Crosstalk to Stromal Fibroblasts Induces Resistance of Lung Cancer to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors

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Abstract

Purpose: Lung cancers with epidermal growth factor receptor (EGFR)–activating mutations show good clinical response to gefitinib and erlotinib, selective tyrosine kinase inhibitors (TKI) to EGFR, but these tumors invariably develop drug resistance. Host stromal cells have been found to have a considerable effect on the behavior of cancer cells. Little is known, however, about the role of host cells on the sensitivity of cancer cells to receptor TKIs. We have therefore assessed the effect of crosstalk between stromal cells and lung cancer cells harboring EGFR mutations on susceptibility to EGFR-TKIs.

Experimental Design: We evaluated the gefitinib sensitivity of lung cancer cells with EGFR-activating mutations, PC-9 and HCC827, when cocultured with fibroblasts and coinjected into severe combined immunodeficient mice. We also examined the effect of lung cancer cells to fibroblast recruitment.

Results: Both human fibroblast cell lines and primary cultured fibroblasts produced various levels of hepatocyte growth factor (HGF). Lung cancer cells markedly recruited fibroblasts. The lung cancer cells became resistant to EGFR-TKIs when cocultured *in vitro* with HGF-producing fibroblasts and coinjected into severe combined immunodeficient mice. Importantly, combined use of gefitinib plus anti-HGF antibody or the HGF antagonist, NK4, successfully overcame the fibroblast-induced EGFR-TKI resistance both *in vitro* and *in vivo*. Colocalization of fibroblasts and HGF was detected in both xenograft tumors in mouse model and lung cancer patient specimens.

Conclusions: These findings indicate that crosstalk to stromal fibroblasts plays a critical role in lung cancer resistance to EGFR-TKIs and may be an ideal therapeutic target in lung cancer with EGFR-activating mutations. (Clin Cancer Res 2009;15(21):6630–8)

Lung cancer is the leading cause of cancer-related death worldwide, with non–small cell lung cancer (NSCLC) accounting for ~80% of lung cancers. The median survival of patients with metastatic NSCLC treated with the most active combination of conventional chemotherapy agents is 8 to 10 months (1, 2). Therefore, recent therapeutic strategies for NSCLC have focused on the development of molecular targeted agents.

Epidermal growth factor receptor (EGFR), a member of a family of closely related growth factor receptor tyrosine kinases, is expressed in a majority of NSCLCs and has been an attractive target for the development of therapeutic agents. Almost 90% of these somatic activating mutations in EGFR consist of in-frame deletions in exon 19 and L858R point mutations in exon 21 (3, 4). These mutations induce oncogenic activity and are closely correlated with sensitivity to small-molecule EGFR tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib. These mutations are more frequently present in females than in males, in nonsmokers than in smokers, in East Asians than in other ethnic groups, and in adenocarcinomas than in other tumor types (5). Several prospective clinical trials have shown that 70% to 75% of patients with tumors harboring these mutations respond to gefitinib or erlotinib (5, 6). However, these findings also indicate that 25% to 30% of NSCLC patients with EGFR-activating mutations show intrinsic resistance to

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Translational Relevance

Lung cancers with epidermal growth factor receptor (EGFR)–activating mutations show good clinical response to gefitinib and erlotinib, selective tyrosine kinase inhibitors (TKI) to EGFR, but these tumors invariably develop drug resistance. In this study, we found that (*a*) lung cancer cells with EGFR-activating mutations recruited human fibroblasts, which could be further upregulated by EGFR-TKI treatment; (*b*) coculture or coinjection of EGFR mutant lung cancer cells with human fibroblasts induced resistance to EGFR-TKIs by activating the MET/phosphoinositide 3-kinase/Akt axis *in vitro* and *in vivo*; (*c*) anti–hepatocyte growth factor (HGF) antibody, a HGF antagonist, NK4, and MET-TKI overcame the resistance to EGFR-TKIs of lung cancer; and (*d*) HGF high-producing fibroblasts were detected in both xenograft tumors in mouse model and lung cancer patient specimens. Our findings indicate that crosstalk to stromal fibroblasts plays a critical role in lung cancer resistance to EGFR-TKIs and may be an ideal therapeutic target in lung cancer with EGFR-activating mutations.

EGFR-TKIs. In addition, almost all NSCLC patients with EGFR mutations who show response to gefitinib or erlotinib ultimately develop resistance to these agents (5). Therefore, it is essential to understand the mechanisms of resistance to gefitinib.

Studies over the last few years have identified two different EGFR-TKI resistance mechanisms, a secondary mutation in EGFR, EGFR T790M, and amplification of the MET oncogene, which have been reported in ~50% and 20%, respectively, of patients acquiring resistance to EGFR-TKIs (7–10). More recently, we identified a third mechanism of gefitinib resistance induced by hepatocyte growth factor (HGF; ref. 11). HGF was originally identified as a mitogenic protein for hepatocytes (12). Its specific receptor is MET, a tyrosine kinase overexpressed in various types of cancer cells, including lung cancer. The binding of HGF to MET induces pleiotropic biological effects in many cell types, including mitogenic, motogenic, morphogenic, and antiapoptotic activities (13, 14). We reported previously that HGF activated MET and directly restored the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, independent of EGFR or ErbB3, thus inducing gefitinib resistance of lung cancer cells with EGFR-activating mutations. This mechanism may be involved in both intrinsic and acquired resistance to gefitinib (11).

It is widely accepted that the behavior of carcinomas, including progression and drug susceptibility, is influenced by crosstalk between tumor cells and the host microenvironment (15). The latter, also called the tumor stroma, includes blood vasculature, inflammatory cells, and fibroblasts. Stroma and tumor cells exchange enzymes and cytokines, which can modify the local extracellular matrix, stimulate migration, and promote proliferation and survival (16, 17). HGF is produced by various stromal cells, especially fibroblasts and endothelial cells (13–15), and plays a definitive role in tumor progression, predominantly as a paracrine mediator derived from stromal fibroblasts (14). We therefore investigated whether the susceptibility to EGFR-TKIs of lung cancer cells with EGFR-activating mutations could be affected by crosstalk to the host microenvironment, focusing on host-cell derived HGF. We found that fibroblasts could be recruited by cancer cells and that fibroblast-derived HGF efficiently induced gefitinib resistance of lung cancer cells with EGFR-activating mutations. We also found that HGF-MET inhibitors, such as anti-HGF antibody and NK4, could circumvent HGF-induced gefitinib resistance.

