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Hepatocyte Growth Factor Reduces Susceptibility to an Irreversible Epidermal Growth Factor Receptor Inhibitor in EGFR-T790M Mutant Lung Cancer

Tadaaki Yamada[1], Kunio Matsumoto[2], Wei Wang[1], Qi Li[1], Yasuhiko Nishioka[3], Yoshitaka Sekido[4], Saburo Sone[3], and Seiji Yano[1,5]

[1)] Divisions of Medical Oncology, Cancer Research Institute, Kanazawa University, 13-1, Takara-machi, Kanazawa, Ishikawa 920-0934, Japan

[2)] Divisions of Tumor Dynamics and Regulation, Cancer Research Institute, Kanazawa University, Japan

[3)] Department of Respiratory Medicine & Rheumatology, Institute of Health Biosciences, The University of Tokushima Graduate School, Japan

[4)] Division of Molecular Oncology, Aichi Cancer Center Research Institute, Japan

[5)] To whom correspondence should be addressed.

E-mail: syano@staff.kanazawa-u.ac.jp

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HGF Reduces Susceptibility to an Irreversible EGFR-TKI

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Abbreviations:

NSCLC, Non-small cell lung cancer: EGFR, Epidermal growth factor receptor: EGFR-TKI, EGFR tyrosine kinase inhibitor: T790M, methionine for threonine at position 790: HGF, hepatocyte growth factor: EGF, epidermal growth factor: IGF-I, insulin-like growth factor I: TGF-α, transforming growth factor α: ERK, extracellular signal-regulated kinase: MET-TKI, MET tyrosine kinase inhibitor: ADCC, antibody-dependent cellular cytotoxicity

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Statement of translational relevance:

The secondary T790M mutation in *EGFR* is the most frequent cause of acquired resistance to the reversible EGFR tyrosine kinase inhibitors (EGFR-TKIs), gefitinib and erlotinib, in lung cancer. Irreversible EGFR-TKIs are potentially useful agents to control the reversible EGFR-TKI-resistance of lung cancer. Although some of these inhibitors are in clinical development and early signs of success have been reported in lung cancer patients who were refractory to the reversible EGFR-TKI, it is clear that resistance may also develop against this class of inhibitors.

In this study, we demonstrated that hepatocyte growth factor (HGF) reduced susceptibility to irreversible EGFR-TKI in lung cancer cells with the secondary T790M mutation in *EGFR*. We further demonstrated that HGF-MET inhibitors could circumvent the HGF-induced hyposensitivity to irreversible EGFR-TKI. Our findings provide a novel insight into the involvement of HGF-MET-mediated signaling in acquired resistance to irreversible EGFR-TKIs in lung cancer harboring T790M mutation in *EGFR*.

Abstract

Purpose

The secondary T790M mutation in epidermal growth factor receptor (*EGFR*) is the most frequent cause of acquired resistance to the reversible EGFR tyrosine kinase inhibitors (EGFR-TKIs), gefitinib and erlotinib, in lung cancer. Irreversible EGFR-TKIs are expected to overcome the reversible EGFR-TKI-resistance of lung cancer harboring T790M mutation in *EGFR*. However, it is clear that resistance may also develop to this class of inhibitors. We demonstrated previously that hepatocyte growth factor (HGF) induced gefitinib resistance of lung cancer harboring *EGFR*-activating mutations. Here, we investigated whether HGF induced resistance to the irreversible EGFR-TKI, CL-387,785 in lung cancer cells (H1975) harboring both L858R activating mutation and T790M secondary mutation in *EGFR.*

Experimental Design

CL-387,785 sensitivity and signal transduction in H1975 cells were examined in the presence or absence of HGF or HGF-producing fibroblasts, with or without HGF-MET inhibitors.

Results

HGF reduced susceptibility to CL-387,785 in H1975 cells. Western blotting and siRNA analyses indicated that HGF-induced hyposensitivity was mediated by the MET/PI3K/Akt signaling pathway, independent of EGFR, ErbB2, ErbB3, and ErbB4. Hyposensitivity of H1975 cells to CL-387,785 was also induced by co-culture with high-level HGF-producing lung fibroblasts. The hyposensitivity

was abrogated by treatment with anti-HGF neutralizing antibody, HGF antagonist NK4, or MET-TKI.

Conclusions

We demonstrated HGF-mediated hyposensitivity as a novel mechanism of resistance to irreversible EGFR-TKIs. It will be clinically valuable to investigate the involvement of HGF-MET-mediated signaling in de novo and acquired resistance to irreversible EGFR-TKIs in lung cancer harboring T790M mutation in *EGFR*.

Introduction

Lung cancer is the most common cause of malignancy-related death worldwide and its incidence is still increasing. Non-small cell lung cancer (NSCLC) accounts for nearly 80% of cases of lung cancer. Median survival of metastatic NSCLC is 8 to 10 months even if treated with the most active combination of conventional chemotherapeutic agents (1, 2). Epidermal growth factor receptor (EGFR) activating mutations, such as deletion in exon 19 and L858R point mutation in exon 21(3), were found in NSCLC. These mutations are predominantly found in female, non-smoking, adenocarcinoma patients, and in patients of East Asian origin, and are associated with favorable response to the reversible EGFR tyrosine kinase inhibitors (EGFR-TKI), gefitinib and erlotinib (4). Several prospective clinical trials showed that 70% to 75% of NSCLC patients with tumors harboring these mutations respond to gefitinib or erlotinib (3, 5). However, even patients who show a marked response to initial treatment also develop acquired resistance to the EGFR tyrosine kinase inhibitors almost without exception after varying periods (3).

Several mechanisms, including T790M secondary mutation in *EGFR* (6, 7), *MET* amplification (8), and overexpression of hepatocyte growth factor (HGF) (9), were reported to induce acquired resistance to reversible EGFR-TKI for NSCLC with EGFR-activating mutations. The first mechanism of acquired resistance described was acquisition of the T790M *EGFR* mutation. The methionine residue at position 790 generates a bulkier side chain that either affects binding of TKIs or enhances the affinity of the EGFR tyrosine kinase

pocket to ATP (7), and this enhanced ATP affinity decreases the effective binding of gefitinib and erlotinib to the tyrosine kinase pocket of EGFR (10). T790M in *EGFR* is found most frequently (~50%) in patients with acquired resistance to EGFR-TKI (7, 11). A minor population of clones with this second mutation (T790M) is thought to exist in the tumor before treatment and to be selected and expand during continuous treatment with gefitinib or erlotinib, and hence develop a resistant phenotype (3,12,13). Resistance mediated by secondary T790M mutation is thought to be manageable by irreversible EGFR inhibitors, such as CL-387,785, PF00299804, HKI-272, and EKB-569, which bind covalently to Cys-797 of EGFR (14-17). Although some of these inhibitors are in clinical development and early signs of success have been reported in lung cancer patients who were refractory to gefitinib or erlotinib (18-20), it is clear that resistance may also develop against this class of inhibitors.

We recently demonstrated that HGF induces gefitinib resistance in lung cancer harboring EGFR-activating mutation by activating its receptor MET and downstream PI3K/Akt pathway. This mechanism can be involved in both intrinsic resistance and acquired resistance to gefitinib (9). HGF was originally identified as a mitogenic protein for hepatocytes and has been shown to have pleiotropic biological activities (21). HGF and its receptor MET are expressed at various levels in various types of cancer cells, including lung cancer (22–25). A recent study demonstrated that HGF was strongly expressed in 5 of 7 specimens with T790M second mutation obtained from lung cancer patients who developed acquired resistance to gefitinib (26), suggesting that these two resistance

mechanisms can coexist in lung cancer patients.

