Tumorigenesis and Neoplastic Progression

Dual Inhibition of Met Kinase and Angiogenesis to Overcome HGF-Induced EGFR-TKI Resistance in EGFR Mutant Lung Cancer

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Acquired resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) is a serious problem in the management of EGFR mutant lung cancer. We recently reported that hepatocyte growth factor (HGF) induces resistance to EGFR-TKIs by activating the Met/PI3K pathway. HGF is also known to induce angiogenesis in cooperation with vascular endothelial growth factor (VEGF), which is an important therapeutic target in lung cancer. Therefore, we hypothesized that dual inhibition of HGF and VEGF may be therapeutically useful for controlling HGF-induced EGFR-TKI–resistant lung cancer. We found that a dual Met/VEGF receptor 2 kinase inhibitor, E7050, circumvented HGF-induced EGFR-TKI resistance in EGFR mutant lung cancer cell lines by inhibiting the Met/Gab1/PI3K/Akt pathway in vitro. HGF stimulated VEGF production by activation of the Met/Gab1 signaling pathway in EGFR mutant lung cancer cell lines, and E7050 showed an inhibitory effect. In a xenograft model, tumors produced by HGF-transfected Ma-1 (Ma-1/HGF) cells were more angiogenic than vector control tumors and showed resistance to gefitinib. E7050 alone inhibited angiogenesis and retarded growth of Ma-1/HGF tumors. E7050 combined with gefitinib induced marked regression of tumor growth. Moreover, dual inhibition of HGF and VEGF by neutralizing antibodies combined with gefitinib also markedly regressed tumor growth. These results indicate the therapeutic rationale of dual targeting of HGF-Met and VEGF–VEGF receptor 2 for overcoming HGF-induced EGFR-TKI resistance in EGFR mutant lung cancer. (Am J Pathol 2012, 181:1034–1043; http://dx.doi.org/10.1016/j.ajpath.2012.05.023)
70% to 80% and significantly longer progression-free survival compared with standard first-line cytotoxic chemotherapy in EGFR mutant lung cancer.\(^5,6\) Patients almost always develop acquired resistance to EGFR-TKIs after varying periods.\(^7\) In addition, 20% to 30% of patients with EGFR-activating mutations exhibit intrinsic resistance to EGFR-TKIs.\(^7\) Therefore, intrinsic and acquired resistance to EGFR-TKIs is a major problem in the management of EGFR mutant lung cancer.

Recent studies documented three clinically relevant mechanisms—EGFR T790M secondary mutation (T790M secondary mutation),\(^8,9\) Met gene amplification,\(^10\) and hepatocyte growth factor (HGF) overexpression\(^11,12\) that induce acquired resistance to EGFR-TKIs in EGFR mutant lung cancer. Of these mechanisms, HGF overexpression is involved in not only acquired resistance but also intrinsic resistance. Moreover, recent studies indicated at least three important roles of HGF in EGFR-TKI resistance in EGFR mutant lung cancer. First, HGF induces resistance to the reversible EGFR-TKIs gefitinib and erlotinib by restoring the mutant lung cancer.\(^5,6\) Patients with lung cancer refractory to gefitinib or erlotinib, irreversibly EGFR inhibitors are in clinical development for use in patients with lung cancer refractory to EGFR-TKIs. Consistently, although some irreversible EGFR inhibitors are in clinical development for use in patients with lung cancer refractory to gefitinib or erlotinib, the early results are disappointing.\(^16,17\) These findings strongly suggest that HGF is an important therapeutic target for overcoming resistance to EGFR-TKIs.

VEGF activates VEGF receptor 2 (VEGFR-2) and plays crucial roles in angiogenesis in non–small-cell lung cancer.\(^18\) HGF is also known to induce angiogenesis in cooperation with VEGF.\(^19,20\) which is thought to be an important therapeutic target in lung cancer.\(^1\) Therefore, we hypothesized that dual inhibition of HGF-Met and VEGF–VEGFR-2 may be therapeutically useful for controlling HGF-induced EGFR-TKI–resistant lung cancer. E7050 is an orally active dual TKI for Met and VEGFR-2.\(^21\) It inhibits proliferation of several Met gene–amplified cancer cells and tumor angiogenesis, resulting in marked tumor regression and prolongation of the lifespan of tumor-bearing mice.\(^21\) Based on these promising preclinical results, E7050 is currently under evaluation in clinical trials. In the present study, we determined the effect of dual inhibition of HGF-Met and VEGF–VEGFR-2 on HGF-induced EGFR-TKI resistance of EGFR mutant lung cancer cells using E7050 as well as anti-HGF Ab and anti-VEGF Ab.