Materials and Methods

Cell culture and reagents. The EGFR mutant human lung adenocarcinoma cell lines PC-9 (del E746_A750) and HCC827 (del E746_A750) were purchased from Immuno-Biological Laboratories and the American Type Culture Collection, respectively. These cell lines have been extensively characterized (9, 18, 19). Human lung embryonic fibroblasts MRC-5 and IMR-90 were obtained from RIKEN Cell Bank. Human endothelial cell lines, human dermal microvascular endothelial cells (HMVEC), and human umbilical vein endothelial cells (HUVEC) were purchased from Kurabo. The PC-9 and HCC827 cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. The MRC-5 (P 30-35) and IMR-90 cell lines were cultured in 10% fetal bovine serum-DMEM. HUVEC and HMVEC cell lines (P 2-5) were maintained in HuMedia-MvG medium with growth supplements (Kurabo).

Gefitinib was obtained from AstraZeneca. Human recombinant HGF and human recombinant NK4 were prepared as described previously (20, 21). The purity of NK4 and HGF was 96.4% and >98%, respectively, as determined by SDS-PAGE and protein staining.

Isolation of fibroblasts from patient lung cancer tissues. Five patients with histologically proven lung cancer and who underwent surgical resection in Kanazawa University Hospital were enrolled. The patient cancer-infiltrating fibroblasts were cultured from lung cancer tissues as described (22). Briefly, cancer tissue samples were collected during surgery and divided into two pieces. One piece was snap-frozen in liquid nitrogen and stored at -80°C. Another piece was minced with scalpels in a tissue culture dish, digested with trypsin, and passed through a cell strainer. The resulting suspension was incubated in RPMI 1640 containing 10% fetal bovine serum and antibiotics. After 48 h, unattached cells were removed and fresh medium was added. After 7 to 10 days, the cells formed homogeneous monolayers morphologically consistent with fibroblast-like cells and were confirmed to consist of >99% type I collagen–positive cells (Supplementary Fig. S1). The study was carried out in accordance with the medical ethical committee guidelines of Kanazawa University.

Cell proliferation assay. Cell proliferation was measured using the MTT dye reduction method (23). Briefly, tumor cells (2×10^3) were plated into each well of 96-well plates with or without gefitinib, cytokines, and/or culture supernatants for 72 h with additional 2 h incubation with MTT solution. The dark blue crystals were dissolved by adding 100 μL DMSO. The absorbance was measured with a microplate reader at test and reference wavelengths of 550 and 630 nm, respectively. Percent growth was determined relative to untreated controls. Each experiment was done at least three times, each with triplicate samples.

Cell migration assay. Cell migration assays were done using the modified Boyden chamber method (24), with an 8 μm pore filter separating the top and bottom Transwell chambers (BD Biosciences). The cells were serum starved for 24 h before the assay. MRC-5 or primary cultured fibroblasts (10^4 cells/200 μL RPMI 1640) were added to the top chamber, and PC-9 or HCC827 cells (5×10^4 cells/500 μL RPMI 1640, with or without 1 μmol/L gefitinib) were added to the bottom chambers. After 48 h incubation at 37°C, the cells that had not migrated were removed from the top surface of the filters with cotton swabs. The cells that had migrated to the bottom surface of the filters were fixed in methanol and stained with H&E. Invasion was quantitated by counting cells in six randomly selected fields on each filter under

a microscope at a ×200 magnification and graphed as the mean of three independent experiments.

Coculture of lung cancer cells with fibroblasts. Cells were cocultured in Transwell chambers separated by 8 μm pore filters. Tumor cells (8×10^3 cells/700 μL) with or without gefitinib (0.3 μmol/L) were placed in the bottom chamber, and fibroblasts (10^4 cells/300 μL), with or without 1 h pretreatment with control IgG or anti-HGF neutralizing antibody (2 μg/mL; R&D Systems), were placed in the top chamber. After 72 h, the top chamber was removed, and cell proliferation was measured with a Cell Counting Kit-8 (Dojindo). Each experiment was done at least three times, each with triplicate samples.

Antibodies and Western blotting. A Western blot analysis was done as described previously (11). The experiment was done in triplicate. The following antibodies were used: anti-Met (25H2), anti–phospho-Met (Y1234/Y1235; 3D7), anti–phospho-EGFR (Y1068), anti-ErbB3 (1B2), anti–phospho-ErbB3 (Tyr^{1289}; 21D3), anti-Akt, or phospho-Akt (Ser^{473}; Cell Signaling Technology) and anti-human EGFR, anti-human/mouse/rat extracellular signal-regulated kinase 1/2, and anti–phospho-extracellular signal-regulated kinase 1/2 (T202/Y204; R&D Systems).

HGF production in cell culture supernatants and tumor tissues. Cells (5×10^6) were incubated for 48 h in 5 mL culture medium. Then, the supernatant was centrifuged and stored at -70°C. Tumors from severe combined immunodeficient (SCID) mice were lysed in mammalian tissue lysis buffer containing a phosphatase and proteinase inhibitor cocktail (Sigma). HGF was quantitated by ELISA in accordance with the manufacturer's procedure (Immunis HGF EIA; Institute of Immunology). The detection limit was 0.1 ng/mL. All samples were run in triplicate.

Xenograft studies in SCID mice. Suspensions of PC-9 cells (5×10^6) with or without MRC-5 (5×10^6) were injected subcutaneously into the backs of 5-week-old female SCID mice (Clea). After 4 days (tumors diameter >4 mm), mice were randomly allocated into groups of five animals to receive gefitinib (25 mg/kg/d) or vehicle only by oral gavage. In some groups, anti-HGF neutralizing antibody (5 mg/kg/d) or NK4 (9 mg/kg/d) was injected intraperitoneally. The tumor area was calculated (width × length). All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval no. AP-081088).

