Met Kinase Inhibitor E7050 Reverses Three Different Mechanisms of Hepatocyte Growth Factor-induced Tyrosine Kinase Inhibitor Resistance in EGFR Mutant Lung Cancer

Clin Cancer Res, version 3

Met Kinase Inhibitor E7050 Reverses Three Different Mechanisms of Hepatocyte Growth Factor-induced Tyrosine Kinase Inhibitor Resistance in EGFR Mutant Lung Cancer

Wei Wang[1], Qi Li[1], Shinji Takeuchi[1], Tadaaki Yamada[1], Hitomi Koizumi[1], Takahiro Nakamura[2], Kunio Matsumoto[2], Naofumi Mukaida[3], Yasuhiko Nishioka[4], Saburo Sone[4], Takayuki Nakagawa[1,5], Toshimitsu Uenaka[5], and Seiji Yano[1].

[1]Division of Medical Oncology, Cancer Research Institute, Kanazawa University. Kanazawa, Ishikawa 920-0934, [2]Division of Tumor Dynamics and Regulation, [3]Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University. Kanazawa, Ishikawa 920-1192, [4]Department of Respiratory Medicine and Rheumatology, University of Tokushima Graduate School, Tokushima, Tokushima 770-8503, [5]Tsukuba Research Laboratories, Eisai co., ltd., Ibaraki, Japan.

Running title:E7050 reverses HGF-induced EGFR-TKI resistance.

Requests for reprints: Seiji Yano, Division of Medical Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-0934, Japan. Phone: 81-76-265-2780, Fax: 81-76-234-4524,
E-mail: syano@staff.kanazawa-u.ac.jp

Translational relevance

Hepatocyte growth factor (HGF) is involved in at least three important steps of epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) resistance in *EGFR* mutant lung cancer, inducing resistance to reversible EGFR-TKIs by restoring Met/Gab1/PI3K/Akt pathways, inducing resistance to next-generation EGFR-TKIs (irreversible TKI and mutant-selective EGFR-TKI), and accelerating the emergence of EGFR-TKI-resistant clones by continuous exposure to HGF. Therefore, HGF may be an ideal target for overcoming EGFR-TKI resistance in *EGFR* mutant lung cancer.

In pre-clinical experiments, we have tested whether a new Met kinase inhibitor, E7050, which is currently under evaluation in clinical trials, could overcome these three HGF-induced EGFR-TKI resistance mechanisms. Our findings suggest that E7050 may be useful for overcoming HGF-induced resistance to gefitinib and next-generation EGFR-TKIs in *EGFR* mutant lung cancer.

Abstract

Purpose: Hepatocyte growth factor (HGF) induces resistance to reversible and irreversible epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) in *EGFR* mutant lung cancer cells by activating Met and the downstream PI3K/Akt pathway. Moreover, continuous exposure to HGF accelerates the emergence of EGFR-TKI-resistant clones. We assayed whether a new Met kinase inhibitor, E7050, which is currently being evaluated in clinical trials, could overcome these three mechanisms of resistance to EGFR-TKIs.

Experimental design: The effects of E7050 on HGF-induced resistance to reversible (gefitinib), irreversible (BIBW2992), and mutant-selective (WZ4002) EGFR-TKIs were determined using the *EGFR*-mutant human lung cancer cell lines PC-9 and HCC827 with an exon 19 deletion, and H1975 with an T790M secondary mutation. PC-9 cells were mixed with HGF-producing fibroblasts, MRC-5 cells, and subcutaneously inoculated into SCID mice and the therapeutic effects of E7050 plus gefitinib were assayed.

Results: E7050 circumvented resistance to all of the reversible, irreversible, and mutant-selective EGFR-TKIs induced by exogenous and/or endogenous HGF in *EGFR* mutant lung cancer cell lines, by blocking the Met/Gab1/PI3K/Akt pathway

in vitro. E7050 also prevented the emergence of gefitinib-resistant HCC827 cells induced by continuous exposure to HGF. In the *in vivo* model, E7050 plus gefitinib resulted in marked regression of tumor growth associated with inhibition of Akt phosphorylation in cancer cells.

Conclusions: A new Met kinase inhibitor, E7050, reverses the three HGF-induced mechanisms of gefitinib resistance, suggesting that E7050 may overcome HGF-induced resistance to gefitinib and next-generation EGFR-TKIs.

Introduction

The reversible epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib show dramatic therapeutic efficacy in patients with *EGFR*-activating mutations, such as in-frame deletions of exon 19 and the L858 point mutation in exon 21 (1, 2). Recent clinical trials have demonstrated that these TKIs induced much higher response rates and longer progression-free survival than standard first-line cytotoxic chemotherapy in patients with *EGFR* mutant lung cancer (3, 4). Almost all patients, however, develop acquired resistance to EGFR-TKIs after varying periods of time (5). In addition, 20% – 30% of patients with *EGFR*-activating mutations show intrinsic resistance to EGFR-TKIs (5). Therefore, intrinsic and acquired resistances to EGFR-TKIs are major problems in the management of *EGFR* mutant lung cancer.

Three clinically relevant mechanisms have been reported to induce acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer—*EGFR* T790M secondary mutation (6, 7), *Met* gene amplification (8), and hepatocyte growth factor (HGF) overexpression (9). We found that HGF overexpression is involved not only in acquired but in intrinsic resistance to EGFR-TKIs (9). HGF has been

shown to play at least three important roles in EGFR-TKI resistance in *EGFR* mutant lung cancer. First, HGF induces resistance to the reversible EGFR-TKIs gefitinib and erlotinib by restoring MetGab1/PI3K/Akt pathways (9, 10). Second, continuous exposure to HGF accelerates the expansion of preexisting *Met*-amplified cancer cells and facilitates *Met* amplification-mediated resistance during EGFR-TKI treatment (10). Third, after lung cancer cells acquire resistance to reversible EGFR-TKIs, HGF induces the resistance of cells with T790M secondary mutation to irreversible EGFR-TKIs (11). These findings indicate that HGF is an ideal target for overcoming EGFR-TKI resistance in *EGFR* mutant lung cancer.

There are several possible strategies for inhibiting HGF-Met signaling, including anti-HGF neutralizing antibody, HGF antagonist (NK4), Met tyrosine kinase inhibitors, and inhibitors of downstream molecules, such as PI3K, Akt, and mTOR (12). Previously, we showed that anti-HGF antibody (13), NK4 (13), and PI3K inhibitors (14) were effective in overcoming HGF-induced gefitinib resistance. Many Met-TKIs have therefore been developed and are expected to reverse HGF-induced resistance to EGFR-TKIs (10, 15).

E7050 is an orally active Met-TKI (16) that has been shown to inhibit the

phosphorylation of Met, including amplified Met, and to suppress the growth of several types of cancer cells with *Met* amplification. Based on favorable pre-clinical data, a phase I clinical trial of E7050 is currently in progress. We have assessed whether E7050 can overcome the three HGF-induced resistance mechanisms to EGFR-TKIs.