Materials and Methods

Cell Culture

The EGFR mutant human lung adenocarcinoma cell lines PC-9\(^{11}\) and Ma-1\(^{12,23}\) were purchased from Immuno-Biological Laboratories Co. (Takasaki, Gunma, Japan) and were gifts from Dr. Noriyuki Masuda, Kinki Central Hospital, Sakai, Japan, respectively. Human dermal microvessel endothelial cell line (HMVEC) was purchased from Kurabo (Osaka, Japan). PC-9 and Ma-1 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 U/mL of streptomycin, and 2 mmol/L glutamine. HMVECs were maintained in HuMedia-MvG with growth supplements (Kurabo) and were used for in vitro assay at passages 2 to 5.

Reagents

E7050 was synthesized by Eisai Co. Ltd., Ibaraki, Japan\(^21\) (see Supplemental Figure S1 at http://ajp.amjpathol.org). Its inhibitory concentration of 50% values for Met in MKN45 cells with amplified Met and for VEGF-2 in human umbilical vein endothelial cells (HUVECs) were 14 and 16 nmol/L, respectively. In addition, oral administration of 25 mg/kg of E7050 inhibited Met phosphorylation and angiogenesis in xenograft tumors.\(^21\) Gefitinib was obtained from AstraZeneca (Cheshire, UK). Recombinant HGF and anti-HGF Ab were prepared as reported previously.\(^24\) The anti-VEGF Ab bevacizumab was obtained from Chugai Pharm (Tokyo, Japan). Recombinant VEGF, basic fibroblast growth factor, and epidermal growth factor (EGF) were obtained from R&D Systems (Minneapolis, MN).

Cell Growth Assay

Cell growth was measured using the MTT dye reduction method.\(^25\) Tumor cells \((2 \times 10^3 \text{ cells/100 } \mu\text{L per well})\) were plated into each well of 96-well plates in RPMI1640 medium with 10% FBS. After 24-hour incubation, various reagents were added to each well and were incubated for a further 72 hours. HMVECs \((5 \times 10^3/200 \mu\text{L per well})\) plated in 96-well plates precoated with 1.5% gelatin were incubated in M131 medium for 24 hours. Next, the cells were washed and incubated with various reagents for 72 hours in fresh minimal essential medium containing 5% FBS. Then, 50 \(\mu\text{L of MTT solution (2 mg/mL; Sigma-Aldrich, St. Louis, MO) was added to all the wells, and incubation was continued for a further 2 hours. The media containing MTT solution were removed, and the dark blue crystals were dissolved by adding 100 \(\mu\text{L of dimethyl sulfoxide. The absorbance was measured using 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 performed at least in triplicate, and three times independently.}

Antibodies and Western Blot Analysis

Cells were lysed in cell lysis buffer containing phosphatase inhibitor cocktail and proteinase inhibitor cocktail (Sigma-Aldrich), and the protein concentrations were determined using a Bicinchoninic Acid Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Total protein (40 \(\mu\text{g})
was resolved by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA), and the proteins were then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). After washing four times, membranes were incubated with Blocking One (Nacalai Tesque Inc., Kyoto, Japan) for 1 hour at room temperature and then were incubated overnight at 4°C with the following primary Abs: anti-Met (25H2), anti-phospho-Met (Y1234/Y1235) (3D7), anti-phospho-EGFR (Y1068), anti-ErbB3 (1B2), anti-phospho-ErbB3 (Tyr1289) (21D3), anti-Gab1 (#3232), phospho-Gab1 (Y627) (C32H2), anti-Akt, or phospho-Akt (Ser473) (736E11), anti-Shc1 (#2432) Abs (Cell Signaling Technology Inc., Beverly, MA) and anti-human EGFR (1 µg/mL) Ab (R&D Systems). After washing three times, membranes were incubated for 1 hour at room temperature with species-specific horseradish peroxidase–conjugated secondary Abs. Immunoreactive bands were visualized using SuperSignal west dura extended duration substrate enhanced chemiluminescent substrate (Pierce Biotechnology). Each experiment was performed at least three times independently.