Cell membrane fluorescence labeling. MRC-5 cells were labeled with red fluorescence using PKH26 red fluorescent cell linker mini kit (Sigma) as described previously (25). Briefly, 2 μmol/L freshly prepared dye was added to 2×10^7 cells/mL diluent C, and the samples were incubated at room temperature for 5 min with gentle mixing. Staining was

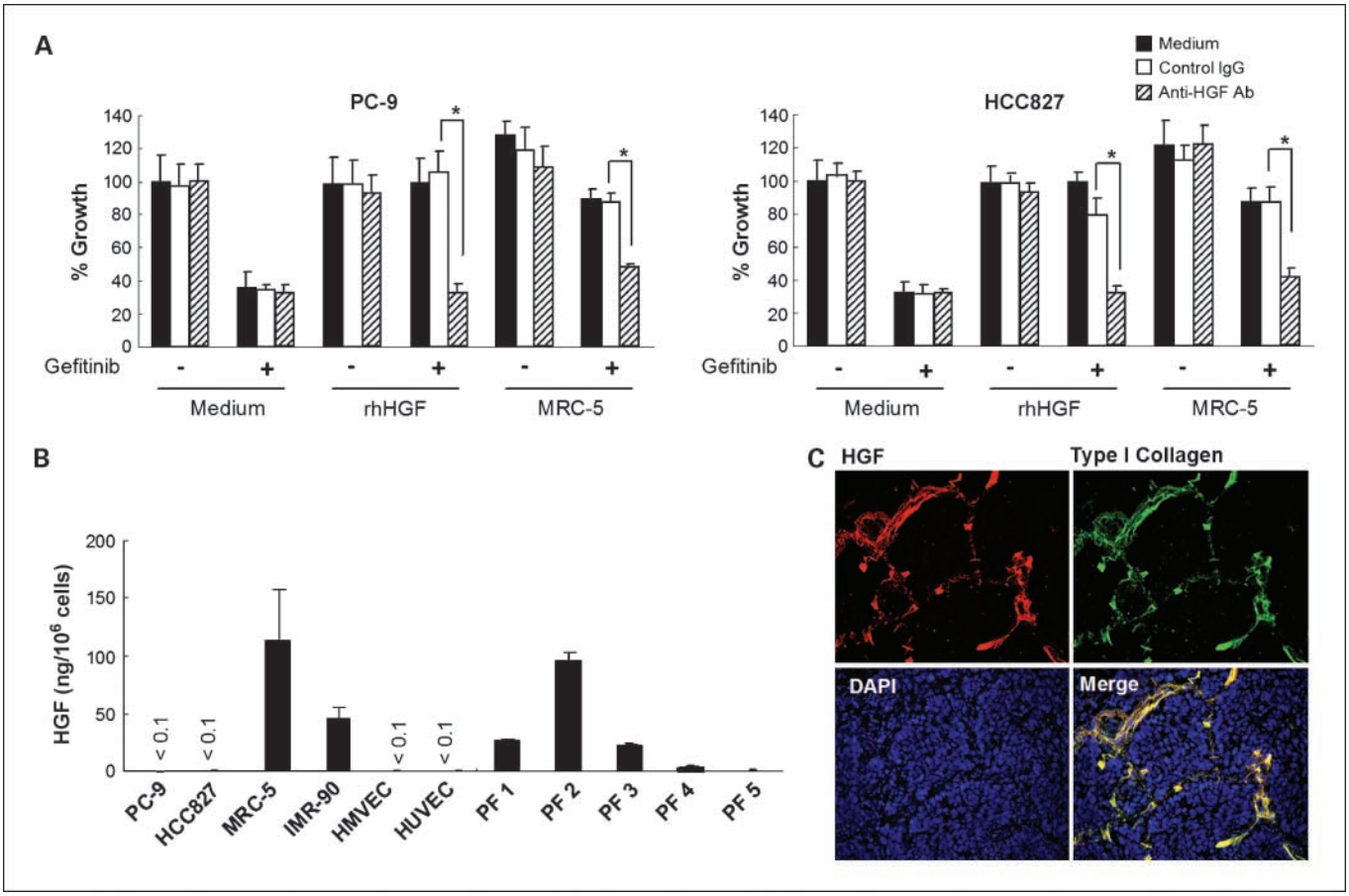

Fig. 1. Fibroblast-derived HGF induces gefitinib resistance in lung cancer cells. *A,* coculture with fibroblasts induces gefitinib resistance in lung cancer cells. The PC-9 and HCC827 lung cancer cell lines were cocultured with MRC-5 cells or HGF (20 ng/mL), with control IgG or anti-HGF neutralizing antibody (1 μg/mL), in the presence or absence of gefitinib (0.3 μmol/L) for 72 h, and lung cancer cell growth was determined with a Cell Counting Kit-8. *, $P < 0.01$ (one-way ANOVA). *B,* HGF production by lung cancer (PC-9 and HCC827), fibroblasts (MRC-5 and IMR-90), and endothelial (HMVEC and HUVEC) cell lines and by primary cultured fibroblasts from tumors of 5 lung cancer patients. Cells were incubated in medium for 48 h, culture supernatants were harvested, and HGF concentrations were determined by ELISA. *C,* colocalization of fibroblasts and HGF in tumor tissue from a lung cancer patient. Frozen tumor specimens were subjected to triple-color immunofluorescence analyses using the combination of anti–type I collagen antibody (*green*), anti-HGF antibody (*red*), and 4′,6-diamidino-2-phenylindole (*blue*) as described in Materials and Methods. The fluorescent images were digitally merged. Representative result from lung cancer patient 2. Original magnification, ×200.