The present study was performed to investigate whether HGF induced resistance to irreversible EGFR-TKI in lung cancer cells with secondary T790M mutation in *EGFR*. We assessed this issue using an irreversible EGFR-TKI, CL-387,785, and human lung cancer cells, H1975, harboring both L858R and T790M mutations in *EGFR*. We found that HGF reduced susceptibility to CL-387,785 in H1975 cells by stimulating the MET/Akt pathway. The resistance was also induced by crosstalk to HGF-producing fibroblast cell lines as well as primary cultured fibroblasts established from lung cancer patients. We further demonstrated that HGF-MET inhibitors could circumvent the HGF-induced hyposensitivity to irreversible EGFR-TKI.

Materials and methods

Cell lines and reagents

The H1975 human lung adenocarcinoma cell line with EGFR L858R/T790M double mutation (10) was kindly provided by Dr. John D. Minna (University of Texas Southwestern Medical Center, Dallas, TX). The PC-9 and HCC827 human lung adenocarcinoma cell lines with *EGFR*-activating mutation (deletion in exon 19) were purchased from Immuno-Biological Laboratories Co. (Takasaki, Gunma, Japan) and American Type Culture Collection (Manassas, VA), respectively. The MRC-5 lung embryonic fibroblast cell line was obtained from RIKEN Cell Bank. H1975, PC-9, and HCC827 cells were cultured in RPMI 1640 medium and MRC-5 (P 30-35) cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (50 µg/mL), in a humidified CO_2 incubator at 37°C. All experiments were performed in medium supplemented with 10% FBS.

Gefitinib was obtained from AstraZeneca (Cheshire, UK). Erlotinib hydrochloride was obtained from Roche Pharma AG (Basel, Switzerland). CL-387,785 and SU-11274 were purchased from Calbiochem (San Diego, CA). Cetuximab was purchased from Merck Serono (Darmstadt, Germany). Recombinant HGF and NK4 were prepared as reported previously (22, 27, 28). The purities of NK4 and HGF were 96.4% and > 98%, respectively, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein staining. Recombinant epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) were obtained from Invitrogen (Carlsbad, CA). Transforming growth

factor α (TGF-α) was from BioSource (San Jose, CA). Goat anti-human HGF neutralizing antibody, monoclonal anti-human EGF neutralizing antibody, goat anti-human TGF-α neutralizing antibody, and control goat IgG were purchased from R&D Systems (Minneapolis, MN).

Isolation of fibroblasts from lung cancer tissue

Primary cultured fibroblasts were established from surgically resected tumors from patients with histologically proven lung cancer at Kanazawa University Hospital, as described previously (29). The protocol was approved by the Medical Ethical Committee of Kanazawa University. Written informed consent was obtained from all patients. To establish primary fibroblasts, fresh lung cancer tissues from patients were minced with a scalpel in tissue culture dishes, digested with trypsin, and passed though a cell strainer. The resulting suspensions were incubated in RPMI1640 supplemented with 10% FBS, penicillin, and streptomycin. After 48 hours, unattached cells were removed and the medium was replaced with fresh medium. After 7 to 10 days, the cells formed a homogenous monolayer morphologically consistent with fibroblast-like cells, which were confirmed to consist of > 90% type I collagen-positive cells.

Cell proliferation assay

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method (30). Tumor cells at 80% confluence were harvested, seeded at 2×10^3

cells per well in 96-well plates, and incubated in RPMI 1640 with 10% FBS. After 24 hours of incubation, several concentrations of gefitinib, erlotinib, CL-387,785, goat anti-human HGF neutralizing antibody, control goat IgG, NK4, SU-11274, and/or cytokines were added to each well, and incubation was continued for a further 72 hours. Then, an aliquot of 50 μL of MTT solution (2 mg/mL; Sigma, St. Louis, MO) was added to each well followed by incubation for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 μL of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 nm 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.

Immunoprecipitation and Western blotting

Tumor cells were incubated in 10 mL of RPMI 1640 with 10% FBS in the presence or absence of HGF and/or CL-387,785. Then, cells were washed twice with PBS, harvested in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and flash-frozen on dry ice. After allowing the cells to thaw, the cell lysates were collected with a rubber scraper, sonicated, and centrifuged at 14000 × *g* (4°C for 20 min). The total protein concentration was measured using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL).

Aliquots of 500 μg of total proteins were immunoprecipitated with the appropriate antibodies. Immune complexes were recovered with Protein G-Sepharose beads (Zymed Laboratories, San Francisco, CA). For Western blotting assay, immunoprecipitates or cell lysates were subjected to SDS-PAGE (Bio-Rad, Hercules, CA) and the proteins were then transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature, and the blots were then incubated at 4°C overnight with anti-Met (25H2), anti-phospho-Met (Y1234/Y1235) (3D7), anti-ErbB2(29D8), anti-ErbB3 (1B2), anti-ErbB4(111B2), anti-phospho-EGFR (Y1068), anti-phospho-EGFR (Y1086), anti-phospho-ErbB2(Tyr1221/1222)(6B12), anti-phospho-ErbB3 (Tyr1289) (21D3), anti-phospho-ErbB4(Tyr1284)(21A9), PI3K p85 (19H8), anti-Akt, or anti-phospho-Akt (Ser473) antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA), and anti-human EGFR (1 μg/mL), anti-human/mouse/rat extracellular signal-regulated kinase (ERK)1/ERK2 (0.2 μg/mL), or anti-phospho-ERK1/ERK2 (T202/Y204) (0.1 μg/mL) antibodies (R&D Systems, Minneapolis, MN). After washing three times, the membranes were incubated for 1 hour at room temperature with secondary Ab (horseradish peroxidase-conjugated species-specific Ab). Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce). Each experiment was performed at least three times independently.

RNA interference

Duplexed Stealth RNAi (Invitrogen) against *MET*, *EGFR*, and *ErbB3* and Stealth RNAi Negative Control Low GC Duplex #3 (Invitrogen) were used for RNA interference (RNAi) assay. Briefly, aliquots of 1×10^5 H1975 cells in 2 mL of antibiotic-free medium were plated on 6-well plates and incubated at 37°C for 24 hours. The cells were then transfected with siRNA (250 pmol) or scramble RNA (siSCR) using Lipofectamine 2000 (5 μL) in accordance with the manufacturer's instructions (Invitrogen). After 24 hours, the cells were washed twice with PBS, and incubated with or without CL-387,785 (300 nM) and/or recombinant human HGF (50 ng/mL) for an additional 72 hours in antibiotic-containing medium. These cells were then used for proliferation assay as described above. *MET*, *EGFR*, and *ErbB3* knockdown were confirmed by Western blotting analysis. The sequences of siRNAs were as follows: *MET*, 5'-UCCAGAAGAUCAGUUUCCUAAUUCA-3' and 5'-UGAAUUAGGAAACUGAUCUUCUGGA-5'; *EGFR*, 5'-UUUAAAUUCACCAAUACCUAUUCCG-3' and 5'-CGGAAUAGGUAUUGGUGAAUUUAAA -5'; *ErbB3*, 5'-GGCCAUGAAUGAAUUCUCUACUCUA-3' and 5'-UAGAGUAGAGAAUUCAUUCAUGGCC-3'. Each experiment was performed at least in triplicate, and three times independently.

HGF production in cell culture supernatant

Cells (2×10^5) were cultured in 2 mL of RPMI 1640 or DMEM with 10% FBS for

24 hours. The cells were washed with PBS and incubated for 48 hours in RPMI 1640 or DMEM with 10% FBS, with or without various concentrations of CL-387,785. The culture medium was then harvested and centrifuged, and the supernatant was stored at –70°C until analysis. For determination of HGF, ELISA was performed in accordance with the manufacturer's recommended procedures (Immunis HGF EIA; B-Bridge International, Mountain View, CA). All samples were run in triplicate. Color intensity was measured at 450 nm with a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves. The detection limit was 0.1 ng/mL.