**Cytokine Production**

Cells (2 x 10^5) were cultured in RPMI1640 medium with 10% FBS for 24 hours. The cells were washed with PBS and were incubated for 48 hours in 2 mL of RPMI1640 medium with 10% FBS. Then, culture medium was harvested and centrifuged, and the supernatant was stored at −70°C until analysis. The concentrations of HGF and VEGF were determined by IMMUNIS HGF enzyme immunoassay (Institute of Immunology, Tokyo, Japan) and Quantikine VEGF enzyme-linked immunosorbent assay (ELISA) (R&D Systems), respectively, according to the manufacturers’ protocols. All the samples were run in duplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves. The detection limits for HGF and VEGF were 100 and 31 pg/mL, respectively.

**HGF Gene Transfection**

One day before transfection, aliquots of 1 x 10^5 Ma-1 cells in 1 mL of antibiotic-free medium were plated on 6-well plates. The full-length HGF cDNA cloned into the BCMGSneo expression vector was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instructions. After 24-hour incubation, the cells were washed with PBS and then were incubated for an additional 72 hours in antibiotic-containing medium. Then, the cells were selected in G418 sulfate (Calbiochem, Jolla, CA). After limiting dilution, HGF-producing cells, Ma-1/HGF, were established. HGF production by Ma-1/HGF was confirmed by ELISA.

**RNA Interference Assay**

Duplexed Stealth RNAi (Invitrogen) against MET, ErbB3, Gab1, and Shc1 and Stealth RNAi negative control low GC duplex #3 (Invitrogen) were used for RNA interference assay. One day before transfection, aliquots of 2 x 10^6 tumor cells in 400 µL of antibiotic-free medium were plated on 24-well plates. After incubation for 24 hours, the cells were transfected with small-interfering RNA (siRNA) (50 pmol) or scramble RNA using Lipofectamine 2000 (1 µL) in accordance with the manufacturer’s instructions. After 24-hour incubation, the cells were washed with PBS and were incubated with or without various reagents for an additional 72 hours in antibiotic-containing medium. Cell growth was measured using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Tokyo, Japan) in accordance with the manufacturer’s instructions. Knockdown of MET, ErbB3, Gab1, and Shc1 was confirmed by Western blot analysis. Each experiment was performed at least in triplicate and three times independently.

**Xenograft Studies in Severe Combined Immunodeficiency Mice**

Suspensions of Ma-1/Vec and Ma-1/HGF cells (3 x 10^6) were injected subcutaneously into the backs of 5-week-old female severe combined immunodeficiency (SCID) mice (Clea, Tokyo, Japan). After 7 days (tumors >5 mm in diameter), mice were randomly allocated into groups of 6 to 10 animals to receive E7050 (50 mg/kg per day) and/or gefitinib (25 mg/kg per day) by oral gavage. In some groups, anti-HGF neutralizing Ab (5 mg/kg per day) and/or anti-VEGF Ab (5 mg/kg three times a week) were injected i.p. The tumor volume was calculated (mm^3 = width^2 x length/2). All the animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center.

**Immunofluorescence for Phosphorylated VEGFR-2**

To detect phosphorylated VEGFR-2 in HMVECs, HMVECs cultured on slides were rinsed with PBS and fixed in cold acetone for 5 minutes. After fixation, the slides were washed briefly in PBS and treated with 5% FBS for blocking. The slides were incubated with rabbit anti-phospho-VEGFR-2 Ab (Tyr1175, 19A10, 1:100 dilution; Cell Signaling Technology Inc.) at 4°C overnight. After washing, the slides were incubated with anti-rabbit Ab conjugated with Alexa Fluor 488 (green, 1:100 dilution; Invitrogen) and at room temperature for 40 minutes.

To detect CD31–phospho-VEGFR-2 double-positive endothelial cells, frozen sections (5 µm thick) of xenograft tumors were fixed with cold acetone and washed with PBS. After blocking with 5% normal horse serum, the slides were incubated overnight at 4°C with rabbit anti-phospho-VEGFR-2 Ab (Tyr1175) (19A10, 1:100 dilution) and rat anti-CD31 Ab (1:100 dilution; Pharmingen, San Diego, CA). After washing with PBS, the slides were stained with matched secondary Abs conjugated with Alexa Fluor 594 (red for CD31, 1:100) or Alexa Fluor 488 (green for phosphor VEGFR-2: 1:100 dilution). The localized green and red fluorescence were detected by fluorescence microscopy.
**Results**

**Met Inhibition Reverses Resistance to EGFR-TKIs Induced by Exogenous HGF**

PC-9 and Ma-1 cells, which have an in-frame deletion in EGFR exon 19, were highly sensitive to gefitinib (Figure 1A).\(^{1,12}\) None of these cell lines produced detectable levels of HGF protein (Figure 2A) or expressed T790M mutation in EGFR or amplified Met. As we reported previously, exogenously added HGF induced resistance to gefitinib in PC-9 and Ma-1 cells.\(^{1,12}\) E7050, a dual TKI for Met and VEGFR-2, did not affect viability of PC-9 or Ma-1 cells at concentrations of <1 µmol/L. However, in combination with gefitinib, E7050 reversed HGF-induced resistance of PC-9 and Ma-1 cells in a dose-dependent manner (Figure 1B). These results indicated that E7050 could reverse EGFR-TKI resistance induced by exogenous HGF in vitro.