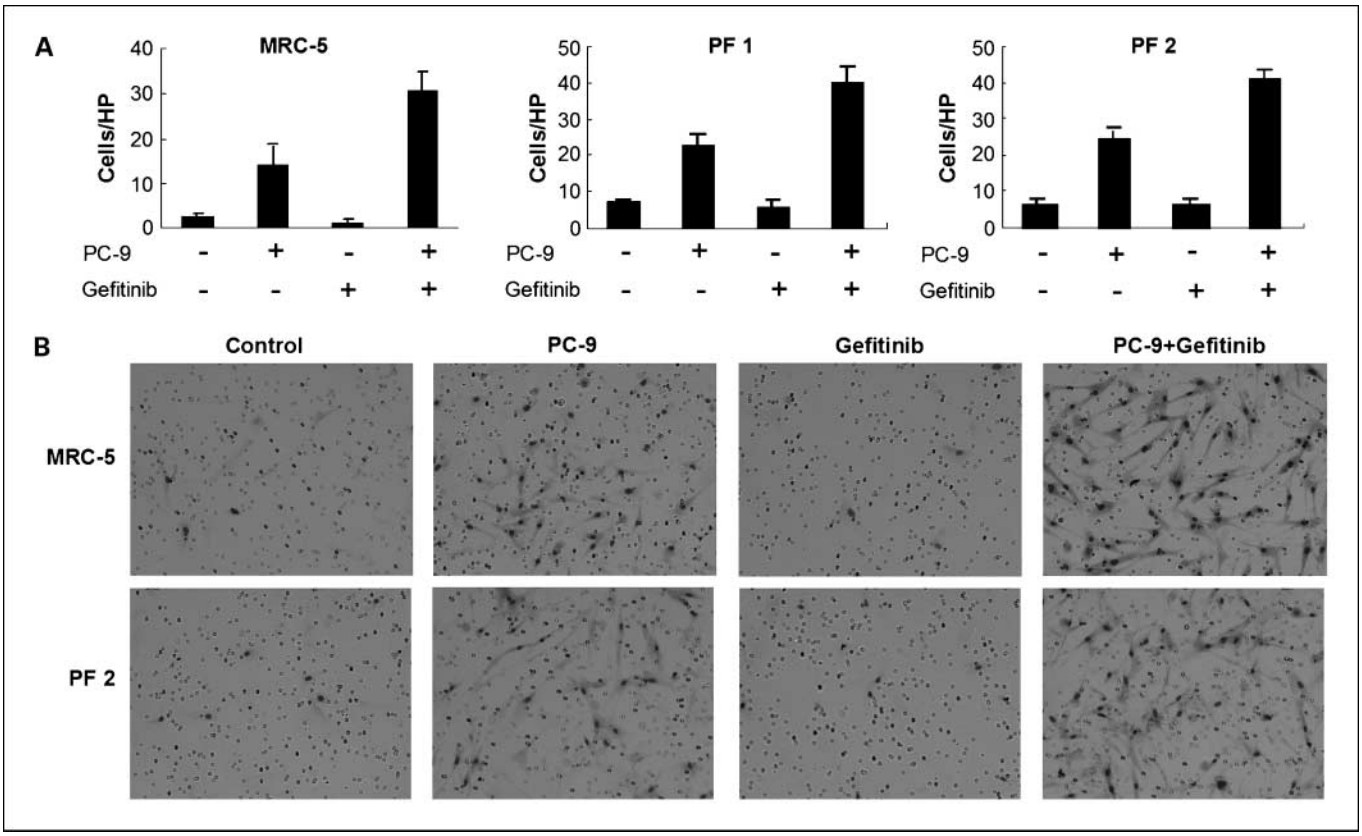

Fig. 2. Migration of fibroblasts in response to PC-9 cells. *A* and *B,* PC-9 cells induce migration of MRC-5 cells or primary cultured fibroblast from lung cancer patients 1 and 2 (PF1 and PF2). The assay was done in triplicate as described in Materials and Methods. Mean and SD.

terminated by addition of four volumes of HBSS containing 10% fetal bovine serum. The labeled MRC-5 cells were coinjected with PC-9 cells subcutaneously into SCID mice.

Immunofluorescence analysis. Frozen tissue sections (4 μm thick) 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 goat anti-human type I collagen antibody (1:200; Southern Biotech) and 3 μg/mL rabbit anti-human HGFα (H163; ref. 26; IBL). After washing with PBS, the slides were stained with matched secondary antibodies conjugated with Alexa Fluor 594 (red) or Alexa Fluor 488 (green; 1:200 dilution; Invitrogen). The localized green and red fluorescence were detected by fluorescence microscope.

Statistical analysis. Differences were compared by one-way ANOVA. All statistical analyses were done using GraphPad Prism version 4.01 (GraphPad Software). $P < 0.05$ was considered significant.

Results

Coculture with fibroblasts induces gefitinib resistance in lung cancer cells. To investigate whether the gefitinib susceptibility of lung cancer cells with EGFR-activating mutations could be affected by crosstalk to the host microenvironment, we cocultured PC-9 and HCC827 cells with fibroblast cell lines using Transwell systems. Whereas PC-9 and HCC827 cells were highly sensitive to gefitinib, exogenously added HGF induced gefitinib resistance in these cells as reported previously (11). Coculture with MRC-5 cells slightly stimulated the proliferation of PC-9 and HCC827 cells. Under these experimental conditions, both PC-9 and HCC827 cells became highly resistant to gefitinib in the presence of MRC-5 cells, but this resistance was canceled by treatment with anti-HGF neutralizing antibody (Fig. 1A). Similar results were obtained using a second fibroblast cell line, IMR-90, but not endothelial cell lines, HUVEC or HMVEC (data not shown). These results suggested that fibroblast-derived HGF induced gefitinib resistance in lung cancer cells with EGFR-activating mutations.

HGF production by lung cancer cells and cancer stromal cells. Then, we examined the production of HGF by various cell lines, including human lung cancer cells, fibroblasts, and endothelial cells. We found that the lung cancer cell lines, PC-9 and HCC827, did not secrete detectable levels of HGF into their culture supernatants nor did HUVEC and HMVEC cells (Fig. 1B). In contrast, the human fibroblast cell lines, MRC-5 and IMR-90, produced high levels of HGF. Because only fibroblast cell lines produced high levels of HGF, we sought to examine HGF production by cancer-associated fibroblasts. We successfully established primary cultures of fibroblasts in tumors from 5 lung cancer patients and found that these cells produced various levels of HGF, with the highest observed in fibroblasts from patient 2 (PF2), 83 ng HGF per million cells. Immunohistochemical staining for HGF revealed that tumor fibroblasts from patient 2 also produced HGF *in vivo* (Fig. 1C).