Co-culture of lung cancer cells with fibroblasts

The co-culture system was performed using Transwell chambers separated by an 8-μm pore size filter. Tumor cells (8×10^3 cells/700 μL) with or without CL-387,785 (300 nM) in the lower chamber were co-cultured with fibroblasts (10^4 cells/300 μL) with or without 2 hours of pretreatment with control IgG (2 μg/mL), anti-HGF neutralizing antibody (2 μg/mL), anti-EGF neutralizing antibody (2 μg/mL), or anti-TGF-α neutralizing antibody (2 μg/mL) in the upper chamber for 72 hours. The upper chamber was then removed and 200 μL of MTT solution (2 mg/mL; Sigma) was added to each well and the cells were incubated for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 400 μL of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric) at test and reference wavelengths of 550 nm 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.

Statistical analysis

All data, expressed as means ± SE, were analyzed by Mann-Whitney U-test, and $P < 0.05$ was considered to indicate statistical significance. All statistical analyses were performed using StatView ver.5.0 (SAS Institute, Cary, NC).

Results

HGF induced hyposensitivity to irreversible EGFR-TKI, CL-387,785, in lung cancer cells with EGFR-L858R/T790M mutations

We first examined the effects of the reversible EGFR-TKI, gefitinib and erlotinib, and irreversible EGF-TKI, CL-387,785, against H1975 cells harboring L858R activating mutation and T790M second mutation in *EGFR* (11). H1975 cells were highly sensitive to CL-387,785 (IC_{50} 0.24 μM), while they were resistant to gefitinib and erlotinib, as reported previously (Fig. 1A)(14, 31). HGF (50 ng/mL) alone had no effect on the proliferation of H1975 cells as well as PC-9 and HCC827cells with activating mutation in *EGFR*. Under these experimental conditions, HGF (50 ng/mL) reduced the degrees of susceptibility of H1975 (IC_{50} 0.48 μM), PC-9, and HCC827 cells to CL-387,785 (Fig. 1B, S1). The effect of HGF in H1975 cells was abrogated by anti-HGF neutralizing antibody (2 μg/mL) but not control IgG (2 μg/mL) (Fig. 1C).

We also examined the effects of other cytokines, including EGF (32), TGF-α (33), and IGF-I (34), reported to be related to EGFR-TKI sensitivity. Although EGF and TGF-α tended to induce hyposensitivity of H1975 cells to CL-387,785, HGF showed the strongest effect in induction of hyposensitivity to CL-387,785 in H1975 cells (Fig. 2).

HGF-induced CL-387,785 hyposensitivity was mediated by restoring phosphorylation of Akt and ERK1/2, but not EGFR or ErbB3

To investigate the molecular mechanism by which HGF induces CL-387,785

hyposensitivity, we examined the protein expression and phosphorylation status of MET, ErbB family proteins, and downstream molecules by Western blotting. H1975 cells expressed EGFR, ErbB2, ErbB3, ErbB4, MET, and PI3K-P85 proteins. Of these, EGFR, ErbB3, and MET were phosphorylated at various levels, but neither ErbB2 nor ErbB4 was phosphorylated in H1975 cells under our experimental conditions. Akt and ERK1/2, the downstream molecules of these receptors, were also phosphorylated. CL-387,785 inhibited the phosphorylation of EGFR, ErbB3, Akt, and ERK1/2, but not MET, showing the selectivity of this compound to the EGFR family. HGF alone stimulated phosphorylation of not only MET, Akt, and ERK1/2 but also EGFR and ErbB3. In the presence of HGF, CL-387,785 inhibited the phosphorylation of EGFR but did not affect phosphorylation of Akt or ERK1/2 (Fig. 3A).

To investigate the mechanism in detail, we immunoprecipitated MET and examined the association with PI3K-related molecules. In H1975 cells, MET was constitutively associated with p85, the binding domain of PI3K, and this association was unaffected by CL-387,785 and/or HGF. MET was slightly associated with ErbB3, and this association was also unaffected by CL-387,785 and/or HGF. On the other hand, MET was constitutively associated with EGFR, and this association was augmented by HGF. Importantly, CL-387,785 disrupted association of MET-EGFR, irrespective of the presence or absence of HGF (Fig. 3B). These results suggest that in the absence of EGFR inhibition, some if not all MET protein is associated with EGFR and HGF stimulates MET-EGFR association and downstream signaling (Akt and ERK 1/2). In contrast, in the

presence of EGFR inhibition, MET may show reduced association with inactivated EGFR. Therefore, HGF may stimulate Akt and ERK1/2 signaling predominantly *via* MET under these conditions.

Specific down-regulation of MET, but not EGFR* or *ErbB3, reversed CL-387,785 hyposensitivity and phosphorylation of Akt and ERK1/2 induced by HGF

To clarify the involvement of MET, EGFR, and ErbB3, we further down-regulated *MET*, *EGFR*, and *ErbB3* expression, respectively, with specific siRNAs using H1975 cells. Down-regulation of EGFR, but not MET or ErbB3, resulted in reduced viability of H1975 cells, similar to irreversible EGFR-TKI treatment, suggesting the importance of EGFR in viability of H1975 cells. Down-regulation of EGFR or ErbB3 did not affect either HGF-induced hyposensitivity to CL-387,785 or phosphorylation of Akt and ERK1/2 restored by HGF in H1975 cells. In parallel experiments, down-regulation of MET expression by *MET*-specific siRNA canceled HGF-induced hyposensitivity to CL-387,785, as well as phosphorylation of Akt and ERK1/2 restored by HGF (Fig. 4). These results indicate that HGF induces CL-387,785 hyposensitivity by activating the Akt and ERK1/2 signaling pathway *via* MET phosphorylation.

Fibroblast-derived HGF induced CL-387,785 hyposensitivity in lung cancer cells

It is well documented that host microenvironments can affect the

chemosensitivity of cancers and that stromal fibroblasts are the major source of HGF (35). We next examined the production of HGF by three human fibroblast cell lines and fibroblasts in primary culture established from the tumors of five different lung cancer patients. Our observations indicated that levels of HGF production by these fibroblasts varied and that the human embryonic lung-derived fibroblasts, MRC-5, and the primary culture fibroblasts from patient 2 (PF2) produced high levels of HGF in their supernatants. On the other hand, H1975 cells did not produce detectable levels of HGF in the culture supernatant with or without various concentrations of CL-387,785 (Fig. 5A, S2). To further investigate whether the susceptibility of H1975 cells to CL-387,785 could be affected by crosstalk to stromal fibroblasts, we co-cultured the H1975 cells with MRC-5 cells or PF2, using Transwell systems. While H1975 cells were highly sensitive to CL-387,785, exogenously added HGF induced CL-387,785 hyposensitivity of H1975 cells as mentioned above. Co-culture with MRC-5 or PF2 cells did not significantly affect the proliferation of H1975 cells. Under these experimental conditions, H1975 cells became hyposensitive to CL-387,785 in the presence of MRC-5 or PF2 cells. This was abrogated by treatment with anti-HGF neutralizing antibody (2 μg/mL), but not the neutralizing antibodies against EGF (2 μg/mL) or TGF-α (2 μg/mL) (Fig. 5B, S3). These results indicate that fibroblast-derived HGF could induce CL-387,785 hyposensitivity of lung cancer cells with *EGFR*-L858R/T790M mutations.