Materials and Methods

Cell culture

The *EGFR* mutant human lung adenocarcinoma cell lines PC-9 and HCC827 were purchased from Immuno-Biological Laboratories Co. (Takasaki, Gunma, Japan) and the American Type Culture Collection (Manassas, VA), respectively. The human embryonic lung fibroblast cell line MRC-5 was purchased from Health Science Research Resources Bank (Sen-nan, Japan). MRC-5 (P 30-35) cells were maintained in DMEM with 10% fetal bovine serum (FBS). PC-9 and HCC827 cells were maintained in RPMI 1640 medium with 10% FBS.

Reagents

E7050 was synthesized by Eizai Co., Ltd., Ibaraki, Japan (16). Gefitinib was obtained from AstraZeneca (Cheshire, UK). The irreversible EGFR-TKI, BIBW2992, and the mutant-selective EGFR-TKI, WZ4002, were purchased from Selleck (Houston, TX). Recombinant HGF and anti-human HGF antibody were prepared as described (17).

Cell growth assay

Cell growth was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium] dye reduction method (18). Tumor cells were plated at a

density of 2×10^3 cells/100 μl/well into 96-well plates in RPMI1640 medium with 10% FBS. After 24-h incubation, various reagents were added to each well, and the cells incubated for a further 72 h, followed by the addition of 50 μl of MTT solution (2 mg/ml; Sigma, St. Louis, MO) to each well and further incubation for 2 h. The media containing MTT solution were removed, and the dark blue crystals were dissolved by adding 100 μl of DMSO. The absorbance of each well was measured with a microplate reader at test and reference wavelengths of 550 and 630 nm, respectively. The percentage of growth is shown relative to untreated controls. Each reagent and concentration was tested at least in triplicate during each experiment, and each experiment was performed at least 3 times.

Antibodies and western blotting

Cells were lysed in cell lysis buffer containing phosphatase and proteinase inhibitor cocktails (Sigma), and protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). For the detection of phosphorylated Met in subcutaneous tumors, 10mg tumor lysates were immunoprecipitaed with anti-Met (25H2) antibody. Total protein (40 μg/lane) was resolved by SDS-polyacrylamide gel electrophoresis, and the proteins were transferred onto PVDF membranes (Bio-Rad, Hercules, CA). After washing 4

times, the membranes were incubated with Blocking One (Nacalai Tesque Inc., Kyoto, Japan) for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies to Met (25H2), phospho-Met (Y1234/Y1235) (3D7), phospho EGFR (Y1068), ErbB3 (1B2), phospho-ErbB3 (Tyr1289) (21D3), Gab1 (#3232), phospho-Gab1 (Y627)(C32H2), Akt, and phospho-Akt (Ser473) (736E11) (1:1000 each; Cell Signaling Technology, Beverly, MA); and anti-human EGFR (1 μg/ml) antibody (R&D Systems, Minneapolis, MN). After washing 3 times, the membranes were incubated for 1 h at room temperature with species-specific horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology). Each experiment was performed at least 3 times independently.

HGF production

Cells (2×10^5) were cultured in RPMI1640 medium with 10% FBS for 24 h, washed with PBS and incubated for 48 h in 2 ml of the same medium. The culture medium was harvested and centrifuged, and the supernatant was stored at –70°C until analysis. HGF concentrations were measured by IMMUNIS HGF EIA (Institute of Immunology, Tokyo, Japan), with a detection limit of 100 pg/ml,

according to the manufacturer's instructions. All culture supernatants were tested in duplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

HGF gene transfection

One day before transfection, aliquots of 1×10^5 HCC827 cells in 1 ml of antibiotic-free medium were plated on 6-well plates. Full-length *HGF* cDNA cloned into the BCMGSneo expression vector (19) was transfected using Lipofectamine 2000 in accordance with the manufacturer's instructions. After 24-h incubation, the cells were washed with PBS and incubated for an additional 72 h in antibiotic-containing medium, followed by selection in G418 sulfate (Calbiochem, La Jolla, CA). After limiting dilution, HGF-producing cells, HCC827/HGF, were established. HGF production by HCC827/HGF cells was confirmed by ELISA.

RNA interference assay

Duplexed Stealth™ RNAi (Invitrogen, Carlsbad, CA) against *MET*, *ErbB3,* and *Gab1,* and Stealth™ RNAi Negative Control Low GC Duplex #3 (Invitrogen) were used for RNA interference assays. One day before transfection, aliquots of

2×10^4 tumor cells in 400 μl of antibiotic-free medium were plated on 24-well plates. After incubation for 24 h, the cells were transfected with siRNA (50 pmol) or scrambled RNA using Lipofectamine 2000 (1 μl) in accordance with the manufacturer's instructions. After 24-h incubation, the cells were washed with PBS and incubated with or without various reagents for an additional 72 h in antibiotic-containing medium. Cell growth was measured using a Cell Counting Kit-8 (Dojin, Tokyo, Japan) in accordance with the manufacturer's instructions. Knockdown of *MET*, *ErbB3, Gab1,* and, *Shc1* was confirmed by western blotting. Each reagent and concentration was tested at least in triplicate during each experiment, and each experiment was performed at least 3 times.

Detection of Met amplification

Cell block sections (4 μm thick) were subjected to dual-color fluorescence *in situ* hybridization (FISH) using a MET/CEP7 probe cocktail (Kreatech Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's instructions. Staining was evaluated as described (20).

Xenograft studies in SCID mice

Suspensions of PC-9 cells (5×10^6) mixed with MRC-5 cells (5×10^6) were injected subcutaneously into the backs of 5-week-old female SCID mice (Clea, Tokyo,

Japan), as described (13). After 4 days (tumor diameter > 5 mm), mice were randomly allocated into groups of 6 animals each to receive E7050 (50 mg/kg/day) and/or gefitinib (25 mg/kg/day) by oral gavage. Tumor volume was calculated as mm^3=$width^2$×length/2. All animal experiments were performed in compliance with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (Approval number: AP-081088).

Immunohistochemistry

Frozen sections (5 μm thick) of xenograft tumors were fixed with cold acetone and washed with PBS. After blocking endogenous peroxidase activity with 3% aqueous H_2O_2 solution for 10 min, the sections were incubated with 5% normal horse serum, followed by overnight incubation at 4°C with anti-phospho-Akt antibody (Ser473) (736E11, 1:100 dilution). The sections were washed with PBS, incubated with biotin-conjugated anti-rabbit IgG (1:200 dilution) for 30 min at room temperature, and incubated for 30 min with avidin–biotin–peroxidase complex (ABC) using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Staining was detected using the DAB (3,3'-diaminobenzidine tetrahydrochloride) Liquid System (DakoCytomation,

Glostrup, Denmark). Samples from which primary antibodies had been omitted served as negative controls.

