EGFR knockdown by restoring the Akt and ERK1/2 signaling pathway.

FIGURE 4. Fibroblast-derived HGF induces resistance to cetuximab in lung cancer cells regardless of EGFR gene status. A, HGF production by lung cancer cell lines (H292, Ma-1) and human embryonic lung fibroblasts (MRC-5). Cells were incubated for 48 hours with or without cetuximab (1 μg/mL), the culture supernatants were harvested, and their concentrations of HGF were determined by enzyme-linked immunosorbent assay (ELISA). All samples were assayed in triplicate. B, Coculture with fibroblasts induced cetuximab resistance in lung cancer cells. Lung cancer H292 and Ma-1 cell lines were cocultured with MRC-5 cells and anti-HGF neutralizing antibody (2 μg/mL) in the presence or absence of cetuximab (1 μg/mL) for 72 hours, and lung cancer cell growth was determined after 72 hours by the MTT assay. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. *p < 0.05 (Mann-Whitney U test).
Fibroblast-Derived HGF Induces Cetuximab Resistance in Lung Cancer Cells

Because host microenvironments can have a profound effect on the chemosensitivity of cancers and because stromal fibroblasts are the major source of HGF,27 we assayed HGF production by human fibroblast cell lines. We found that the human embryonic lung-derived fibroblast cell line MRC-5 secreted high levels of HGF into the supernatant. In contrast, H292 and Ma-1 cells did not secrete detectable levels of HGF into the culture supernatant (Figure 4A). To further investigate whether the resistance of H292 and Ma-1 cells to cetuximab may be affected by crosstalk with stromal fibroblasts, we cocultured these lung cancer cells with MRC-5 cells using Transwell systems. We found that coculture of H292 and Ma-1 cells with MRC-5 cells did not significantly affect the proliferation of the former. In the presence of MRC-5 cells, however, H292 and Ma-1 cells became resistant to cetuximab, a resistance abrogated by treatment with anti-HGF neutralizing antibody (2 μg/mL) (Figure 4B). These results indicate that fibroblast-derived HGF could induce cetuximab resistance in lung cancer cells regardless of EGFR gene status.

Cell-Derived HGF Induces Cetuximab Resistance in Lung Cancer Cells

To determine whether cell-derived HGF induces cetuximab resistance in lung cancer cells, we established Ma-1/HGF cells, which stably express human HGF (Figure 4A). Unlike Ma-1 cells, Ma-1/HGF cells were resistant to cetuximab (Figure 5A), but this resistance was abrogated by treatment with anti-HGF neutralizing antibody (2 μg/mL) (Figure 5B). When we assayed the level of expression and phosphorylation status of Met protein and its downstream molecules by Western blotting, we found that Met, but not Gab1, was phosphorylated in Ma-1/HGF cells. We could not specify the reason for weak phosphorylation in Gab1 in Ma-1/HGF cells, compared with Ma-1 cells stimulated with HGF. Akt and ERK1/2, the molecules downstream of these receptors, were also phosphorylated. Addition of cetuximab to Ma-1/HGF cells had no effect on the phosphorylation of Akt and ERK1/2 (Figure 5C), indicating that cell-derived HGF could induce cetuximab resistance of lung cancer cells by means of the Akt and ERK1/2 signaling pathways.

![Figure 5](https://example.com/figure5.png)

**FIGURE 5.** Cell-derived HGF induces resistance to cetuximab in lung cancer cells through phosphorylation of Akt and ERK1/2. A, Ma-1 and Ma-1/HGF cells were incubated for 72 hours with increasing concentrations of cetuximab, and cell growth was determined by the MTT assay. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. B, Ma-1/HGF cells were incubated for 72 hours with or without cetuximab (1 μg/mL) and/or control IgG (2 μg/mL) in the presence or absence of anti-HGF neutralizing antibody (2 μg/mL), and cell growth was determined by the MTT assay. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. *p < 0.05 (Mann-Whitney U test). C, Ma-1 and Ma-1/HGF cells were incubated with or without cetuximab (1 μg/mL) for 1 hour and in the presence or absence of cetuximab (1 μg/mL) and/or HGF (20 ng/mL) for 15 minutes. The cells were lysed, and the indicated proteins were detected by immunoblotting. Results shown are representative of three independent experiments.
HGF Derived from Fibroblasts Induces In Vivo Cetuximab Resistance of Lung Cancer Cells with Wild-Type EGFR

To investigate whether the cetuximab sensitivity of lung cancer cells with wild-type EGFR could be affected by fibroblasts in vivo, we inoculated H292 cells, with or without MRC-5 cells, into SCID mice subcutaneously as described.28 The tumors in mice injected with H292 and MRC-5 cells grew slightly faster than those in mice injected with H292 cells alone. Cetuximab treatment, beginning on day 4, caused marked regression of tumors in mice injected with H292 cells alone. The same treatment prevented the enlargement of tumors in mice injected with H292 and MRC-5 cells, but it did not cause tumor regression, indicating that these tumors were resistant to cetuximab treatment in vivo (Figure 6). These results indicate that HGF, produced presumably by fibroblasts (MRC-5), induced in vivo cetuximab resistance in lung cancer cells with wild-type EGFR.