We next examined the effects of E7050 on signal transduction by Western blot analysis. Gefitinib inhibited the phosphorylation of EGFR and ErbB3 and, thereby, inhibited the phosphorylation of Akt and ERK1/2; however, gefitinib did not show this effect in the presence of HGF. E7050 suppressed constitutive phosphorylation of Met and its adaptor protein Gab1 but not EGFR, ErbB3, or downstream Akt and ERK1/2. HGF stimulated phosphorylation of Met and Gab1. E7050 successfully inhibited HGF-induced phosphorylation of Met and Gab1 and strongly suppressed phosphorylation of Akt combined with gefitinib (Figure 1C).

**Immunohistochemical Analysis**

Frozen sections (5 µm thick) of xenograft tumors were fixed with cold acetone and washed with PBS. After blocking the endogenous peroxidase activity with 3% aqueous H₂O₂ solution for 10 minutes, the sections were treated with 5% normal horse serum. The sections were then reacted with primary Ab (anti-CD31 Ab, 1:100 dilution) at 4°C overnight. After washing with PBS, anti-CD31 Ab–treated sections were incubated with peroxidase-conjugated anti-rat IgG for 40 minutes. The DAB (3,3′-diaminobenzidine tetrahydrochloride) liquid system (DakoCytomation, Glostrup, Denmark) was used to detect immunostaining. Omission of primary Ab served as a negative control.

**Statistical Analysis**

The statistical significance of differences was analyzed by one-way analysis of variance. In cases in which the P values for the overall comparisons were less than 0.05, post hoc pairwise comparisons were performed by the Newman-Keuls multiple comparison test. Statistical analyses were performed using GraphPad Prism software version 4.01 (GraphPad Software Inc., San Diego, CA).

**Met Inhibition Overcomes Resistance to EGFR-TKIs Induced by Endogenous HGF**

A previous study demonstrated that HGF is mainly detected in cancer cells in patients with non–small-cell lung cancer and acquired resistance to EGFR-TKIs and that transient HGF gene transfection into PC-9 cells resulted in resistance to EGFR-TKIs.\(^{11}\) Herein, we further generated stable HGF gene transfectants (Ma-1/HGF) using Ma-1 cells to assess the role of continuously produced endogenous HGF. Ma-1/Vec were used as control cells.
transfected with vector alone. Ma-1/HGF, but not Ma-1 or Ma-1/Vec, secreted high levels of HGF and became resistant to gefitinib (Figure 2, A and B). Anti-HGF Ab reversed gefitinib resistance of Ma-1/HGF cells (data not shown), indicating that endogenously produced HGF induced gefitinib resistance in this cell line. E7050 combined with gefitinib (Figure 2C). E7050 inhibited phosphorylation of Akt in combination with gefitinib (Figure 2C). Akt inhibition by E7050 overcomes resistance to EGFR-TKIs induced by endogenous HGF. A: Tumor cells (1 × 10^6/10 mL) were incubated for 48 hours. Concentration of HGF in the culture supernatants was determined by ELISA. B: Ma-1/Vec and Ma-1/HGF cells were incubated with various concentrations of gefitinib with or without E7050. Cell growth was determined by MTT assay. C: Ma-1/HGF cells were incubated with E7050 (1 μmol/L) and/or gefitinib (1 μmol/L) for 1 hour. Then, cell lysates were harvested, and phosphorylation of the indicated proteins was determined by Western blot analysis. D: Ma-1/HGF cells were treated with or without scramble, ErbB3, Met, or Gab1 siRNA for 12 hours and were further incubated in medium for 48 hours. Then, cell lysates were harvested, and expression of the indicated proteins was determined by Western blot analysis. E: Ma-1/HGF cells were treated with siRNA as in D. After 72-hour incubation in medium, cell growth was determined by MTT assay. *P < 0.05 versus medium alone (one-way ANOVA). Bars indicate SD.