Statistical analysis

Between group differences were analyzed by one-way ANOVA, with *P*-values <0.05 for overall comparisons tested by *post hoc* pairwise comparisons using the Newman-Keuls multiple comparison test. All statistical analyses were performed using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA).

Results

E7050 reverses resistance to EGFR-TKIs induced by exogenous HGF

PC-9 and HCC827 cells were highly sensitive to gefitinib (Fig. 1A), while exogenously added HGF induced resistance to gefitinib in both cell lines (9, 13, 14). Although E7050 did not affect the growth of PC-9 or HCC827 cells at concentrations <3 μM, the combination of E7050 with gefitinib reversed HGF-induced resistance of both cell lines in a concentration-dependent manner (Fig. 1B).

We previously reported that stromal fibroblasts are a source of exogenous HGF for EGFR-TKI naïve NSCLC and that fibroblast-derived HGF induces resistance to gefitinib and erlotinib in PC-9 and HCC827 cells (13). Although E7050 had no effect on the growth or production of HGF or VEGF by MRC-5 cells (HGF-high producing fibroblasts) or PC-9 cells (data not shown), it reversed the gefitinib resistance of PC-9 cells induced by co-culturing with MRC-5 cells (Fig. 1C), indicating that E7050 can reverse the EGFR-TKI resistance induced by exogenous HGF *in vitro*.

E7050 reverses resistance to EGFR-TKIs induced by endogenous HGF

We have shown that HGF is present in tumor cells of NSCLC patients with

acquired resistance to EGFR-TKIs, and that transient HGF gene transfection into PC-9 cells resulted in resistance to EGFR-TKIs (9). We therefore generated a stable HGF gene transfectant in HCC827 cells (HCC827/HGF) and assessed the effects of continuously produced endogenous HGF. HCC827/HGF, but not HCC827 or the vector control HCC827/Vec, cells secreted high levels of HGF and became resistant to gefitinib (Fig. 2A, B). Anti-HGF antibody reversed the gefitinib resistance of HCC827/HGF cells (Supplementary Fig. S1), indicating that endogenously produced HGF induced gefitinib resistance in this cell line. Although the combination of E7050 plus gefitinib successfully reversed the resistance of HCC827/HGF cells, E7050 alone did not inhibit the proliferation of HCC827/HGF cells (Fig. 2B).

Using western blotting, we examined the effects of E7050 on signal transduction in HCC827/Vec and HCC827/HGF cells. We found that gefitinib inhibited the phosphorylation of EGFR and ErbB3 in HCC827/Vec cells, thereby inhibiting the phosphorylation of Akt and ERK1/2. However, gefitinib failed to inhibit phosphorylation of Akt in the presence of HGF. E7050 suppressed the constitutive phosphorylation of Met, but not of EGFR, ErbB3, and downstream Akt and ERK1/2. While HGF stimulated the phosphorylation of Met, E7050 plus

gefitinib inhibited this HGF-induced Met phosphorylation, and strongly suppressed the phosphorylation of Gab1, Akt, and ERK1/2 (Fig. 2C).

The amount of Met protein was decreased in HCC827/HGF cells, compared with HCC827/Vec cells. This could be a result of Met downregulation by persistent HGF stimulation, as also observed in a previous report (21). In contrast, the degree of Met phosphorylation was higher in HCC827/HGF than in HCC827/Vec cells. Gefitinib inhibited the phosphorylation of EGFR and ErbB3, but not of Akt in HCC827/HGF cells. The combination of E7050 and gefitinib inhibited the phosphorylation of both Met and Akt (Fig. 2C). These results suggest that E7050 reversed HGF-induced gefitinib resistance by inhibiting the Met/Gab1/PI3K/Akt pathway.

To confirm that the E7050 reversal of gefitinib resistance in HCC827/HGF cells was due to the inhibition of Met/Gab1, we transfected cells with siRNA specific for *Met* or *Gab1*. Transfection of *ErbB3*, *Met, or Gab1* siRNA successfully knocked down the expression of the corresponding protein (Fig. 2D). While scrambled or *ErbB3* siRNA did not reverse the gefitinib resistance of HCC827/HGF cells, siRNAs for *Met* and *Gab1* sensitized these cells to gefitinib (Fig. 2D), indicating that E7050 reverses gefitinib resistance in HCC827/HGF

cells by inhibiting the Met/Gab1 pathway.

E7050 reverses HGF-induced resistance to next-generation EGFR-TKIs in H1975 cells

Next-generation EGFR-TKIs, irreversible TKIs (22 – 24) and mutant *EGFR* selective TKIs (25) have been developed to treat gefitinib-resistant tumors caused by the *EGFR* T790M secondary mutation. H1975 cells with the *EGFR* mutations L858R and T790M mutations were resistant to reversible EGFR-TKIs, gefitinib and erlotinib (data not shown), but were sensitive to BIBW2992, an irreversible EGFR-TKI, and WZ4002, a mutant-selective EGFR-TKI (Fig. 3). HGF markedly induced resistance to BIBW2992 and WZ4002, whereas E7050 efficiently reversed the HGF-induced resistance to both BIBW2992 and WZ4002. These results indicate that E7050 can overcome HGF-induced resistance not only to gefitinib but to next-generation EGFR-TKIs, including irreversible and mutant-selective EGFR-TKIs.

E7050 prevents emergence of gefitinib-resistant HCC827 cells induced by continuous exposure to HGF

As HGF has been reported to accelerate the expansion of preexisting *Met*-amplified HCC827 cells and to facilitate *Met* amplification-mediated

resistance during EGFR-TKI treatment (10), we examined the effects of E7050 on these phenomena. Although HCC827 cells did not produce viable colonies after 30 days of continuous exposure to gefitinib alone (Fig. 4A, B), these cells produced many colonies after exposure to both HGF and gefitinib. In contrast to previous findings (10), the percentage of cells with *Met* amplification was not increased when compared with parental HCC827 cells. The reason for this discrepancy remains unclear. Western blot analyses revealed that while the resultant cells expressed the same level of Met and Gab1 proteins compared with parental HCC827 cells, they expressed much higher levels of phosphorylated Met and Gab1 (Supplementary fig S2).

Importantly, E7050 prevented the emergence of viable clones even under conditions of continuous exposure to gefitinib and HGF (Fig. 4B). These results suggest the potential of E7050 to abrogate the effects resulting from continuous exposure to HGF.

E7050 circumvents HGF-induced resistance when combined with gefitinib *in vivo*

To investigate the therapeutic efficacy of E7050 *in vivo,* we used the gefitinib resistance model previously described (13). We mixed PC-9 cells with the

HGF-high producing fibroblast cell line, MRC-5, and inoculated SCID mice subcutaneously with this mixture. Oral treatment with gefitinib and/or E7050 was started after the establishment of solid tumors on day 4. Consistent with previous observations, we found that treatment with gefitinib alone prevented the enlargement of tumors produced by the mixture of PC-9 and MRC-5 cells, but did not cause tumor regression. As gefitinib induces shrinkage of PC-9 tumors (13, 14), our results suggest that MRC-5 cells induced gefitinib resistance *in vivo*. Under these experimental conditions, treatment with E7050 alone did not inhibit tumor growth, whereas the combination of E7050 and gefitinib induced marked tumor regression (Fig. 5A and B).