HGF-induced hyposensitivity was abrogated by addition of HGF-MET

inhibitors

Recently, several inhibitors of HGF-MET signaling have been developed. Therefore, to establish the novel therapeutic modality against HGF-mediated resistance to irreversible EGFR-TKI, we treated H1975 cells with CL-387,785 in combination with anti-HGF neutralizing antibody, HGF antagonist, or MET tyrosine kinase inhibitor (MET-TKI). The MET-TKI, SU11274, moderately reversed the HGF-induced CL-387,785 hyposensitivity at the non-toxic concentration of 1 μM. Both anti-HGF neutralizing antibody (2 μg/mL) and HGF antagonist, NK4 (300 nM), completely abrogated the CL-387,785 hyposensitivity at non-toxic concentrations (Fig. 6), suggesting the promising potential of these compounds to overcome HGF-induced resistance.

Discussion

In the present study, we demonstrated that HGF reduced susceptibility to an irreversible EGFR-TKI, CL-387,785, in human lung cancer cells harboring a secondary T790M mutation. HGF-induced CL-387,785 hyposensitivity was mediated by activation of the PI3K/Akt pathway *via* phosphorylation of MET, independent of EGFR, ErbB2, ErbB3, or ErbB4. The reduced susceptibility was also caused by co-culture with an HGF-producing fibroblast cell line as well as primary cultured fibroblasts established from a lung cancer patient. In addition, HGF-MET inhibitors could circumvent the HGF-induced hyposensitivity to irreversible EGFR-TKI.

H1975 is a human lung adenocarcinoma cell line, which has both L858R and T790M as an activating mutation and secondary resistance mutation, respectively, in *EGFR*. This cell line is widely used as a model to develop novel targeting drugs, including irreversible EGFR-TKI, which overcome T790M-mediated drug resistance (14, 15). More recently, this cell line was also used to identify a novel mechanism of resistance to irreversible EGFR-TKI. Yu *et al.* carried out a cell-based *in vitro* random mutagenesis screen to identify an *EGFR* mutation that induced resistance to CL-387,785 (15). They found several mutations in *EGFR* that induced resistance to CL-387,785 in H1975 cells, although the mechanisms by which these mutations caused resistance remained unclear. In the present study, we demonstrated another mechanism showing that HGF reduced susceptibility to CL-387,785 in this cell line by activating the MET/PI3K/Akt pathway to send a survival signal. In addition, HGF-induced

CL-387,785 hyposensitivity was also observed in PC-9 and HCC827 cells, which had *EGFR*-activating mutation (deletion in exon 19) alone. Lung cancer cells with *EGFR*-activating mutations, with or without T790M mutation, seem to be dependent on the signal from the mutated EGFR for their survival (36). Therefore, an alternative signal pathway *via* MET may be important for their survival when the EGFR signal is blocked by inhibitors. In fact, Tang *et al*. demonstrated the efficacy of dual receptor tyrosine kinase-targeted inhibition against MET (SU11274) and EGFR (erlotinib or CL-387,785) as a strategy to achieve optimized inhibition in T790M-EGFR-mediated erlotinib resistance (37).

To overcome T790M mutation-mediated resistance, several agents, such as irreversible EGFR-TKIs, anti-EGFR antibody (38), and Hsp90 inhibitors (39), have been developed and evaluated with regard to their efficacy in pre-clinical and clinical trials. Of the irreversible EGFR-TKIs, PF00299804, HKI-272, EKB-569, and BIBW2992 are currently in clinical trials, while commercially available CL-387,785 is not (19, 40). Yoshimura *et al.* reported promising results showing that EKB-569 caused partial responses in 2 cases of lung cancer that acquired resistance during treatment with reversible EGFR-TKI, gefitinib (18). Other groups also reported early signs of success with HKI-272 and PF00299804 in gefitinib- or erlotinib-refractory cancers (19, 20). Anti-EGFR chimeric antibody, cetuximab, has also been reported to block the downstream signal of EGFR and potently induce antibody-dependent cellular cytotoxicity (ADCC), and thus show antitumor activity against several cell lines, including H1975 (38). However, even though these agents show favorable responses in

tumors with T790M mutation, it is clear that resistance can also develop against this class of inhibitors. We demonstrated that HGF could induce hyposensitivity to irreversible EGFR-TKI. In addition, our preliminary experiments indicated that while cetuximab inhibited the growth of H1975 cells, HGF caused hyposensitivity to cetuximab (data not shown). Thus, it may be useful to investigate the involvement of HGF-MET-mediated signaling in acquired resistance to irreversible EGFR-TKIs as well as anti-EGFR antibody in lung cancer harboring T790M mutation in *EGFR*.

MET is known to be only one specific receptor for HGF (41, 42). MET activated by HGF binding forms a homodimer and transduces strong signals to various pathways, including PI3K/Akt, MAPK/ERK, and STAT (43). MET is also known to form heterodimers with other growth receptors, including EGFR and ErbB3 (8). Engelman *et al*. reported that amplified MET associated with ErbB3 and caused gefitinib resistance in lung cancer cells (8). Recent reports further indicated the important interaction between MET and EGFR (44, 45). These two receptors mediate collaborative signaling with receptor cross-activation (43). However, direct interaction between MET and EGFR with T790M mutation has not been reported previously. In the present study, we found that MET directly associated with EGFR harboring T790M mutation in H1975 cells, and that this association was enhanced by HGF resulting in augmented phosphorylation of Akt and ERK1/2. These observations indicate that MET interacts closely with EGFR harboring T790M mutation and regulates these important signal pathways. Therefore, simultaneous inhibition of MET and EGFR with T790M mutation may

be useful not only for overcoming HGF-induced TKI resistance, but also for controlling the progression of TKI-naïve tumors with T790M mutation in *EGFR*. Although we could not perform *in vivo* experiments in this study because of the limited availability of CL-387,785, further *in vivo* experiments are warranted to assess the therapeutic effects of irreversible EGFR-TKI combined with HGF-MET inhibitors.

The tumor microenvironment is important for tumor progression and drug sensitivity (46). Fibroblastic stromal cells have been linked to several activities that promote tumor progression, including angiogenesis, epithelial to mesenchymal transition, progressive genetic instability, and deregulation of antitumor immune responses, enhanced metastasis, and prevention of apoptosis induced by chemotherapeutic agents. Stromal fibroblasts are one of the major sources of various cytokines, including HGF (35). In the present study, we confirmed that fibroblast cell lines and primary cultured fibroblasts produced various levels of HGF and irreversible EGFR-TKI-hyposensitivity could be induced by HGF derived from both fibroblast cell lines and primary cultured fibroblasts by a paracrine mechanism. Therefore, it is possible that tumor-associated fibroblasts are involved in resistance to irreversible EGFR-TKI in lung cancer patients harboring *EGFR* T790M mutation.

In summary, we reported a novel mechanism of resistance to irreversible EGFR-TKI in lung cancer harboring secondary T790M mutation in *EGFR*. HGF induced hyposensitivity to the irreversible EGFR-TKI, CL-387,785, by activating the PI3K/Akt pathway *via* phosphorylation of MET independent of EGFR family

proteins. The hyposensitivity was also induced by co-culture with HGF-producing fibroblasts, suggesting the possible involvement of microenvironments in resistance to irreversible EGFR-TKIs. Moreover, we demonstrated that HGF-MET inhibitors could circumvent the HGF-induced hyposensitivity to irreversible EGFR-TKI. Therefore, it will be clinically valuable to investigate the involvement of HGF-MET-mediated signaling in acquired resistance to irreversible EGFR-TKIs in lung cancer harboring T790M mutation in *EGFR*.