Lung cancer cells induce recruitment of fibroblasts. Cancer-associated fibroblasts have been shown to possess heterogeneous origins and characteristics (26). These cells originate from local resident fibroblasts and from bone marrow–derived stromal cells. We speculated that lung cancer cells might affect

the behavior of fibroblasts, particularly their recruitment. We therefore assessed the effect of lung cancer cells on fibroblast migration. In the presence of medium alone, few MRC-5 cells migrated through the filters; in contrast, PC-9 (Fig. 2) and HCC827 (data not shown) cells dramatically induced MRC-5 cell migration. PC-9 cells also induced migration of the primary cultured fibroblasts, which was not abrogated by imatinib (inhibitor of platelet-derived growth factor receptors) or antibodies against fibronectin, fibroblast growth factor-2, platelet-derived growth factor-AA, platelet-derived growth factor-BB, stromal cell–derived factor-1, heparin-binding epidermal growth factor, or interleukin-8 (data not shown). These data suggest that lung tumors may recruit fibroblasts to make these tumors resistant to gefitinib.

Fibroblast supernatants induce gefitinib resistance in lung cancer cells. To further confirm the effect of HGF derived from fibroblasts, we examined the effect of supernatants of fibroblasts and endothelial cells on the gefitinib sensitivity of PC-9 and HCC827 cells. The culture supernatants of MRC-5 and IMR-90 cells made PC-9 and HCC827 cells highly resistant to gefitinib. Pretreatment of these supernatants with anti-HGF neutralizing antibody, but not control IgG, entirely abrogated the resistance induced by the supernatants of MRC-5 and IMR-90 cells (Fig. 3A). In contrast, the supernatants of HMVEC or HUVEC did not affect the gefitinib sensitivity of PC-9 or HCC827 cells (data not shown). Moreover, supernatants of primary cultured fibroblasts obtained from lung cancer patients induced gefitinib resistance in PC-9 cells (Fig. 3B), with the level of resistance correlating with HGF production by fibroblasts. The supernatants of PF2 made PC-9 cells completely resistant to gefitinib, whereas the supernatants of primary cultured fibroblasts obtained from other patients induced only partial resistance to gefitinib. Importantly, anti-HGF antibody significantly restored the sensitivity of PC-9 cells to gefitinib in all 5 patients.

In parallel experiments, HGF induced resistance of PC-9 cells to erlotinib (Fig. 3C).

HGF derived from fibroblasts restores PI3K/Akt pathway via MET but not EGFR or ErbB3. We reported previously that, in the presence of gefitinib, HGF phosphorylated MET and restored the signal for Akt and extracellular signal-regulated kinase 1/2 independent of EGFR or ErbB3 in lung cancer cells harboring EGFR-activating mutations (11). We found that culture supernatants of PF2 cells containing 80 ng/mL HGF did not affect the phosphorylation of EGFR or ErbB3 (Fig. 4). In contrast, these HGF-containing supernatants stimulated the phosphorylation of MET and restored the phosphorylation of Akt and extracellular signal-regulated kinase 1/2 that had been inhibited by gefitinib. These effects were abrogated by pretreatment of the culture supernatants with anti-HGF neutralizing antibody, indicating that HGF produced by PF2 cells restored the phosphorylation of PI3K/Akt via MET, but not via EGFR