To confirm that E7050 inhibits Met/PI3K/Akt signaling *in vivo*, we assessed expression of phosphorylated Met and Akt in the xenograft tumors. Immunoprecipitation revealed that phosphorylated Met was detected in control tumors and gefitinib-treated tumors but not in tumors treated with E7050 monotherapy or E7050 plus gefitinib (Fig 5C), indicating efficacy of E7050 as a Met kinase inhibitor. Moreover, we observed higher levels of phosphorylated Akt in control cancer cells, with this phosphorylation slightly decreased by either E7050 or gefitinib alone and markedly inhibited by the combination of E7050 and

gefitinib (Fig. 5D). In addition, there were no discernible differences in HGF concentrations between control and treated groups, when HGF protein concentrations were determined by EIA using lysates of tumors obtained after 5 days of treatment (Supplementary fig S3). These results suggest that E7050 overcame the gefitinib resistance associated with inhibition of the Met/Akt pathway.

Discussion

HGF is a multifunctional cytokine that can be produced not only by cancer cells but also by stromal cells, such as fibroblasts. The HGF receptor, Met, and EGFR interact with each other and mediate redundant signaling (26). Elevated serum concentrations of EGFR ligands and HGF were detected in patients with NSCLC, and HGF expression has been associated with poor prognosis in patients resected for NSCLC (27-28). While the role of HGF in *EGFR* mutant lung cancer remained unclear, we observed HGF-induced EGFR-TKI resistance in *EGFR* mutant lung cancers (9). Moreover, many studies have shown the important roles of HGF in sensitivity to molecular targeted drugs. Our observations regarding EGFR-TKI in lung cancer were confirmed by subsequent studies (10, 29) and the concentrations of HGF in peripheral blood were found to be inversely correlated with clinical responses to EGFR-TKIs, in both *EGFR* mutant and wild-type lung cancer (30, 31). HGF was also found to cause resistance to sunitinib, a multi-kinase inhibitor, in renal cell carcinoma by compensating for inhibited angiogenesis (32). Taken together, these findings indicate the importance of HGF as a therapeutic target for drug resistance in cancer.

We have shown here that a new Met-TKI, E7050, reversed three HGF-induced resistance mechanisms in *EGFR* mutant lung cancer. First, E7050 reversed HGF-induced gefitinib resistance by inhibiting Met phosphorylation and thereby suppressing the downstream PI3K/Akt pathway. Second, E7050 inhibited the HGF-induced resistance to next-generation EGFR-TKIs, irreversible EGFR-TKIs and mutant-selective EGFR-TKIs. Third, E7050 prevented the emergence of resistant clones induced by continuous exposure to HGF.

An interaction between HGF and *Met* amplification has been associated with EGFR-TKI resistance in lung cancer (10). In the presence of gefitinib, continuous exposure to HGF accelerated the expansion of preexisting *Met* amplified HCC827 cells. Unexpectedly, when we cultured HCC827 cells with gefitinib and HGF for 30 days, we found that the percentage of cells with *Met* amplification was not increased. The reason we failed to detect expansion of clones with *Met* amplification, however, remains unclear. Transfection of the HGF gene into HCC827 cells produced HCC827/HGF cells, which constitutively produce HGF. These cells, however, were selected in the presence of geneticin but not gefitinib, with several clones showing amplification of *Met* (data not shown). Therefore,

this phenomenon may be unique to a population of *EGFR* mutant lung cancer cells observed only under selection pressure with gefitinib plus an as yet unknown concentration of HGF.

Met was shown to be constitutively phosphorylated in human lung cancer cell lines, with the degree of phosphorylation not always correlated with susceptibility to EGFR-TKIs (33). Indeed, previous studies reported that the level of Met phosphorylation was higher in HCC827 cells than in other *EGFR* mutant cell lines (9, 10, 13, 29). Similar to these results, we also observed that the level of Met phosphorylation was higher in HCC827 cells than in PC-9 and Ma-1 cells (Supplementary fig S4). While the bands for pMet in our study seem to be weaker than those in a previous study (34), ours and previous studies constantly showed that Met phosphorylation in HCC827 cells was higher than that in other *EGFR* mutant cells. While the difference in the intensity of pMet bands between our study and the previous is unclear, it might be due to minor differences in experimental conditions, including the exposure time at western blot and the cell culture conditions. Regarding HGF-triggered EGFR-TKI resistance, previous studies also support our findings that while HCC827 cells were highly sensitive to EGFR-TKIs, further Met activation or phosphorylation resulted in inducing

resistance to EGFR-TKIs (10, 29, 35). We confirmed that knockdown of Met by siRNA canceled HGF-induced resistance in HCC827 cells (9). Moreover, it was reported that *Met* amplification resulted in increased level of Met phosphorylation and caused resistance to EGFR-TKIs in HCC827 cells (8). This accumulating evidence indicates that constitutive Met phosphorylation is insufficient and further activation by HGF or *Met* amplification may be necessary to induce EGFR-TKI resistance in HCC827 cells. Therefore, there may be a threshold level for Met phosphorylation to sufficiently cause EGFR-TKI resistance.

E7050 inhibits both Met and VEGFR2 kinases (16). *In vitro*, PC-9 and HCC827 cells express little VEGFR2 (data not shown). E7050 did not significantly inhibit the growth of these cell lines, and the anti-VEGF antibody bevacizumab did not augment the susceptibility of these cell lines to gefitinib (data not shown). These results suggest that the *in vitro* anti-tumor effects of E7050, when combined with gefitinib and HGF, may be largely due to Met inhibition. *In vivo*, we found that very high concentrations of HGF, obtained by HGF gene transfection into cancer cells, increased intratumor vessel density (submitted for publication elsewhere). However, HGF concentrations were lower in our xenograft model of mixed PC-9 and MRC-5 cells (fibroblasts) than in

xenograft tumors produced by HGF-gene transfected lung cancer cells. We observed no difference in intratumor vessel density between tumors induced by PC-9 cells alone and tumors induced by PC-9 and MRC-5 cells (Supplementary fig S5). In addition, E7050 did not affect significantly the vessel density in tumors induced by PC-9 and MRC-5 cells. Collectively, these observations suggest that the anti-tumor effects of E7050 in this resistance model may not be predominantly due to angiogenesis inhibition.