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Figure legends

Figure 1

HGF induced CL-387,785 hyposensitivity of lung adenocarcinoma cells with *EGFR-L858R/T790M* mutations.

(A) H1975 cells were highly sensitive to CL387,785 (IC_{50} 300nM), and were resistant to gefitinib and erlotinib. Tumor cells were incubated with increasing concentrations of CL-387,785, gefitinib, or erlotinib and cell growth was determined after 72 hours of treatment by MTT assay.

(B) HGF induced CL-387,785 hyposensitivity of H1975 cells with EGFR-L858R/T790M mutation. Tumor cells were incubated with increasing concentrations of CL-387,785 and/or HGF at 50 ng/mL, and cell growth was determined in the same way as in A.

(C) Pretreatment of HGF with anti-HGF antibody abrogated HGF-induced hyposensitivity of H1975 cells to CL-387,785. HGF (50 ng/mL) was pretreated with control IgG (2 μg/mL) or anti-HGF antibody (2 μg/mL) at 37°C for 1 hour. The resultant solutions were added to the cultures of tumor cells with or without CL-387,785 (300 nM). Cell growth was determined in the same way as in A. *, $P < 0.01$ (Mann-Whitney *U* test).

Figure 2

HGF was most potent in induction of CL-387,785 hyposensitivity of H1975 cells.

H1975 cells were incubated with or without CL-387,785 and/or 50 ng/mL of HGF, EGF, TGF-α, or IGF-I. Cell growth was determined after 72 hours of treatment. The percentage of growth is shown relative to untreated controls. *, $P < 0.05$ (Mann-Whitney *U* test).

Figure 3

HGF induces CL-387,785 hyposensitivity of lung adenocarcinoma cells with *EGFR-T790M* mutation by restoring phosphorylation of Akt and ERK1/2, but not EGFR and ErbB3.

(A) CL-387,785 inhibited the phosphorylation of EGFR but did not affect phosphorylation of Akt and ERK1/2 in the presence of HGF. Tumor cells were treated with or without CL-387,785 (300 nM) and/or HGF (50 ng/mL) for 1 hour. Cells were lysed, and the indicated proteins were detected by immunoblotting. **(B)** Cell extracts were immunoprecipitated with an antibody to MET. The precipitated proteins were determined by immunoblotting with the indicated antibodies.

Figure 4

Specific down-regulation of *MET*, but not *EGFR* and *ErbB3*, reversed CL-387,785 hyposensitivity and phosphorylation of Akt and ERK1/2 induced by HGF.

(A) siRNA specific for *MET*, but not for *EGFR* or *ErbB3*, reversed CL-387,785 hyposensitivity by HGF. Control, *MET*-specific, *EGFR*-specific, or *ErbB3*-specific

siRNAs were introduced into H1975 cells. The growth of cells with or without CL-387,785 (300 nM) and/or HGF (50 ng/mL) was measured by MTT assay. *, $P < 0.01$ (Mann-Whitney *U* test).

(B) Down-regulation of *MET*, but not *EGFR* and *ErbB3*, by specific-siRNA inhibited restored Akt and ERK1/2 phosphorylation by HGF in cells treated with CL-387,785. Control, *MET*-specific, *EGFR*-specific, or *ErbB3*-specific siRNAs were introduced into H1975 cells. After 48 hours, the cells were treated with or without CL-387,785 (300 nM) and/or HGF (50 ng/mL) for 1 hour, and then cell extracts were prepared and immunoblotted with the indicated antibodies.

Figure 5

Fibroblast-derived HGF induces CL-387,785 hyposensitivity in lung cancer cells with *EGFR*-T790M mutations.

(A) HGF production by lung cancer cell line (H1975), human embryonic lung fibroblasts (MRC-5), and primary cultured fibroblasts from the tumor of lung cancer patient 2 (PF2). The cells were incubated in medium for 48 hours, the culture supernatants were harvested, and their HGF concentrations were determined by ELISA.

(B) Co-culture with fibroblasts induced CL-387,785 hyposensitivity in lung cancer cells. The lung cancer H1975 cell line was co-cultured with MRC-5 or PF2 cells, with medium, anti-HGF neutralizing antibody (2 μg/mL) in the presence or absence of CL-387,785 (300 nM) for 72 hours, and lung cancer cell growth was determined after 72 hours of treatment by MTT assay. *, $P < 0.01$

(Mann-Whitney *U* test).

Figure 6

Anti-HGF antibody, NK4, or SU11274 abrogated HGF induced CL-387,785 hyposensitivity in lung cancer cells with *EGFR-T790M* mutation.

H1975 cells were treated for 72 hours with or without CL-387,785 (300 nM) and/or HGF (50 ng/mL) in the presence or absence of anti-HGF neutralizing antibody (2 μg/mL), NK4 (300 nM), or SU11274 (1 μM). Cell growth was determined by MTT assay. *, $P < 0.01$ (Mann-Whitney *U* test).

Supplemental Figure 1

HGF induced CL-387,785 hyposensitivity of lung adenocarcinoma cells with *EGFR-L858R/T790M* mutations.

HGF induced CL-387,785 hyposensitivity of PC-9 and HCC827 cells with *EGFR*-activating mutation (deletion in exon 19). Tumor cells were incubated with increasing concentrations of CL-387,785 and/or HGF at 50 ng/mL, and cell growth was determined in the same way as described in Figure 1-A. *, $P < 0.05$ *vs*. medium alone (Mann-Whitney *U* test).

Supplemental Figure 2

CL-387,785 did not affect HGF production in lung cancer cells with *EGFR*-T790M mutations.

HGF production by the lung cancer cell line H1975 with or without various doses

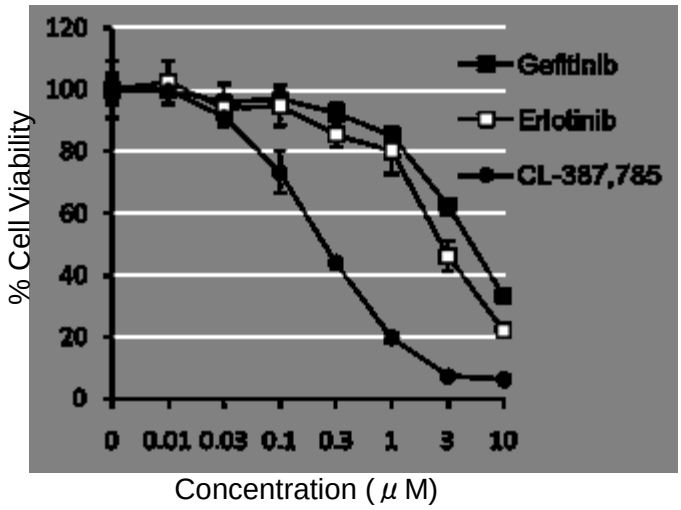
(0.03, 0.1, 0.3 μM) of CL-387,785, and human embryonic lung fibroblasts (MRC-5). The cells were incubated in medium for 48 hours, the culture supernatants were harvested, and their HGF concentrations were determined by ELISA.

Supplemental Figure 3

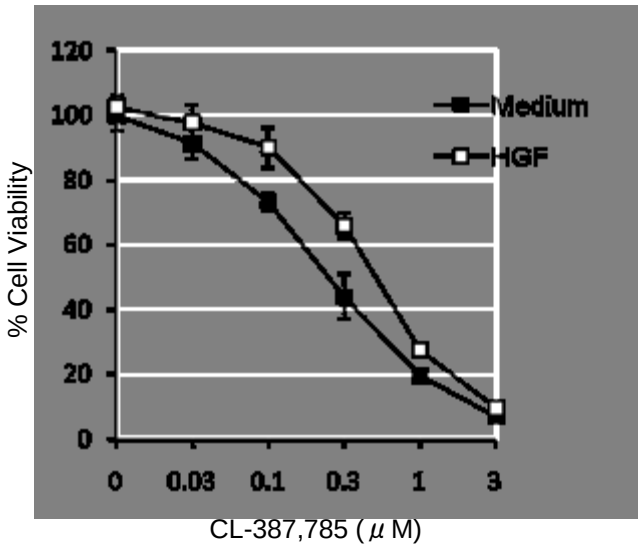
Fibroblast-derived HGF, but not EGF or TGF-α, induces CL-387,785 hyposensitivity in lung cancer cells with *EGFR*-T790M mutations.