**HGF Stimulates VEGF Production by EGFR Mutant Lung Cancer Cells and E7050 Shows an Inhibitory Effect**

PC-9 and Ma-1 cells constitutively produced detectable levels of VEGF, and HGF stimulated VEGF production (Figure 4A). Consistent with these observations, Ma-1/HGF cells produced higher levels of VEGF (mean ± SD) than did Ma-1/Vec cells (2110 ± 40 versus 1170 ± 50 pg/10^6 per 48 hours). As HGF was reported to enhance VEGF production mediated by Shc1, an adaptor protein for Met, in endothelial cells, we next evaluated the involvement of Shc1 compared with Gab1, a common adaptor of Met, using specific siRNA. Treatment with siRNA for Met, Shc1, and Gab1 successfully knocked down expression of the corresponding protein (Figure 2D; see also Supplemental Figure S2 at http://ajp.amjpathol.org). Silencing of Met and Gab1, but not Shc1, canceled the augmented VEGF production by HGF, indicating that HGF stimulates VEGF production by activating Met predominantly using Gab1, not Shc1, as an adaptor (Figure 4B). Moreover, E7050 successfully canceled HGF-induced VEGF production (Figure 4C). Immunoblotting with immunoprecipitation showed that E7050 showed an inhibitory effect (Figure 3, B and C). E7050 slightly inhibited constitutive viability of HMVEC cells (Figure 3D). HMVEC growth was stimulated by VEGF and basic fibroblast growth factor but not by HGF or EGF. E7050 inhibited VEGF-stimulated HMVEC growth in a dose-dependent manner (Figure 3D). E7050 was less effective against basic fibroblast growth factor–stimulated HMVEC growth. These results indicated selective activity of E7050 against VEGF–VEGFR-2.

**Antiangiogenic Effect of E7050 by Inhibition of VEGFR-2**

We next explored the potential of E7050 against VEGFR-2. Western blot analysis indicated that HMVECs expressed VEGFR-2 protein at high levels, whereas the human EGFR mutant lung cancer cell lines PC-9, Ma-1, Ma-1/Vec, and Ma-1/HGF did not (Figure 3A). VEGFR-2 was phosphorylated by VEGF stimulation in HMVECs,
Gab1 was constitutively associated with Met and weakly phosphorylated in Ma-1 cells. HGF remarkably augmented phosphorylation of Gab1 associated with Met (Figure 4D). Although E7050 did not affect constitutive association between Met and Gab1, it inhibited HGF-induced Gab1 phosphorylation (Figure 4D). These findings indicated that Gab1 constitutively associates with Met and mediates important signaling for not only induction of EGFR-TKI resistance but also stimulation of VEGF production.

**E7050 Circumvents HGF-Induced Resistance When Combined with Gefitinib in Vivo**

To investigate the therapeutic efficacy of E7050, we inoculated Ma-1/Vec or Ma-1/HGF cells into SCID mice subcutaneously. Gefitinib treatment (25 mg/kg) induced regression of tumors formed by Ma-1/Vec cells, and E7050 treatment (50 mg/kg) showed a slight inhibitory effect (Figure 5A). Under these experimental conditions, gefitinib treatment inhibited the growth of Ma-1/HGF tumors only marginally, indicating gefitinib resistance in vivo caused by endogenous HGF (Figure 5B). E7050 treatment alone also retarded tumor growth, indicating mild antitumor activity of E7050 as monotherapy. However, E7050 combined with gefitinib resulted in marked growth regression of Ma-1/HGF tumors (Figure 5B).

Western blot analyses using in vivo tumors indicated that phosphorylation of Akt, an important survival signaling in EGFR mutant lung cancer, was inhibited only by combined treatment with gefitinib and E7050 in Ma-1/HGF tumors (Figure 5C; see also Supplemental Figure S3 at http://ajp.amjpathol.org). Histologic examinations showed that tumors produced by HGF-transfected Ma-1 (Ma-1/HGF) cells were more angiogenic than those produced by Ma-1/Vec cells (see Supplemental Figure S4 at http://ajp.amjpathol.org). In Ma-1/HGF tumors, wh-