The secondary T790M mutation in *EGFR* is the most prominent mechanism of acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer, with this mutation detected in about 50% of these patients (4). The T790M mutation increases the affinity of EGFR for ATP, decreasing the binding of EGFR to EGFR-TKIs, and inducing resistance to the latter agents (36). *EGFR* mutant lung cancer cells with the T790M secondary mutation, however, remain susceptible to EGFR mediating signaling and are thought to be manageable by inhibition of EGFR-mediated signaling (37). Preclinical studies have shown that next-generation EGFR-TKIs, irreversible TKIs and mutant EGFR-selective TKIs have activity against gefitinib-resistant tumors with EGFR T790M secondary mutation (21 – 23). However, several irreversible EGFR-TKIs, including

BIBW2992 (38) and HKI-272 (39), failed to meet primary endpoints in clinical trials of patients with EGFR-TKI refractory lung cancer. High concentrations of HGF have been frequently detected in tumors with *EGFR*-T790M secondary mutations showing acquired resistance (10, 40, 41). In addition, we found previously (11) and confirmed here that HGF induces resistance to irreversible EGFR-TKIs in *EGFR* mutant lung cancer cells. Taken together, these observations suggest that HGF expressed in tumors with acquired resistance and *EGFR* T790M secondary mutations induce resistance to irreversible EGFR-TKI. As E7050 reversed the resistance to irreversible and mutant-selective EGFR-TKIs, it may augment the therapeutic efficacy of next-generation EGFR-TKIs in *EGFR* mutant lung cancer patients with acquired resistance to the *EGFR* T790M secondary mutation. These ideas further illustrate the necessity of methods to select patients who develop EGFR-TKI resistance due to HGF.

In conclusion, we have presented preclinical evidence showing that a new Met kinase inhibitor, E7050, may overcome HGF-induced resistance in *EGFR* mutant lung cancer. Further evaluation of E7050 in clinical trials is warranted to improve the outcomes of patients with *EGFR* mutant lung cancer.

Acknowledgments

We are grateful to Mr. Kenji Kita for technical assistance and fruitful discussion.

Grant support

This study was supported by Grants-in-Aid for Cancer Research (S. Yano, 21390256) and Scientific Research on Innovative Areas “Integrative Research on Cancer Microenvironment Network” (S. Yano, 22112010A01) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129-39.

2. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497-500.

3. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol 2010;11:121-8.

4. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010;362:2380-8.

5. Jackman D, Pao W, Riely GJ, Engelman JA, Kris MG, Janne PA, et al. Clinical definition of acquired resistance to epidermal growth factor

receptor tyrosine kinase inhibitors in non-small-cell lung cancer. J Clin Oncol 2010;28:357-60.

6. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786-92.

7. Pao W, Miller VA, Politi KA, Janne PA, Kocher O, Meyerson M, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:e73.

8. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039-43.

9. Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma cells with EGF receptor mutations. Cancer Res 2008;68:9479-87.

10. Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell 2010;17:77-88.

11. Yamada T, Matsumoto K, Wang W, Li Q, Nishioka Y, Sekido Y, et al. Hepatocyte growth factor reduces susceptibility to an irreversible epidermal growth factor receptor inhibitor in EGFR-T790M mutant lung cancer. Clin Cancer Res 2010;16:174-83.

12. Matsumoto K, Nakamura T. Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions. Int J Cancer 2006;119:477-83.

13. Wang W, Li Q, Yamada T, Matsumoto K, Matsumoto I, Oda M, et al. Crosstalk to stromal fibroblasts induces resistance of lung cancer to EGFR tyrosine kinase inhibitors. Clin Cancer Res 2009;15;6630-8.

14. Donev IS, Wang W, Yamada T, Li Q, Takeuchi S, Matsumoto K, et al. Transient PI3K inhibition induces apoptosis and overcomes HGF-mediated resistance to EGFR-TKIs in EGFR mutant lung cancer. Clin Cancer Res 2011; 17:2260-9.

15. Kim ES, Salgia R. MET pathway as a therapeutic target. J Thorac Oncol 2009;4:444-7.

16. Nakagawa T, Tohyama O, Yamaguchi A, Matsushima T, Takahashi K, Funasaka S, et al. E7050: a dual c-Met and VEGFR-2 tyrosine kinase inhibitor promotes tumor regression and prolongs survival in mouse xenograft models. Cancer Sci 2010;101:210-5.

17. Montesano R, Matsumoto K, Nakamura T, Orci L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell 1991;67:901-8.

18. Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. J Immunol Methods 1984;70:257-68.

19. Nishioka Y, Yano S, Fujiki F, Mukaida N, Matsushima K, Tsuruo T, et al. Combined therapy of multidrug-resistant human lung cancer with anti-P-glycoprotein antibody and monocyte chemoattractant protein-1 gene transduction: the possibility of immunological overcoming of multidrug resistance. Int J Cancer 1997;71:170-7.

20. Cappuzzo F, Marchetti A, Skokan M, Rossi E, Gajapathy S, Felicioni L, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. J Clin Oncol 2009;27:1667-74.

21. Navab R, Liu J, Seiden-Long I, Shih W, Li M, Bandarchi B, et al. Co-overexpression of Met and hepatocyte growth factor promotes systemic metastasis in NCI-H460 non-small cell lung carcinoma cells. Neoplasia. 2009;11:1292-300.

22. Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, Chilieac LR, et al. BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. Oncogene 2008;27:4702-11.

23. Kobayashi S, Ji H, Yuza Y, Meyerson M, Wong KK, Tenen DG, et al. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. Cancer Res 2005;65:7096-101.

24. Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci U S A 2005;102:7665-70.

25. Zhou W, Ercan D, Chen L, Yun CH, Capellletti M, Cortot AB, et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. Nature 2009;462:1070-4.

26. Stabile LP, Rothstein ME, Keohavong P, Lenzner D, Land SR, Gaither-Davis AL, et al. Targeting of both EGFR and c-Met pathways results in additive inhibition of lung tumorigenesis in transgenic mice. Cancers, 2010, 2 2153-2170.

27. Siegfried JM, Weissfeld LA, Singh-Kaw P, Weyant RJ, Testa JR, Landreneau RJ. Association of immunoreactive hepatocyte growth factor with poor survival in resectable non-small cell lung cancer. Cancer Res 1997;57:433-439.

28. Ma PC, Tretiakova MS, MacKinnon AC, Ramnath N, Johnson C, Dietrich S, et al. Expression and mutational analysis of MET in human solid cancers. Genes, Chromosomes & Cancer 2008; 47:1025-1037.

29. Benedettini E, Sholl LM, Peyton M, Reilly J, Ware C, Davis L, et al. Met activation in non-small cell lung cancer is associated with de novo resistance to EGFR inhibitors and the development of brain metastasis. Am J Pathol 2010;177:415-23.

30. Kasahara K, Arao T, Sakai K, Matusmoto K, Sakai A, Kimura H, et al. Impact of serum hepatocyte growth factor on treatment response to epidermal growth factor receptor tyrosine kinase inhibitors in patients with non-small cell lung adenocarcinoma. Clin Cancer Res 2010;16:4616-24.