Co-culture with fibroblasts induced CL-387,785 hyposensitivity in lung cancer cells. The lung cancer H1975 cell line was co-cultured with MRC-5 cells, with medium, anti-HGF neutralizing antibody (2 μg/mL), anti-EGF neutralizing antibody (2 μg/mL), or anti-TGF-α neutralizing antibody (2 μg/mL) in the presence or absence of CL-387,785 (300 nM) for 72 hours, and lung cancer cell growth was determined after 72 hours of treatment by MTT assay. *, $P < 0.01$ (Mann-Whitney *U* test).

Fig. 1 A


120
100
80
60
40
20
0
% Cell Viability
Gefitinib
Erlotinib
CL-387,785
0 0.01 0.03 0.1 0.3 1 3 10
Concentration (μ M)

B


120
100
80
60
40
20
0
% Cell Viability
Medium
HGF
0 0.03 0.1 0.3 1 3
CL-387,785 (μ M)

C

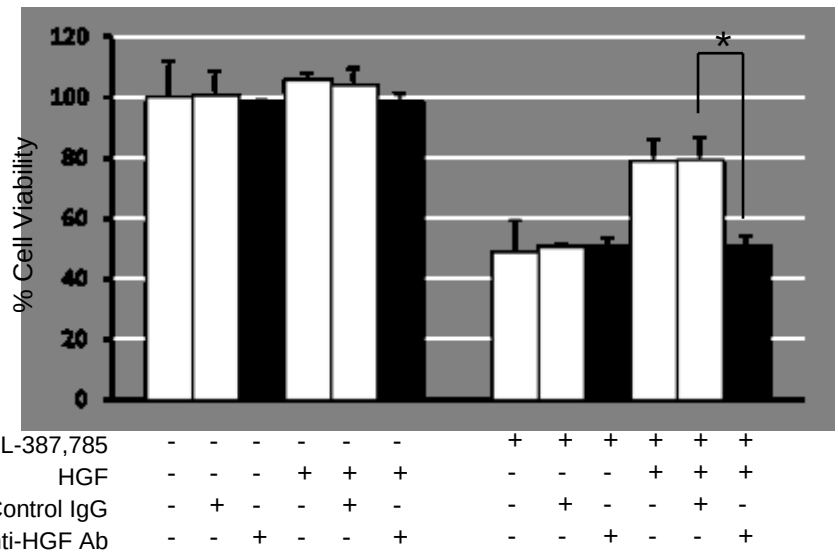

120
100
80
60
40
20
0
% Cell Viability
*
CL-387,785 - - - - - - + + + + + +
HGF - - - + + + - - - + + +
Control IgG - + - - + - - + - - + -
Anti-HGF Ab - - + - - + - - + - - +

Fig. 2

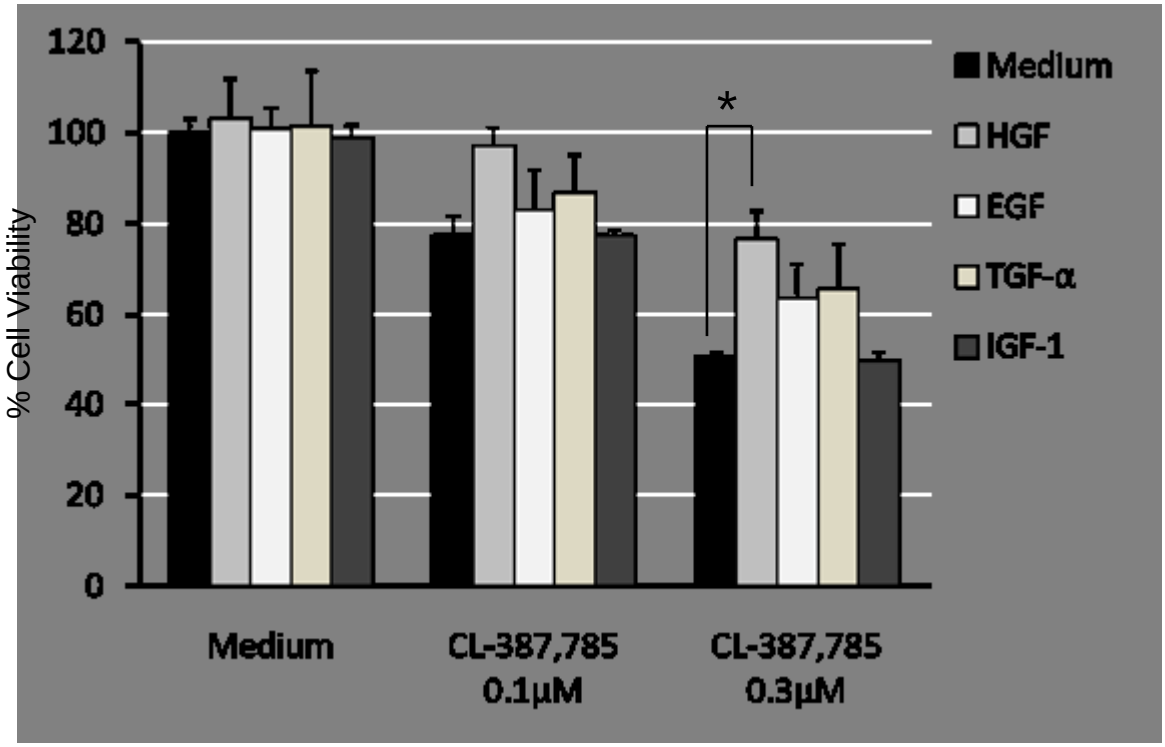
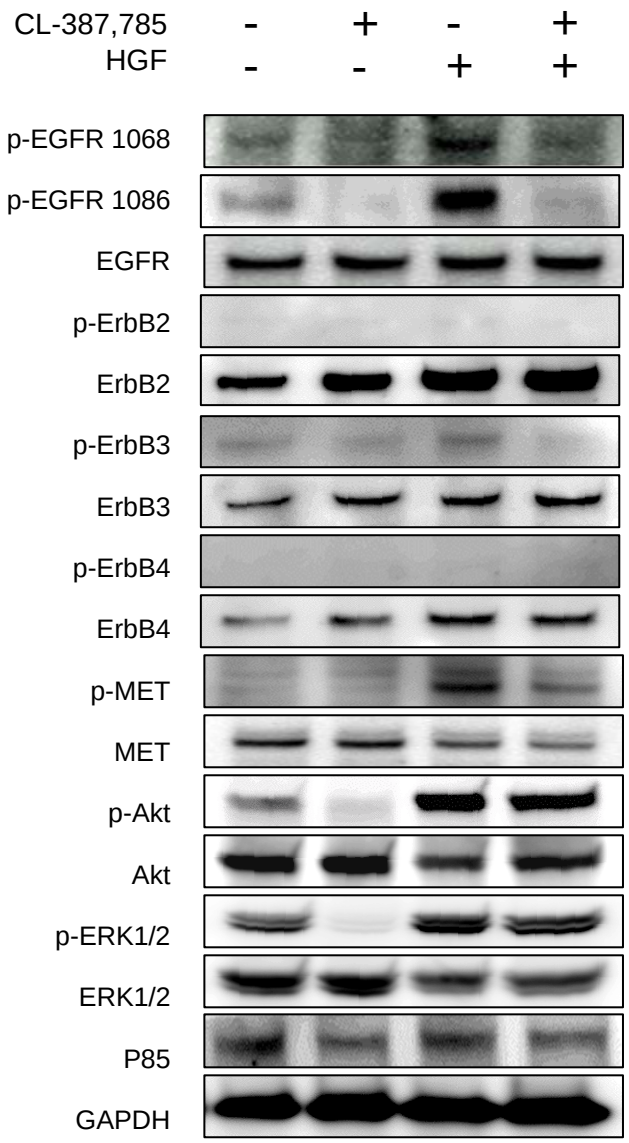