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Figure 3. E7050 inhibits VEGFR-2 phosphorylation and suppresses growth of HMVECs. **A:** VEGFR-2 protein expression in HMVEC and EGF mutant lung cancer cell lines was examined by Western blot analysis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B:** HMVECs were cultured with or without E7050 (1 μmol/L) for 1 hour, and then 50 ng/mL of VEGF and HGF were added. After 10 minutes, the cells were lysed, and the indicated proteins were detected by immunoblotting. **C:** HMVECs were cultured with or without E7050 (1 μmol/L) for 1 hour, and then VEGF was added. After 10 minutes, the cells were fixed and stained for phosphorylated VEGFR-2. Phosphorylated VEGFR-2 was detected on the plasma membrane after VEGF stimulation. E7050 prevented expression of phosphorylated VEGFR-2 even after VEGF stimulation. **D:** HMVECs were incubated with various concentrations of E7050 for 72 hours with or without 50 ng/mL of VEGF, basic fibroblast growth factor (bFGF), HGF, or EGF. Cell growth was determined by MTT assay. Bars indicate SD.

Figure 4. HGF stimulates VEGF production by EGF mutant lung cancer cells. **A:** PC-9 and Ma-1 cells (2 × 10^7/2 mL) were incubated with or without HGF for 48 hours. Concentration of VEGF in the culture supernatants was determined by ELISA. *P < 0.05 versus medium alone. Bars indicate SD. **B:** PC-9 and Ma-1 cells (2 × 10^7/2 mL) were treated with scramble, Met, Gab1, or Shc1 siRNA for 12 hours and then were further incubated in medium for 48 hours. Then, the concentration of VEGF in the culture supernatants was determined by ELISA. *P < 0.05 versus medium alone. **C:** PC-9 and Ma-1 cells (2 × 10^7/2 mL) were incubated with or without HGF (50 ng/mL) for 48 hours. Concentration of VEGF in the culture supernatants was determined by ELISA. *P < 0.05 versus medium alone. **D:** IP-Met or IP-Gab1. Treatment of PC-9 and Ma-1 cells with E7050 (1 μmol/L) for 1 hour and then further incubated with or without HGF (50 ng/mL) for 10 minutes. Then, cell lysates were harvested and immunoprecipitated with the indicated antibody. Protein association was determined by Western blot analysis.
whereas gefitinib treatment did not affect the number of CD31-positive endothelial cells, E7050 with or without gefitinib dramatically inhibited the number of CD31-positive endothelial cells (Figure 5D; see also Supplemental Figure S5 at http://ajp.amjpathol.org), indicating potent antiangiogenic efficacy of E7050.

Moreover, control- or gefitinib-treated tumor contained CD31–phosphorylated VEGFR-2 double-positive cells but not in tumors treated with E7050 alone or gefitinib+E7050 (Figure 5D), indicating an association between antiangiogenic effect of E7050 and inhibition of VEGFR-2 phosphorylation in vivo. We also assessed the effects of gefitinib and/or E7050 on cell proliferation (Ki-67) and apoptosis (TUNEL). We found that gefitinib with or without E7050 suppressed cell proliferation in Ma-1/Vec tumors, whereas E7050 alone did not (see Supplemental Figures S5 and S6 at http://ajp.amjpathol.org). In Ma-1/HGF tumors, monotherapy with gefitinib or E7050 tended to reduce the number of proliferating cells, whereas combination treatment markedly inhibited cell proliferation. The number of apoptotic cells was increased in Ma-1/Vec tumors treated with gefitinib or E7050 and was markedly increased in tumors treated with both (see Supplemental Figures S5 and S7 at http://ajp.amjpathol.org). The number of apoptotic cells in Ma-1/HGF tumors was increased by E7050, unaffected by gefitinib, and markedly increased by combined gefitinib and E7050. These results suggest that monotherapy with the angiogenesis inhibitor E7050 was not sufficient for regressing Ma-1/HGF tumors, whereas its combination with gefitinib, which blocks EGFR signaling, was necessary for tumor regression.

**Dual Inhibition of HGF and VEGF by Specific Abs Reverses HGF-Induced Resistance When Combined with Gefitinib in Vivo**

To confirm the effects of dual targeting of HGF/Met and VEGF/VEGFR-2 on HGF-induced resistance, we finally examined the effects of anti-HGF Ab and anti-VEGF Ab with gefitinib against Ma-1/HGF tumors in vivo. Anti-HGF Ab and gefitinib slightly inhibited the growth of Ma-1/HGF tumors (Figure 6A). However, combined use of anti-HGF Ab and gefitinib further inhibited the growth, suggesting that anti-HGF Ab neutralized HGF and sensitized Ma-1/HGF cells to gefitinib in vivo (Figure 6A). In a parallel experiment, monotherapy with anti-VEGF Ab or combined treatment with anti-HGF Ab and anti-VEGF Ab inhibited tumor growth as effectively as com-
Ma-1/HGF cells (3 × 10⁶) were injected subcutaneously into SCID mice. From day 7, oral daily treatment with gefitinib (25 mg/kg), i.p. daily treatment with anti-HGF Ab (5 mg/kg per day), and/or i.p. treatment with anti-VEGF Ab (5 mg/kg per day, three times a week), were given. Data show the mean ± SE of six tumors on day 17. *P < 0.05. Bars indicate SD.