31. Tanaka H, Kimura T, Kudoh S, Mitsuoka S, Watanabe T, Suzumura T, et al. Reaction of plasma hepatocyte growth factor levels in non-small cell lung cancer patients treated with EGFR-TKIs. Int J Cancer 2011 Sep 15;129:1410-6.

32. Shojaei F, Lee JH, Simmons BH, Wong A, Esparza PA, Plumlee PA, et al. HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. Cancer Res. 2010;70:10090-100.

33. Kubo T, Yamamoto H, Lockwood WW, Valencia I, Soh J, Peyton M, et al. MET gene amplification or EGFR mutation activate MET in lung cancers untreated with EGFR tyrosine kinase inhibitors. Int J Cancer. 2009; 124:1778-84.

34. Guo A, Villén J, Kornhauser J, Lee KA, Stokes MP, Rikova K, et al. Signaling networks assembled by oncogenic EGFR and c-Met. Proc Natl Acad Sci U S A. 2008;105:692-7.

35. Okamoto W, Okamoto I, Tanaka K, et al. TAK-701, a humanized monoclonal antibody to hepatocyte growth factor, reverses gefitinib resistance induced by tumor-derived HGF in non-small cell lung cancer with an EGFR mutation. Mol Cancer Ther 2010;9:2785–2792.

36. Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc Natl Acad Sci U S A 2008;105:2070-5.

37. Nguyen KS, Kobayashi S, Costa DB. Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancers dependent on the epidermal growth factor receptor pathway. Clin Lung Cancer 2009;10:281-9.

38. Miller VA, Hirsh V, Cadranel J, Chen YM, Park K, Kim SW, et al. Phase IIB/III double-blind randomized trial of AFATINIB (BIBW 2992), an irreversible inhibitor of EGFR/HER1 and HER2) + best supportive care (BSC) versus placebo plus BSC in patients with NSCLC failing 1-2 lines of chemotherapy and ERLOTINIB or GEFITINIB (LUX-lung 1). Ann Oncol 2010;21(Supplement 8):viii1- viii2.

39. Sequist LV, Besse B, Lynch TJ, Miller VA, Wong KK, Gitlits B, et al. Neratinib, an irreversible pan-ErbB receptor tyrosine kinase inhibitor: results of a phase II trial in patients with advanced non-small-cell lung cancer. J Clin Oncol 2010;28:3076-83.

40. Onitsuka T, Uramoto H, Nose N, Takenoyama M, Hanagiri T, Sugio K, et al. Acquired resistance to gefitinib: the contribution of mechanisms other than the T790M, MET, and HGF status. Lung Cancer 2010;68:198-203.

41. Yano S, Yamada T, Takeuchi S, Tachibana K, Minami Y, Yatabe Y, et al. Hepatocyte growth factor expression in EGFR mutant lung cancer with intrinsic and acquired resistance to tyrosine kinase inhibitors in a Japanese cohort. J Thorac Oncol 2011;6:2011-7.

Legends for figures

Figure 1. E7050 reverses resistance to EGFR-TKIs induced by exogenous HGF. **A,** PC-9 and HCC827 cells were incubated with various concentrations of gefitinib, with or without HGF (20 ng/ml). **B,** PC-9 and HCC827 cells were incubated with various concentrations of E7050, with or without HGF (20 ng/ml) and/or gefitinib (0.3 μM). **C,** Cells were cocultured in Transwell chambers separated by 8 μm pore filters. PC-9 and HCC827 cells (8×10^3 cells/700 μL) with gefitinib and or E7050 (0.3 μM) were placed in the lower chambers, and MRC-5 fibroblasts (10^4 cells/300 μL), producing high concentrations of HGF, were placed in the upper chambers. After 72 h, the upper chambers were removed, and cell growth was measured using the MTT-assay. Bars indicate SD. *$p<0.01$.

Figure 2. E7050 reverses resistance to EGFR-TKIs induced by endogenous HGF.

A, Cells (2×10^5/2 ml) were incubated for 48 h, and concentrations of HGF in the culture supernatants were determined by ELISA. **B,** HCC827/Vec and HCC827/HGF cells were incubated with various concentrations of gefitinib, with

or without E7050. Cell growth was determined by MTT assays. **C,** HCC827/Vec and HCC827/HGF cells were incubated with HGF (20 ng/ml), E7050 (1 μM), and/or gefitinib (1 μM) for 1 h. The cell lysates were harvested and phosphorylation of indicated proteins was determined by western blotting. **D,** HCC827/HGF cells were treated with or without *ErbB3*, *Met*, or *Gab1* siRNA or scrambled siRNA for 24 h, followed by further incubation in medium for 48 h. The cell lysates were harvested and western blotting was performed to determine the expression of the indicated proteins. Cell growth after 72h was determined using MTT assays. Bars indicate SD. *p<0.01.

Figure 3. E7050 reverses HGF-induced resistance to next-generation EGFR-TKIs in H1975 cells. H1975 cells were incubated for 72 h with various concentrations of BIBW2992 **(A),** an irreversible EGFR-TKI, and WZ4002 **(B)**, a mutant-selective EGFR-TKI, in the presence or absence of HGF (20 ng/ml) and/or E7050 (1 μM). Cell growth was determined by MTT assays. Bars indicate SD.

Figure 4. E7050 prevents the emergence of gefitinib-resistant HCC827 cells

with amplified *Met* induced by continuous exposure to HGF. A, HCC827 cells were incubated with or without gefitinib (1 μM), HGF (20 ng/ml), and/or E7050 (1 μM), changing the medium every 3 days. After 30 days, viable colonies were stained with crystal violet. **B,** Representative cultures.

Figure 5. E7050 circumvents HGF-induced resistance when combined with gefitinib *in vivo.* A, PC-9 cells (5×10^6) with or without MRC-5 cells (5×10^6) were inoculated subcutaneously into SCID mice (N=6) on day 0. The mice began treatment with oral gefitinib (25 mg/kg/day) and/or E7050 (50 mg/kg/day), on day 4. The tumor area was measured every 3 or 4 days and the tumor volume was calculated as described in *Materials and Methods*. Bars show SE of the means ±SD. $p<0.01$. **B,** Macroscopic appearance of treated tumors harvested on day 17. **C,** Expression of phosphorylated Met in harvested tumors. Met protein was immunoprecipitated by anti-Met antibody. Then, phosphorylated Met and Met protein were detected by anti-phosphotyrosine antibody and anti-Met antibody, respectively. **D,** Expression of phosphorylated Akt in the harvested tumors. Frozen sections were immunohistochemically stained with anti-phospho Akt antibody. Original magnification, ×200.