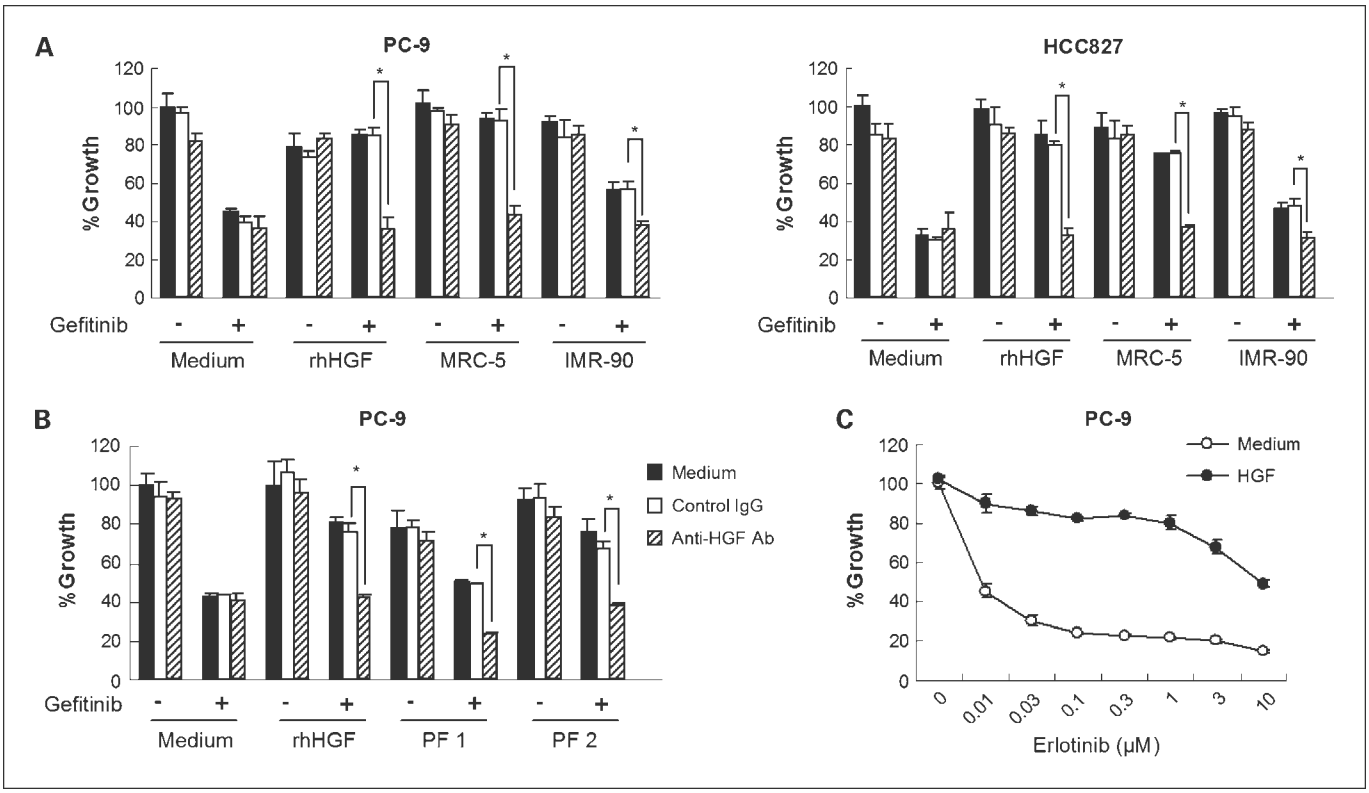

Fig. 3. Fibroblast supernatants induce gefitinib resistance in lung cancer cells. *A,* supernatants of human fibroblast cell lines, MRC-5 and IMR-90, induce gefitinib resistance in PC-9 and HCC827 cells. Lung cancer cells were incubated for 72 h with or without gefitinib (0.3 μmol/L) in the presence of culture supernatants of MRC-5 or IMR-90 cells (ratio of 1:2) with or without 1 h pretreatment with control IgG or anti-HGF neutralizing antibody (1 μg/mL). Lung cancer cell growth was determined by MTT assays. *, $P < 0.01$ (one-way ANOVA). *B,* supernatants of primary cultured fibroblasts from lung cancer patients (PF) induce gefitinib resistance in lung cancer cells. Lung cancer cells were incubated for 72 h with or without gefitinib (0.3 μmol/L) in the presence of culture supernatants of PF1 or PF2 (ratio of 1:2) with or without 1 h pretreatment by control IgG or anti-HGF neutralizing antibody (1 μg/mL). Lung cancer cell growth was determined by MTT assays. *, $P < 0.01$ (one-way ANOVA). *C,* HGF induces lung cancer cell resistance to erlotinib. PC-9 cells were incubated for 72 h with various concentrations of erlotinib and/or HGF (20 ng/mL), and cell growth was determined by MTT assays.

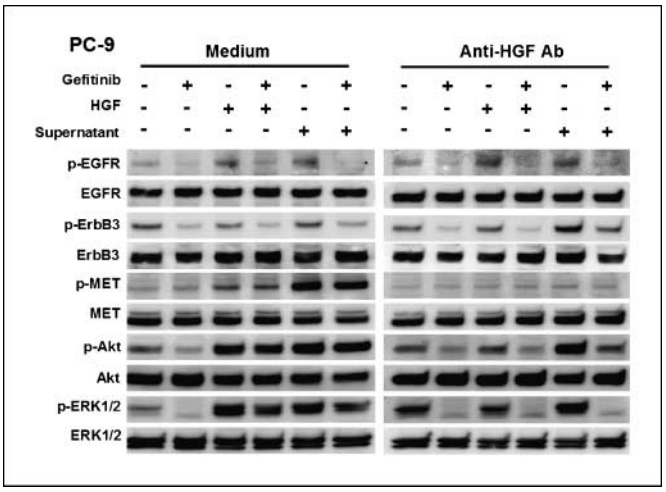

Fig. 4. Fibroblast-derived HGF restores PI3K/Akt pathway via MET but not EGFR or ErbB3. PC-9 cells were treated with or without gefitinib (0.3 μmol/L) and/or HGF (20 ng/mL) or the supernatant of PF2 in the presence or absence of anti-HGF neutralizing antibody (1 μg/mL) for 1 h. Cells were lysed, and the indicated proteins were detected by immunoblotting.

or ErbB3, in lung cancer cells harboring EGFR-activating mutations. These results confirm the importance of fibroblast-derived HGF in the gefitinib resistance of lung cancer cells with EGFR-activating mutations and further indicate that HGF may be a therapeutic target for overcoming resistance to gefitinib.

Several inhibitors of HGF-MET have been developed recently, including anti-HGF antibody, natural antagonist NK4, and MET-TKI. We therefore tested whether these inhibitors could overcome gefitinib resistance induced by fibroblast-derived HGF. We found that 3 μmol/L (nontoxic concentration) of the MET-TKI, SU11274, moderately reversed the HGF-induced gefitinib resistance of PC-9 cells. In contrast, both anti-HGF neutralizing antibody (1 μg/mL) and the natural HGF inhibitor NK4 (300 nmol/L) completely overcome the gefitinib resistance at nontoxic concentrations (Fig. 5).

***HGF derived from fibroblasts induces gefitinib resistance of lung adenocarcinoma cells* in vivo.** To investigate whether the gefitinib sensitivity of lung cancer cells with EGFR-activating mutations could be affected by fibroblasts *in vivo*, we inoculated PC-9 cells, with or without MRC-5 cells, into SCID mice subcutaneously. The tumors in mice injected with PC-9 plus MRC-5 cells grew slightly faster than the tumors in mice injected with PC-9 cells alone. Gefitinib treatment, beginning on day 4, caused marked regression of tumors in mice injected with PC-9 cells alone. The same treatment prevented enlargement of tumors in mice injected with PC-9 and MRC-5 cells, but it did not cause tumor regression, indicating resistance of the tumors to gefitinib treatment *in vivo* (Fig. 6A).

To further elucidate the role of HGF *in vivo*, the mice bearing tumors caused by injection of PC-9 and MRC-5 cells were treated with anti-HGF neutralizing antibody or NK4 in the presence or absence of gefitinib. Treatment with anti-HGF antibody or NK4 alone marginally reduced the tumor growth. Treatment with gefitinib alone prevented tumor enlargement, but it did not cause significant regression of tumor size. Importantly, the combination of gefitinib with anti-HGF antibody or NK4 caused marked tumor regression (Fig. 6B). These results indicate that HGF, produced presumably by fibroblasts (MRC-5), induced *in vivo* gefitinib resistance in lung cancer cells with EGFR-activating mutations.

In the final set of experiments, we confirmed HGF production by MRC-5 cells *in vivo*. Whereas the tumors in mice injected with PC-9 cells alone did not produce detectable levels of HGF, the tumors in mice injected with PC-9 and MRC-5 cells produced high levels of HGF on day 4, which decreased on day 7 (Fig. 6C). The decrease of HGF levels in the tumors may be the reason for incomplete resistance to gefitinib induced by the coinjected MRC-5 cells. On the other hand, we could not detect human HGF in the serum of mice implanted with PC-9 and MRC-5 cells (data not shown), suggesting that HGF level in the tumor area may be more important to predict the resistance to EGFR-TKIs. To confirm the presence of MRC-5 cells in the tumor, red fluorescence–labeled MRC-5 cells were mixed with PC-9 cells before inoculation and shown to be present in these tumors (Supplementary Fig. S2A). Compared with PC-9 alone tumors, more infiltrating fibroblast-like cells were found in the coinjected tumors (Supplementary Fig. S2B). Moreover, double staining for type I collagen and human HGF clearly showed that the infiltrating fibroblasts produced HGF (Fig. 6D). These results indicate that the human fibroblast cell line MRC-5, when inoculated along with PC-9 cells, produced HGF and hence induced gefitinib resistance as tumor-associated fibroblasts.