**Figure 6.** Dual inhibition of HGF and VEGF by specific antibodies reversed HGF-induced resistance when combined with gefitinib in vitro. A: Ma-1/HGF cells (3 × 10⁶) were inoculated subcutaneously into SCID mice. From day 7, oral daily treatment with gefitinib (25 mg/kg), i.p. daily treatment with anti-HGF Ab (5 mg/kg per day), and/or i.p. treatment with anti-VEGF Ab (5 mg/kg per day, three times a week), were given. Data shown are the mean ± SE of six tumors on day 17. *P < 0.05. Bars indicate SD. B: Scheme showing the role of HGF in EGFR mutant lung cancer cells. HGF induces EGFR-TKI resistance and VEGF production by activating the Met/Gab1 axis.

**Discussion**

HGF is a mediator that regulates multiple biological functions, including cell motility, invasion, and angiogenesis. Moreover, recent studies indicated considerable roles of HGF on sensitivity to anticancer agents. For example, HGF was reported to induce cisplatin resistance in lung adenocarcinoma cells by reducing the expression of apoptosis-inducing factor. It inhibited Fas-induced apoptosis by activating the PI3K/Akt pathway and, thus, inhibiting Fas death-inducing signaling complex formation in hepatocellular carcinoma. HGF also suppressed anoikis by increasing cyclooxygenase-2 expression through activation of Erk and AP-1 in head and neck squamous cell carcinoma. Regarding sensitivity to molecularly targeted drugs, we demonstrated that HGF induces EGFR-TKI resistance in EGFR mutant lung cancer. Thus, accumulating evidence suggests that HGF is an ideal therapeutic target for drug resistance in various types of cancer.

In the present study, we found that HGF stimulates VEGF production of EGFR mutant lung cancer cells by predominantly activating the Met/Gab1 axis in the same way as induction of EGFR-TKI resistance (Figure 6B). Moreover, the present findings using mainly E7050, a dual inhibitor of Met and VEGFR-2 kinases, indicated the rationale of dual inhibition of the HGF-Met axis and the VEGF-VEGFR-2 axis on treatment of HGF-induced EGFR-TKI resistance in EGFR mutant lung cancer. This strategy was supported by recent studies indicating that blockade of VEGF signaling caused hypoxia by inhibition of angiogenesis and that hypoxia is likely to enhance HGF-Met signaling and, thereby, promote tumor survival and metastasis. In fact, in renal cell carcinoma, HGF was reported to induce resistance to sunitinib, an inhibitor of multiple kinases, including VEGFR-2, by compensating for inhibited angiogenesis. In a model of pancreatic neuroendocrine cancer, inhibition of VEGF caused shrinkage of the primary tumor, but it also made the surviving cancer more aggressive with more metastatic behavior. Clinical studies indicated a correlation between the presence of hypoxic regions in neoplastic lesions and poor prognosis. Hypoxic conditions induced transcriptional activation of Met and subsequent amplification of HGF-Met signaling, thereby increasing the invasiveness of cancer cells. Therefore, dual blockade of the HGF-Met and VEGF-VEGFR axes may be valuable for overcoming not only EGFR-TKI resistance but also angiogenesis inhibitor resistance.