Figure 1

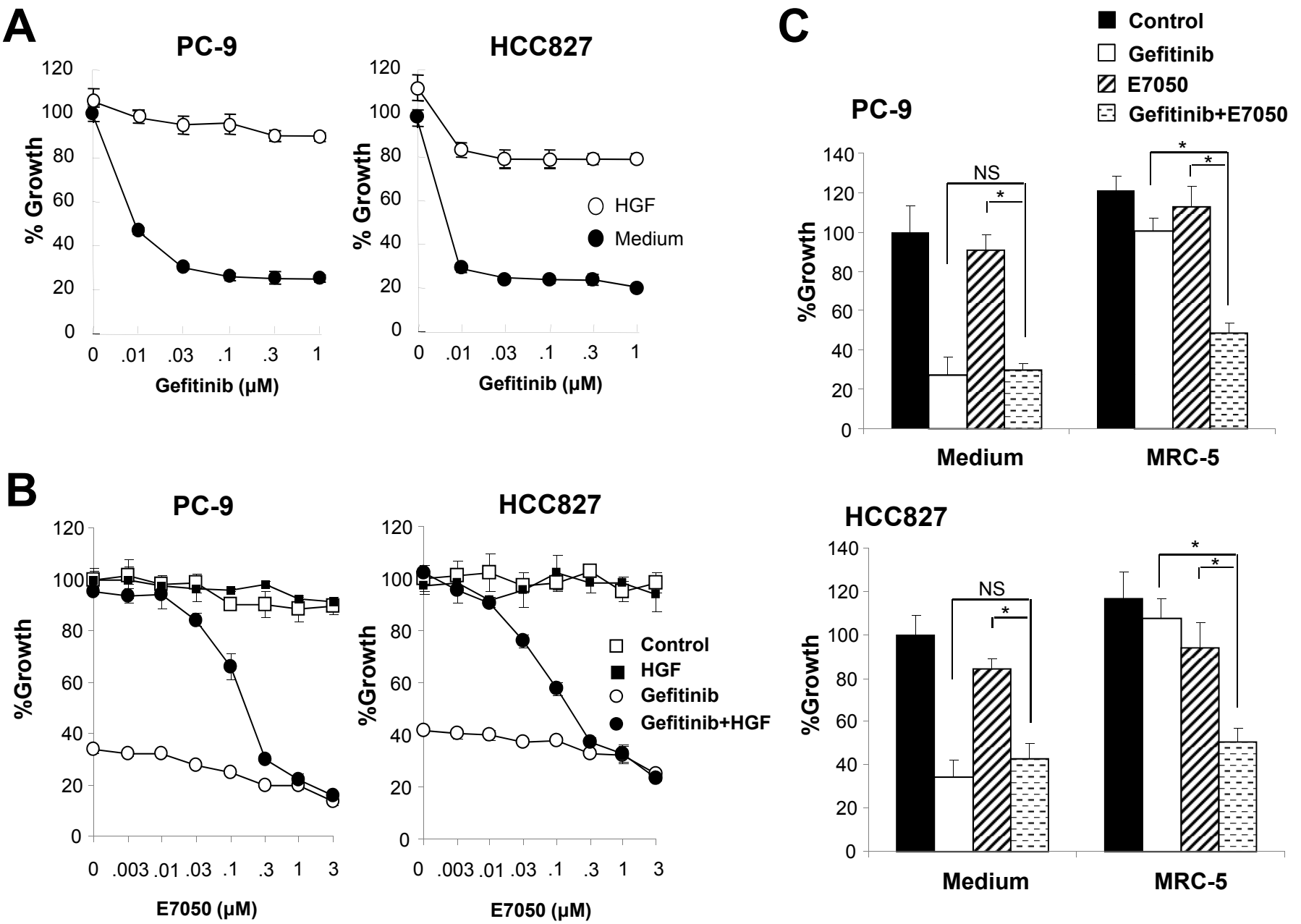

Figure 2

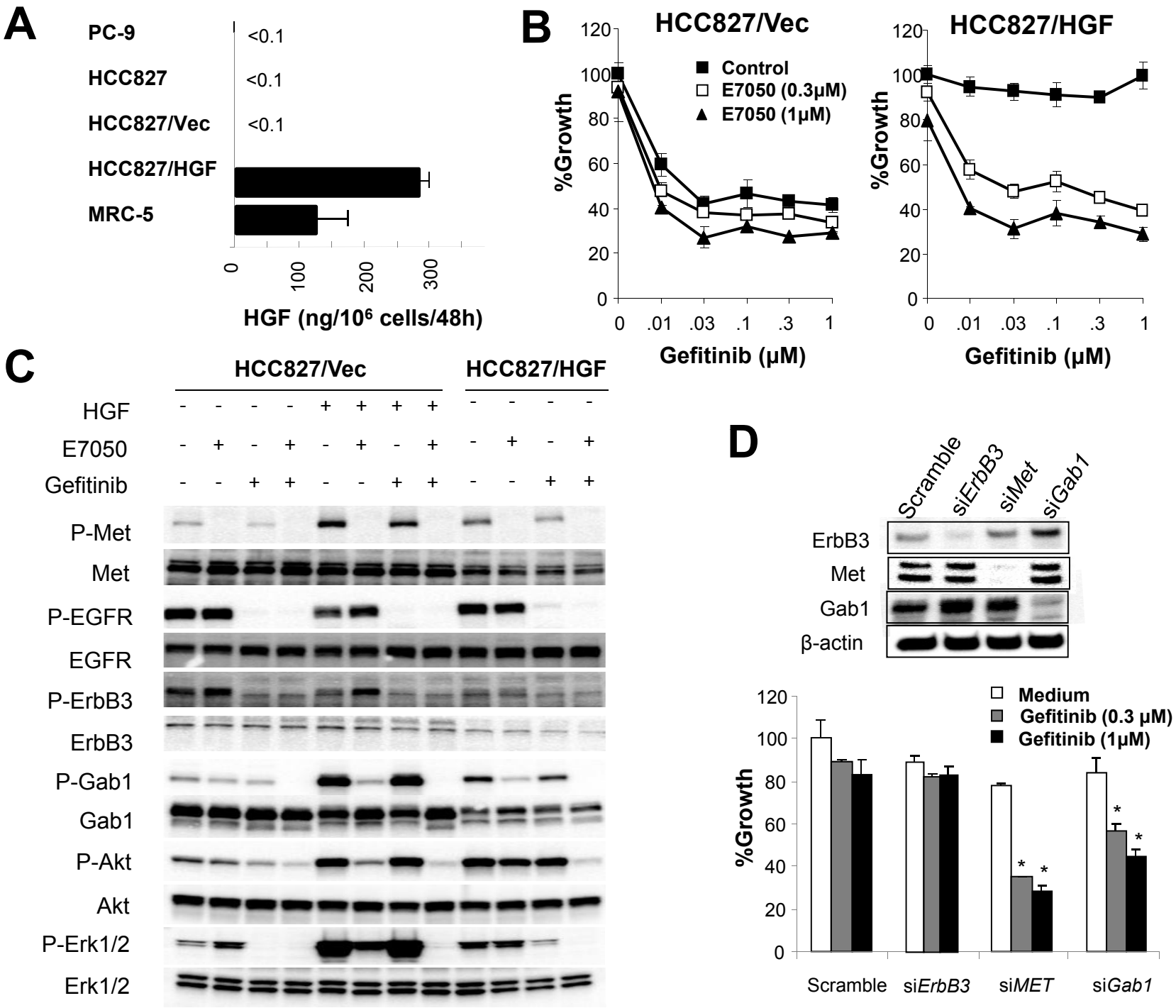