Discussion

We have shown here that a novel crosstalk between tumor and stromal fibroblasts is responsible for inducing resistance to TKIs in lung cancers harboring EGFR-activating mutations. Cancer cells recruit fibroblasts, which produce HGF that induces EGFR-TKI resistance in lung cancer cells harboring EGFR-activating mutations by activating the MET/PI3K/Akt axis. We further showed that EGFR-TKI resistance induced by fibroblast-derived HGF could be circumvented by HGF-MET inhibitors in both *in vitro* and *in vivo* experimental models. These results indicate that a novel strategy, targeting the crosstalk between tumor cells and stromal fibroblasts, may be important for circumventing the EGFR-TKI resistance of lung cancers harboring EGFR-activating mutations.

The tumor microenvironment is important for tumor progression. Fibroblastic stromal cells have been linked to several activities that promote tumor progression, including angiogenesis,

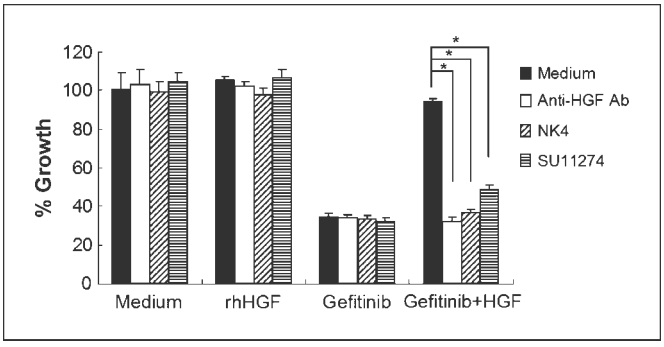

Fig. 5. Anti-HGF antibody, NK4, or SU11274 abrogate HGF-induced gefitinib resistance in lung cancer cells. PC-9 cells were treated for 72 h with or without gefitinib (0.3 μmol/L) and/or HGF (20 ng/mL) or supernatant of PF2 in the presence or absence of anti-HGF neutralizing antibody (1 μg/mL), NK4 (300 nmol/L), or SU11274 (0.3 μmol/L). Cell growth was determined by MTT assays. *, $P < 0.01$ (one-way ANOVA).