In endothelial cells, HGF was reported to induce VEGF production using Shc1 as an adaptor for Met. In contrast, silencing of Shc1 did not abrogate HGF-induced VEGF production in EGFR mutant lung cancer cells, although similar to Gab1, Shc1 constitutively associated with Met, as well as with Gab1. On the other hand, Gab1 is thought to be an important adaptor protein for Met-mediating signaling in various cell types. The present data show that Gab1 constitutively associated with Met and HGF markedly stimulated phosphorylation of Gab1 in EGFR mutant lung cancer cells. Silencing of Gab1 successfully canceled HGF-stimulated VEGF production and combined treatment with anti-HGF Ab and gefitinib in this model. The addition of gefitinib to anti-VEGF Ab further inhibited growth. Combined use of anti-HGF Ab, anti-VEGF Ab, and gefitinib showed the strongest inhibitory effect on the growth of Ma-1/HGF tumors, indicating the usefulness of dual inhibition of HGF and VEGF for overcoming HGF-induced resistance to EGFR-TKIs (Figure 6A). This was further supported by results of histologic analysis with Ma-1/HGF tumors. Anti-HGF Ab tended to decrease the number of proliferating tumor cells and tumor vessels and increase the number of apoptotic cells (see Supplemental Figures S5–S8 at http://ajp.amjpathol.org). Anti-HGF Ab with gefitinib discernibly reduced proliferating cell numbers, tumor vessel numbers, and phosphorylation of Akt and increased apoptotic cell numbers, but their effects were less than those of E7050 (dual inhibitor of HGF/Met and VEGFR-2) with gefitinib.
HGF-induced EGFR-TKI resistance. These findings suggest that Gab1 may be a novel ideal target for controlling EGFR mutant lung cancer.

These data suggest that E7050 regressed tumors with HGF-induced resistance by several mechanisms. First, E7050 inhibited phosphorylation of Met expressed in cancer cells, blocked the downstream Gab1/Pi3K/Akt pathway, and, hence, sensitized the cancer cells. Second, E7050 inhibited HGF-induced VEGF production by cancer cells and may indirectly suppress angiogenesis. Third, E7050 inhibited phosphorylation of VEGFR-2 in endothelial cells and directly suppressed angiogenesis. VEGF inhibition was suggested to normalize tumor vascular structure and permeability, decrease tumor interstitial pressure, and increase penetration of anticancer drugs into tumors. Therefore, it is also possible that E7050 increased its own penetration into tumors by inhibiting VEGF-2 phosphorylation and normalizing tumor vascular structure and permeability. Recently, neuropilin-1, the co-receptor for VEGF165 isoform, was reported to associate with Met and to mediate VEGF-triggered expression of myeloid cell leukemia-1, a member of the Lin-1, the co-receptor for VEGF165 isoform, was reported to associate with Met and to mediate VEGF-triggered expression of myeloid cell leukemia-1, a member of the Bcl-2 family, which inhibits cell apoptosis, in prostate cancer cells. In Ma-1/HGF tumors, E7050 treatment decreased the expression of myeloid cell leukemia-1 and survivin, another member of the Bcl family (see Supplemental Figure S9 at http://apj.amipathol.org). Thus, E7050 might also inhibit VEGF/neuropilin-1–triggered antiapoptotic protein expression by suppressing Met phosphorylation.

A recent study indicated an interaction between HGF and Met amplification in EGFR-TKI resistance in lung cancer. In the presence of gefitinib, continuous exposure to HGF accelerates expansion of preexisting MET-amplified cancer cells, although this was observed only in HCC2827 cells. We established Ma-1 cells (Ma-1/HGF) constitutively producing HGF by HGF gene transfection. However, Ma-1/HGF did not show Met amplification (data not shown). Therefore, this phenomenon may be uniquely observed in a population of EGFR mutant lung cancer cells.

It is important to define the incidence rate of HGF-induced resistance to EGFR-TKI in EGFR mutant lung cancer. In a previous study with 10 clinical specimens of EGFR mutant lung cancer, we reported the presence of patients whose resistance was associated with high HGF expression but not EGFR-T790M second mutation or Met amplification. This was confirmed by another group. Moreover, it was reported that HGF is frequently co-expressed in tumors with EGFR-T790M secondary mutation or Met amplification. Although there are considerable ethnic differences in the incidence rates of EGFR mutation in lung cancer, it is still unclear whether there are ethnic differences in the incidence of resistance mechanisms, such as EGFR-T790M secondary mutation, Met amplification, and HGF overexpression. We are currently engaged in a multicenter trial to elucidate the incidence of HGF-induced EGFR-TKI resistance and the involvement of angiogenesis in a Japanese cohort. Recent studies indicated that blood levels of HGF and/or VEGF are associated with clinical response to EGFR-TKIs in patients with lung cancer, suggesting key roles of HGF and VEGF and the possibility of noninvasive diagnosis of HGF-induced resistance to EGFR-TKIs.

In conclusion, we provided preclinical evidence showing the rationale of dual inhibition of HGF-Met and VEGF-VEGFR-2 in HGF-induced EGFR-TKI resistance of EGFR mutant lung cancer. Further studies are needed to determine whether dual targeting therapy will be clinically effective in patients with EGFR mutant lung cancer who develop HGF-induced resistance.

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References