Figure 3

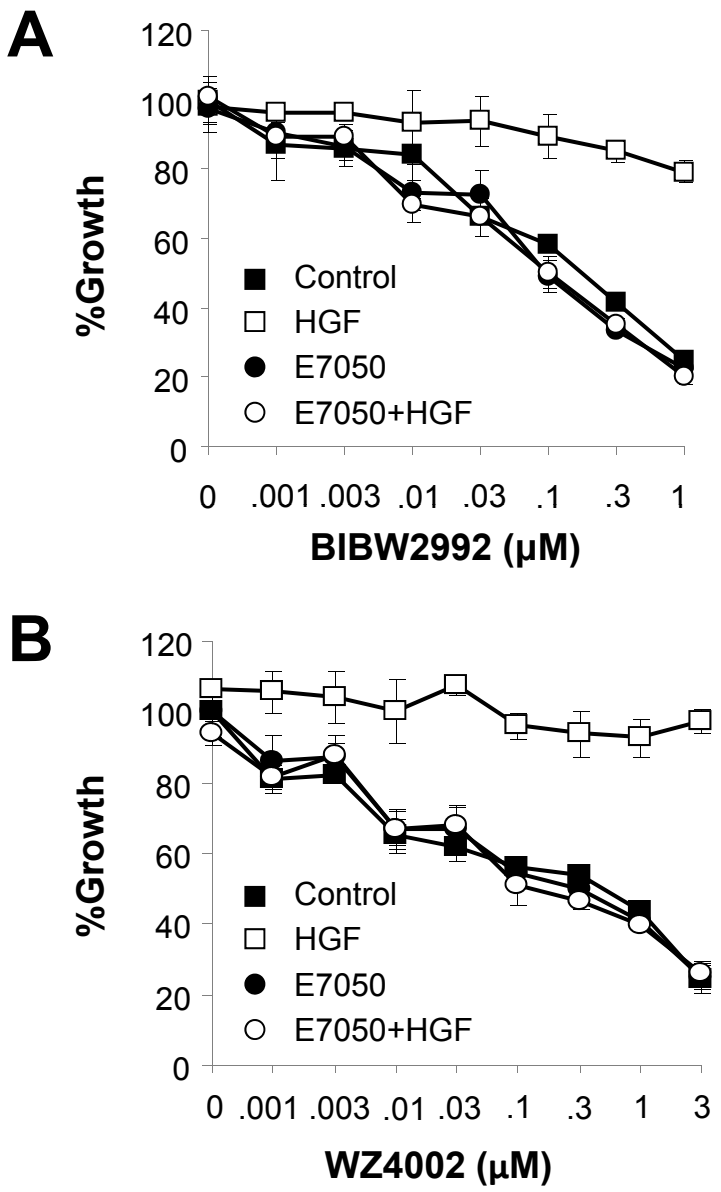

Figure 4

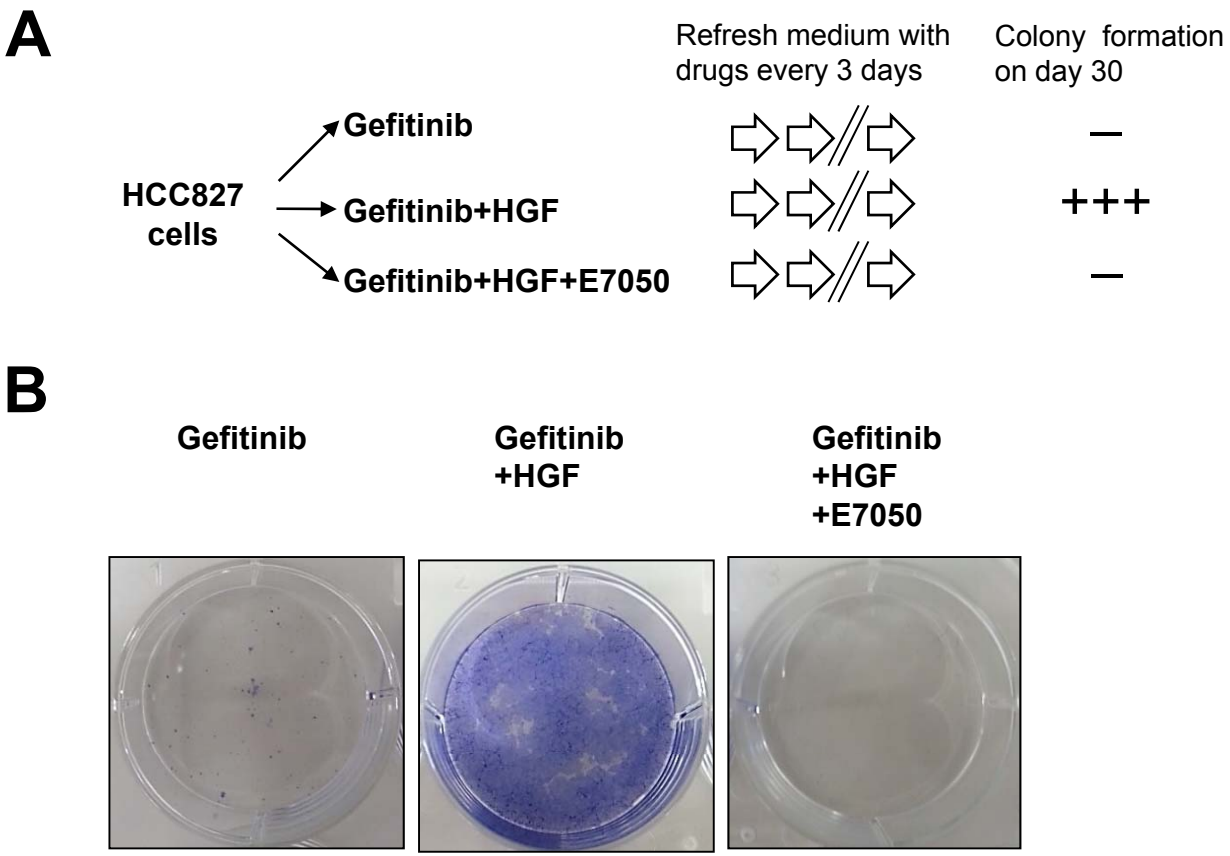

Figure 5