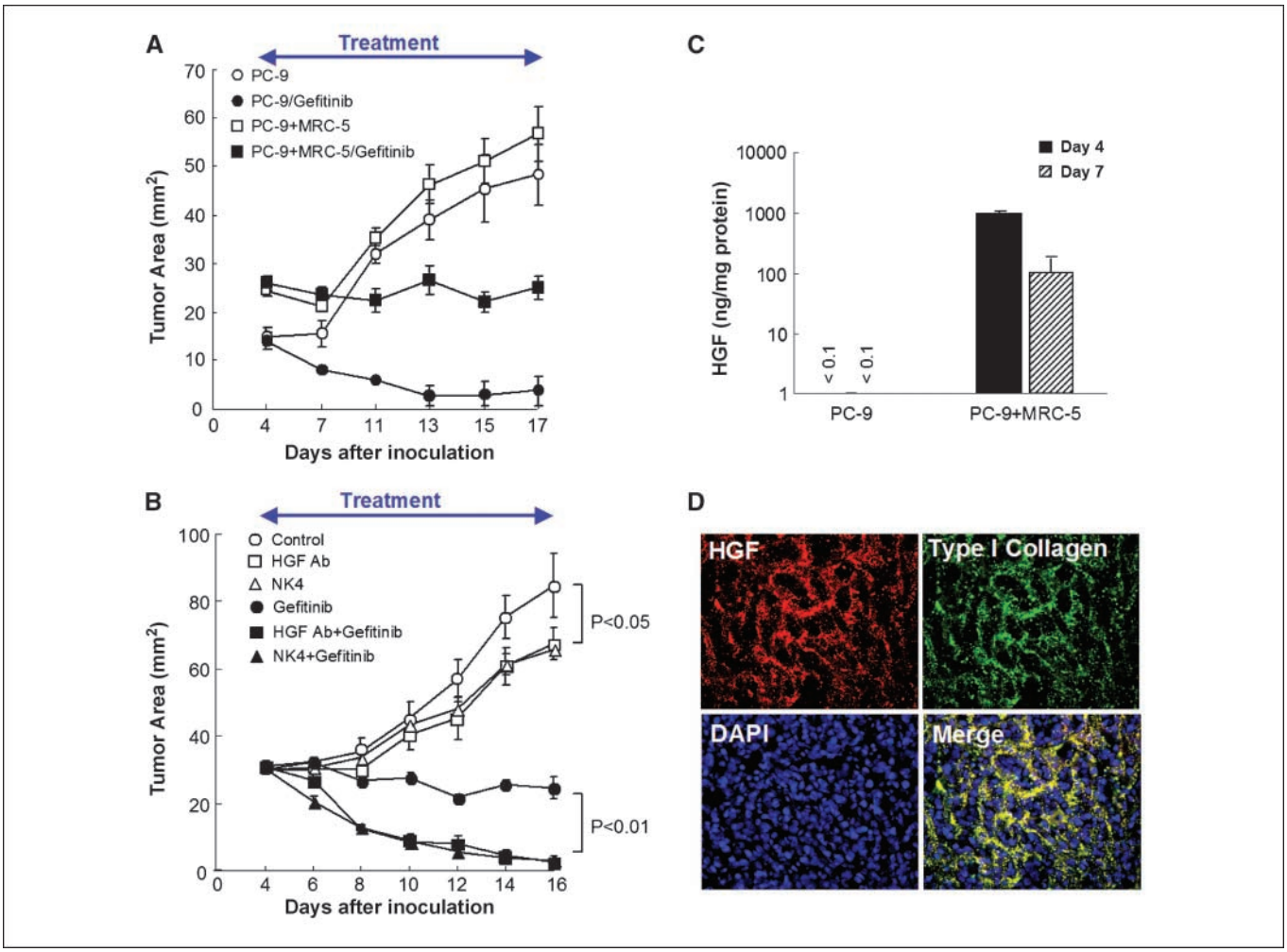

Fig. 6. Fibroblast-derived HGF induces gefitinib resistance in PC-9 tumors in SCID mice. PC-9 cells (5×10^6) with or without MRC-5 cells (5×10^6) were inoculated subcutaneously into SCID mice on day 0. Mice received oral gefitinib (25 mg/kg/d) or vehicle only, starting on day 4. *A,* coinjection with MRC-5 cells induces resistance of PC-9 tumors to gefitinib treatment. The tumor area was measured every 3 or 4 d and calculated as described in Materials and Methods. Bar, SE. *B,* anti-HGF antibody or NK4 reverses gefitinib resistance in tumors caused by injection of PC-9 cells mixed with MRC-5 cells. Mice received oral gefitinib (25 mg/kg/d), with or without anti-HGF neutralizing antibody (5 mg/kg/d) or NK4 (9 mg/kg/d) intraperitoneally daily, starting on day 4. Bar, SE. *C,* HGF production in tumor tissues. The tumors were harvested on day 4 or 7 and lysed. HGF production in tumor tissue lysate was examined by ELISA. *D,* colocalization of MRC-5 cells and HGF in tumors caused by injection of PC-9 and MRC-5 cells. Tumors were harvested on day 14. Frozen tumor specimens were subjected to triple-color immunofluorescence analyses using the combination of anti–type I collagen antibody (*green*), anti-HGF antibody (*red*), and 4′,6-diamidino-2-phenylindole (*blue*) as described in Materials and Methods. The fluorescent images were digitally merged. Original magnification, ×200.

epithelial-to-mesenchymal transition, progressive genetic instability, deregulation of antitumor immune responses, enhanced metastasis, and enhanced growth. Stromal fibroblasts act as a major source of chemoattractants, which facilitate tumor cell motility and metastasis. In a contrasting situation, fibroblasts frequently infiltrate into tumors, but the mechanism underlying this infiltration is still poorly understood (27, 28). We found that the lung cancer cell lines, PC-9 and HCC827, induced recruitment of fibroblasts but that this recruitment was not significantly affected by inhibition of fibronectin, fibroblast growth factor-2/fibroblast growth factor receptor-1, platelet-derived growth factors/platelet-derived growth factor receptors, stromal cell–derived factor-1, interleukin-8, or stromal cell–derived factor-1 plus interleukin-8 (data not shown). Interestingly, this recruitment was further enhanced by addition of gefitinib to the culture. Taken together, these findings indicate that PC-9 and HCC827 cells damaged by gefitinib treatment released unknown chemoattractants, which led to enhanced fibroblast recruitment. Thus, dying lung cancer cells likely release chemoattractants to recruit fibroblasts that secrete HGF, thus protecting live lung cancer cells from gefitinib. Further examinations are ongoing in our laboratory to identify the chemotactic factor(s) responsible for this phenomenon.

We reported previously that HGF, either exogenously added or overexpressed in lung cancer cells harboring EGFR-activating mutations, caused gefitinib resistance by restoring the MET/PI3K/Akt axis (11). In the present study, we confirmed these findings and further extended these observations, showing that gefitinib resistance could also be induced by HGF derived from both fibroblast cell lines and primary cultured fibroblasts by a paracrine mechanism. Fibroblast cell lines have been found to produce higher concentrations of HGF than other cell types,

including endothelial cells and lung cancer cell lines (11, 29). Moreover, primary cultured fibroblasts established from tumors of lung cancer patients produced various levels of HGF *in vitro*. Therefore, it is highly possible that tumor-associated fibroblasts are involved in both intrinsic and acquired resistance to gefitinib and erlotinib observed in lung cancer patients harboring EGFR-activating mutations.

Several prospective studies indicate that, although lung cancer patients harboring EGFR-activating mutations (superresponders) show dramatic responses to gefitinib or erlotinib, the rate of complete response (radiographic disappearance of tumors) is very low (3–6). Many superresponders retain a small tumor remnant, which is maintained for months to years. Two mechanisms, T790M second mutation in EGFR (8) and MET amplification (9), have been reported associated with acquired resistance to gefitinib or erlotinib in lung cancer patients with EGFR-activating mutations. Continuous treatment with gefitinib or erlotinib may select minor clones with a T790M second mutation and/or MET amplification. Alternatively, cancer cells may acquire these alterations during continuous treatment with gefitinib or erlotinib. However, if the majority of cancer cells in the small tumor remnant have T790M second mutation and/or MET amplification, the tumor remnant will enlarge in a short period. Therefore, it would seem difficult for these two mechanisms to explain why the size of the tumor remnant is maintained for months to years during treatment with gefitinib or erlotinib. We found that fibroblast-derived HGF contributed to the persistence of tumors during gefitinib treatment. These findings suggest that fibroblasts recruited to tumors may protect tumors by producing HGF until minor clones with T790M and/or MET amplification become dominant. Further studies are required to assess this hypothesis.

The results of our previous (11) and present studies showed that HGF derived from cancer cells or stromal fibroblasts induce gefitinib resistance in lung cancers harboring EGFR-activating mutations. Therefore, HGF-MET may be an ideal target for circumventing this type of resistance. We found that several HGF-MET inhibitors, including anti-HGF antibody, the natural inhibitor NK4, and MET-TKI (14, 30–34), could abrogate HGF-induced gefitinib resistance, with anti-HGF neutralizing antibody and NK4 completely abrogating HGF-induced resistance to gefitinib *in vitro* and *in vivo*. In contrast, a nontoxic concentration of the MET-TKI, SU11274, had a smaller effect than either anti-HGF antibody or NK4. Newer MET-TKIs, with higher activity, are currently being evaluated in clinical trials against various cancers, including gastric cancers and renal cell carcinomas with MET mutations or amplification (35, 36). Therefore, these new MET-TKIs may be more effective in lung cancer patients with EGFR-TKI resistance. Additional studies are required to determine the relative efficacy and safety of agents in treating lung cancer patients with HGF-induced gefitinib resistance.

In summary, we showed that a novel crosstalk between tumor cells and stromal fibroblasts is responsible for the resistance to EGFR-TKIs of lung cancers harboring EGFR-activating mutations. These results suggest that new strategies targeting this crosstalk may be important for circumventing resistance to EGFR-TKIs of lung cancers harboring EGFR-activating mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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