120
100
80
60
40
20
0
% Cell Viability
*
Medium
HGF
EGF
TGF-α
IGF-1
Medium
CL-387,785
0.1μM
CL-387,785
0.3μM

Fig. 3

A


CL-387,785 - + - +
HGF - - + +
p-EGFR 1068
p-EGFR 1086
EGFR
p-ErbB2
ErbB2
p-ErbB3
ErbB3
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MET
p-Akt
Akt
p-ERK1/2
ERK1/2
P85
GAPDH

B

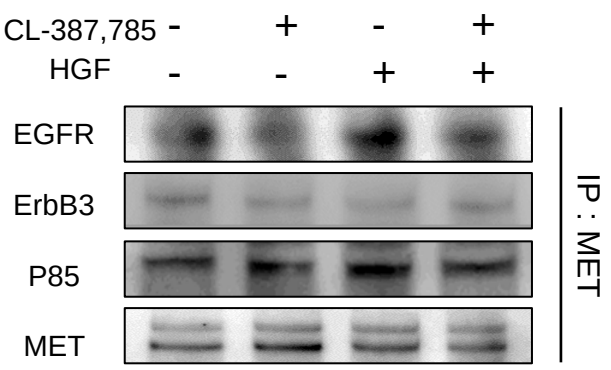
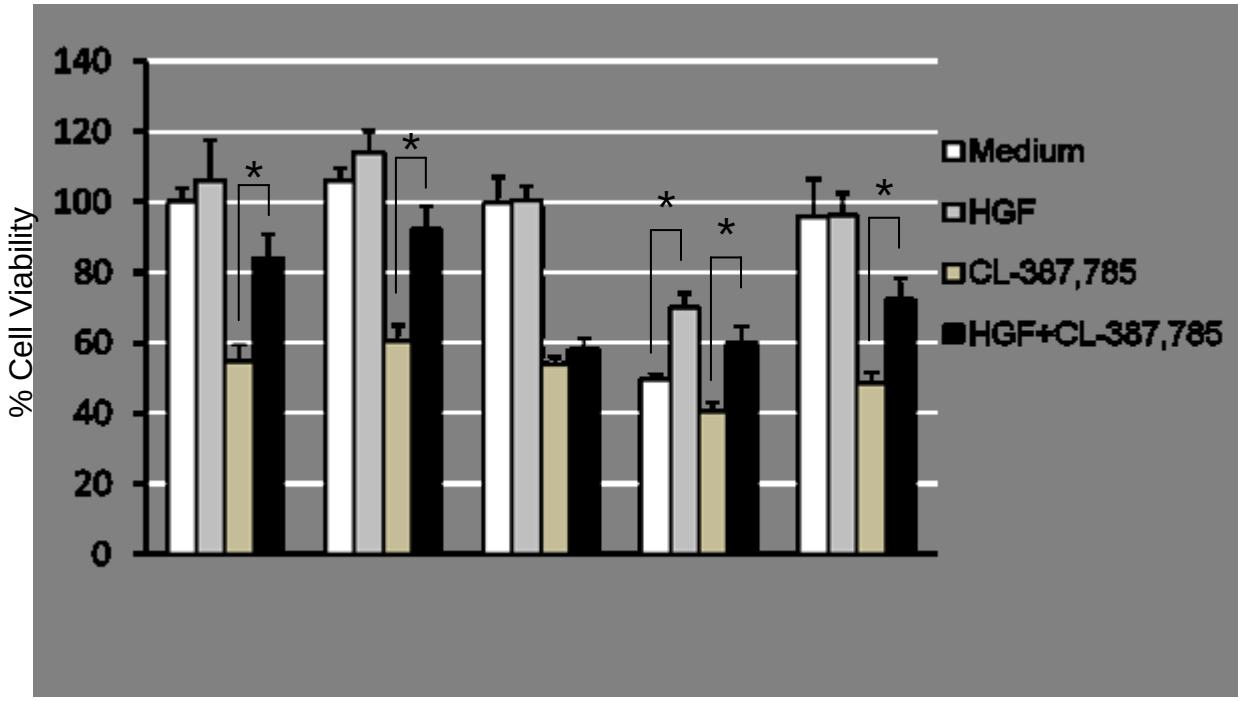

CL-387,785 - + - +
HGF - - + +
EGFR
ErbB3
P85
MET
IP : MET

Fig. 4

A


% Cell Viability
140
120
100
80
60
40
20
0
*
*
*
*
*
Medium
HGF
CL-387,785
HGF+CL-387,785

B

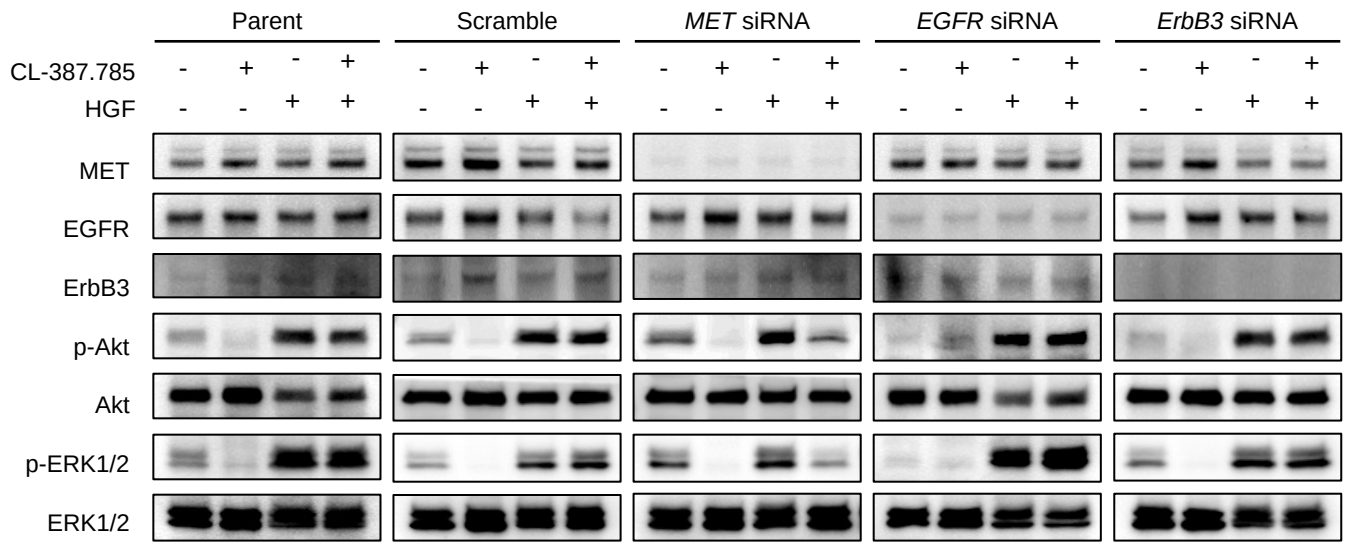
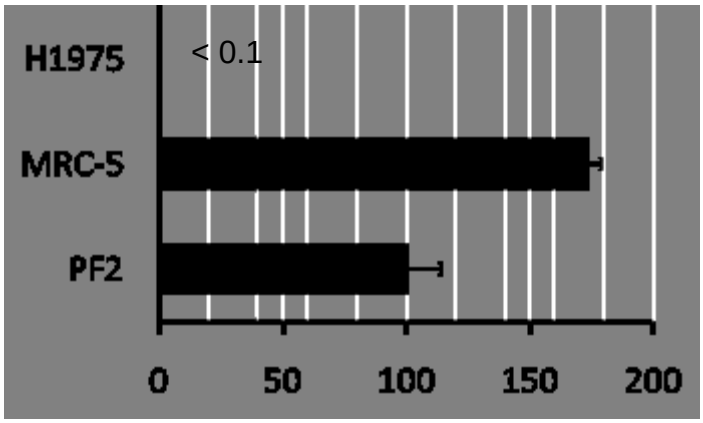