DISCUSSION

EGFR is an attractive target for treatment of various cancers including lung cancer. Several agents targeting EGFR have been developed, including anti-EGFR antibodies and small-molecule EGFR-TKIs. Most patients who show a dramatic response to initial treatment, however, acquire resistance to these agents after varying periods of time. To overcome this resistance to EGFR inhibitors, it is necessary to clarify its molecular mechanisms.

Among the mechanisms associated with resistance to reversible EGFR-TKIs in NSCLCs with EGFR-activating mutations are the T790M secondary mutation in EGFR and Met amplification.31 NSCLC resistance to the anti-EGFR antibody cetuximab has been associated with increased PTEN instability, Met amplification, and strong activation of Met.32,33

We focused on the role of HGF in NSCLC resistance to cetuximab, in cell lines with or without EGFR mutations. HGF was originally identified as a mitogenic protein for hepatocytes and has been shown to have pleiotropic biological activities.23 We and other researchers previously demonstrated that HGF induces resistance to reversible and irreversible EGFR-TKIs in lung cancer cells harboring EGFR-activating mutations by activating its receptor Met and the downstream Gab1/Akt pathway.21,22,34 In addition, HGF induced resistance to cetuximab in colorectal cancer cells through Met activation.35 Thus, HGF may be a candidate molecule for resistance to EGFR inhibitors in cancers, including colorectal and lung cancers.

We found that HGF induced resistance to cetuximab in lung cancer cells, both in vitro and in vivo. We also found that HGF acted by restoring the Met/Gab1/Akt pathway in these cells and that inhibitors of HGF, Met, and PI3K could overcome this resistance. Several HGF-Met targeting agents are being tested clinically. These compounds may be promising in overcoming resistance to cetuximab.

EGFR mutations have been shown to correlate with the efficacy of EGFR-TKIs in several clinical trials of patients with NSCLC. Anti-EGFR agents, including erlotinib and cetuximab,17 have shown particular efficacy in patients with wild-type EGFR. To our knowledge, this report is the first to show that HGF induced resistance to EGFR blocking, both with EGFR inhibitors and gene knockdown, in NSCLCs with EGFR wild type. These findings suggest that HGF induces resistance to EGFR inhibitors in NSCLC containing both wild-type and mutant EGFR. However, further studies are required to determine which type of resistance, primary and/or acquired, is involved in the HGF-triggered resistance to cetuximab in NSCLC.

Several factors have been reported to be biomarkers for the effectiveness of cetuximab in patients with colorectal cancer, including K-Ras mutation, levels expression of EGFR ligands, and absence of PTEN.37–39 In contrast, biomarkers predictive of the effects of cetuximab in patients with NSCLC have not yet been established. Moreover, the cetuximab is less effective in NSCLC than in colorectal cancer. Thus,
dependence on EGFR signaling for growth and survival and subsequent EGFR inhibition may differ in NSCLC and colorectal cancer, indicating a need for novel, tumor-specific approaches for the use of cetuximab in NSCLC.

Serum and plasma concentrations of HGF may predict the effects of EGFR-TKIs in NSCLC, regardless of EGFR gene status.40–42 We showed that resistance to cetuximab was also induced by crosstalk with HGF-producing fibroblast cell lines (paracrine model) and by stable transfection of an HGF gene (autocrine model). Therefore, HGF in the cancer microenvironment may be predictive of sensitivity to cetuximab in NSCLC. Further studies are needed to identify markers predictive of cetuximab benefit in NSCLC containing wild-type EGFR.

In summary, we have described a novel mechanism of resistance to cetuximab in lung cancer cells, irrespective of EGFR gene status. HGF induced resistance to cetuximab by activating the Gab1/Akt pathway through phosphorylation of Met. This resistance was also induced by transfecting cells with the HGF gene, resulting in an autocrine effect, and by coculture with HGF-producing fibroblasts, suggesting that microenvironments may be associated with resistance to cetuximab. Thus, regardless of EGFR gene status, the HGF-Met-triggering pathway may be of importance in lung cancer resistance to cetuximab.

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