Parent
Scramble
MET siRNA
EGFR siRNA
ErbB3 siRNA
CL-387.785
HGF
MET
EGFR
ErbB3
p-Akt
Akt
p-ERK1/2
ERK1/2

Fig. 5 A


H1975
< 0.1
MRC-5
PF2
0
50
100
150
200
HGF (ng/2 × 10^5cells)

B

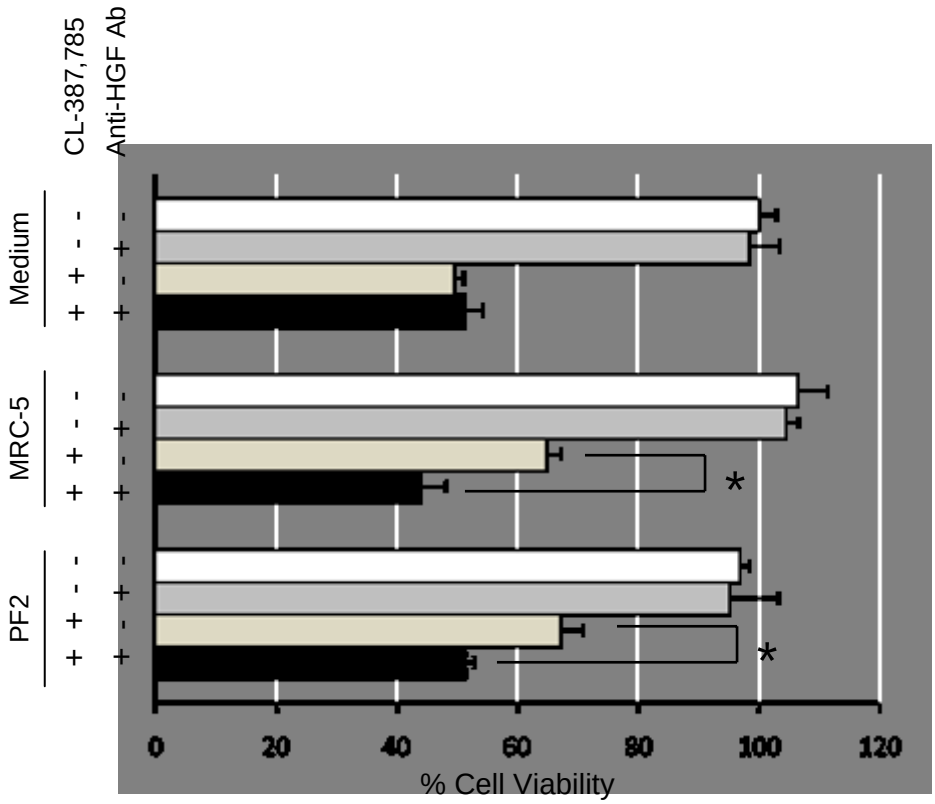
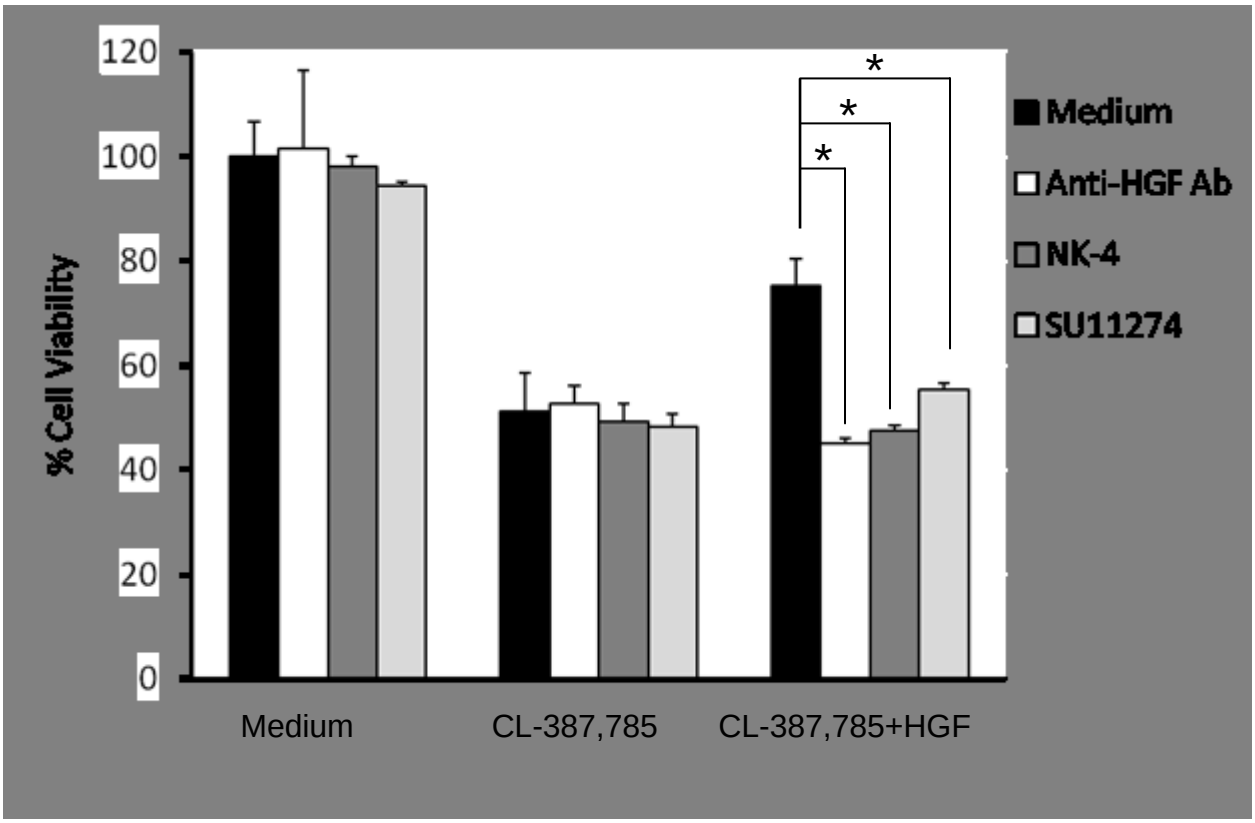

CL-387,785
Anti-HGF Ab
Medium
MRC-5
PF2
*
*
0
20
40
60
80
100
120
% Cell Viability

Fig. 6


120
100
80
60
40
20
0
% Cell Viability
Medium
CL-387,785
CL-387,785+HGF
*
*
*
Medium
Anti-HGF Ab
NK-4
SU11274