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Clinical Cancer Research

Cancer Therapy: Preclinical

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

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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-TKI), 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 showed 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 small interfering RNA analyses indicated that HGF-induced hyposensitivity was mediated by the MET/phosphoinositide 3-kinase/Akt signaling pathway independent of EGFR, ErbB2, ErbB3, and ErbB4. Hyposensitivity of H1975 cells to CL-387,785 was also induced by coculture 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 showed 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*. *Clin Cancer Res; 16(1); 174–83. ©2010 AACR.*

Lung cancer is the most common cause of malignancy-related death worldwide and its incidence is still increasing. Non–small cell lung cancer accounts for ~80% of cases of lung cancer. Median survival of metastatic non–small cell lung cancer is 8 to 10 months even if treated with the most active combination of conventional chemotherapeutic agents (1, 2). Epidermal growth factor (EGF) receptor (EGFR)–activating mutations, such as deletion in exon 19 and L858R point mutation in exon 21 (3), were found in non–small cell lung cancer. These mutations are predominantly found in female, nonsmoking, 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 non–small cell lung cancer 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 TKIs 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; ref. 9), were reported to induce acquired resistance to reversible EGFR-TKI for non–small cell lung cancer 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

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Translational Relevance

The most frequent cause of acquired resistance to the reversible epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI), gefitinib and erlotinib, in lung cancer is the secondary T790M mutation in EGFR. Irreversible EGFR-TKIs have the potential to be useful in controlling 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 showed that hepatocyte growth factor (HGF) reduced susceptibility to irreversible EGFR-TKI in lung cancer cells with the secondary T790M mutation in *EGFR*. We further showed 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*.

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 showed that HGF induces gefitinib resistance in lung cancer harboring EGFR-activating mutation by activating its receptor MET and downstream phosphoinositide 3-kinase (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 showed 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 done 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 showed 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). The PC-9 and HCC827 human lung adenocarcinoma cell lines with *EGFR*-activating mutation (deletion in exon 19) were purchased from Immuno-Biological Laboratories and American Type Culture Collection, 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 and MRC-5 (P 30-35) cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (50 μg/mL), in a humidified CO_2 incubator at 37°C. All experiments were done in medium supplemented with 10% FBS.

Gefitinib was obtained from AstraZeneca. Erlotinib hydrochloride was obtained from Roche Pharma. CL-387,785 and SU11274 were purchased from Calbiochem. Cetuximab was purchased from Merck Serono. 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 SDS-PAGE and protein staining. Recombinant EGF and insulin-like growth factor-I were obtained from Invitrogen. Transforming growth factor-α (TGF-α) was from BioSource. 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.

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

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RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin. After 48 h, 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 MTT dye reduction method (30). Tumor cells at 80% confluence were harvested, seeded at 2×10^3 per well in 96-well plates, and incubated in RPMI 1640 with 10% FBS. After 24 h of incubation, several concentrations of gefitinib, erlotinib, CL-387,785, goat anti-human HGF neutralizing antibody, control goat IgG, NK4, SU11274, and/or cytokines were added to each well, and incubation was continued for a further 72 h. Then, an aliquot of 50 μL MTT solution (2 mg/mL; Sigma) was added to each well followed by incubation for 2 h at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 μL DMSO. Absorbance was measured with a MTP-120 microplate reader (Corona Electric) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage of growth is shown relative to untreated controls. Each experiment was done at least in triplicate and three times independently.

Immunoprecipitation and Western blotting. Tumor cells were incubated in 10 mL 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 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, and 1 mmol/L 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 14,000 × *g* (4°C for 20 min). The total protein concentration was measured using a Pierce BCA Protein Assay kit (Pierce). Aliquots of 500 μg total proteins were immunoprecipitated with the appropriate antibodies. Immune complexes were recovered with protein G-Sepharose beads (Zymed Laboratories). For Western blotting assay, immunoprecipitates or cell lysates were subjected to SDS-PAGE (Bio-Rad) and the proteins were then transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with Blocking One (Nacalai Tesque) for 1 h 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 ($Tyr^{1221/1222}$; 6B12), anti–phospho-ErbB3 (Tyr^{1289}; 21D3), anti–phospho-ErbB4 (Tyr^{1284}; 21A9), PI3K p85 (19H8), anti-Akt, or anti–phospho-Akt (Ser^{473}) antibodies (1:1,000 dilution; Cell Signaling Technology) and anti-human EGFR (1 μg/mL), anti-human/mouse/rat extracellular signal-regulated kinase 1/2 (ERK1/2; 0.2 μg/mL), or anti–phospho-ERK1/2 (T202/Y204; 0.1 μg/mL) antibodies (R&D Systems). After washing three times, the membranes were incubated for 1 h at room temperature with secondary antibody (horseradish peroxidase–conjugated species-specific antibody). Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce). Each experiment was done at least three times independently.

RNA interference. Duplexed Stealth RNA Interference (Invitrogen) against *MET*, *EGFR*, and *ErbB3* and Stealth RNA Interference Negative Control Low GC Duplex 3 (Invitrogen) were used for RNA interference assay. Briefly, aliquots of 1×10^5 H1975 cells in 2 mL antibiotic-free medium were plated on 6-well plates and incubated at 37°C for 24 h. The cells were then transfected with small interfering RNA (siRNA; 250 pmol) or scramble RNA (siSCR) using Lipofectamine 2000 (5 μL) in accordance with the manufacturer's instructions (Invitrogen). After 24 h, the cells were washed twice with PBS and incubated with or without CL-387,785 (300 nmol/L) and/or recombinant human HGF (50 ng/mL) for an additional 72 h 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′, and *ErbB3* 5′-GGCCAUGAAUGAAUUCUCUACUCUA-3′ and 5′-UAGAGUAGAGAAUUCAUUCAUGGCC-3′. Each experiment was done at least in triplicate and three times independently.

HGF production in cell culture supernatant. Cells (2×10^5) were cultured in 2 mL RPMI 1640 or DMEM with 10% FBS for 24 h. The cells were washed with PBS and incubated for 48 h 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 done in accordance with the manufacturer's recommended procedures (Immunis HGF EIA; B-Bridge International). 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.

Coculture of lung cancer cells with fibroblasts. The coculture system was done using Transwell chambers separated by an 8-μm pore size filter. Tumor cells (8×10^3 per 700 μL) with or without CL-387,785 (300 nmol/L) in the bottom chamber were cocultured with fibroblasts (10^4 cells per 300 μL) with or without 2 h 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 top chamber for 72 h. The top chamber was then removed and 200 μL MTT solution (2 mg/mL; Sigma) was added to each well and the cells were incubated for 2 h at 37°C. The medium was removed and the dark blue crystals in each well

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were dissolved in 400 μL DMSO. Absorbance was measured with a MTP-120 microplate reader (Corona Electric) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage of growth is shown relative to untreated controls. Each experiment was done at least in triplicate and three times independently.

Statistical analysis. All data, expressed as mean ± SE, were analyzed by Mann-Whitney *U* test, and $P < 0.05$

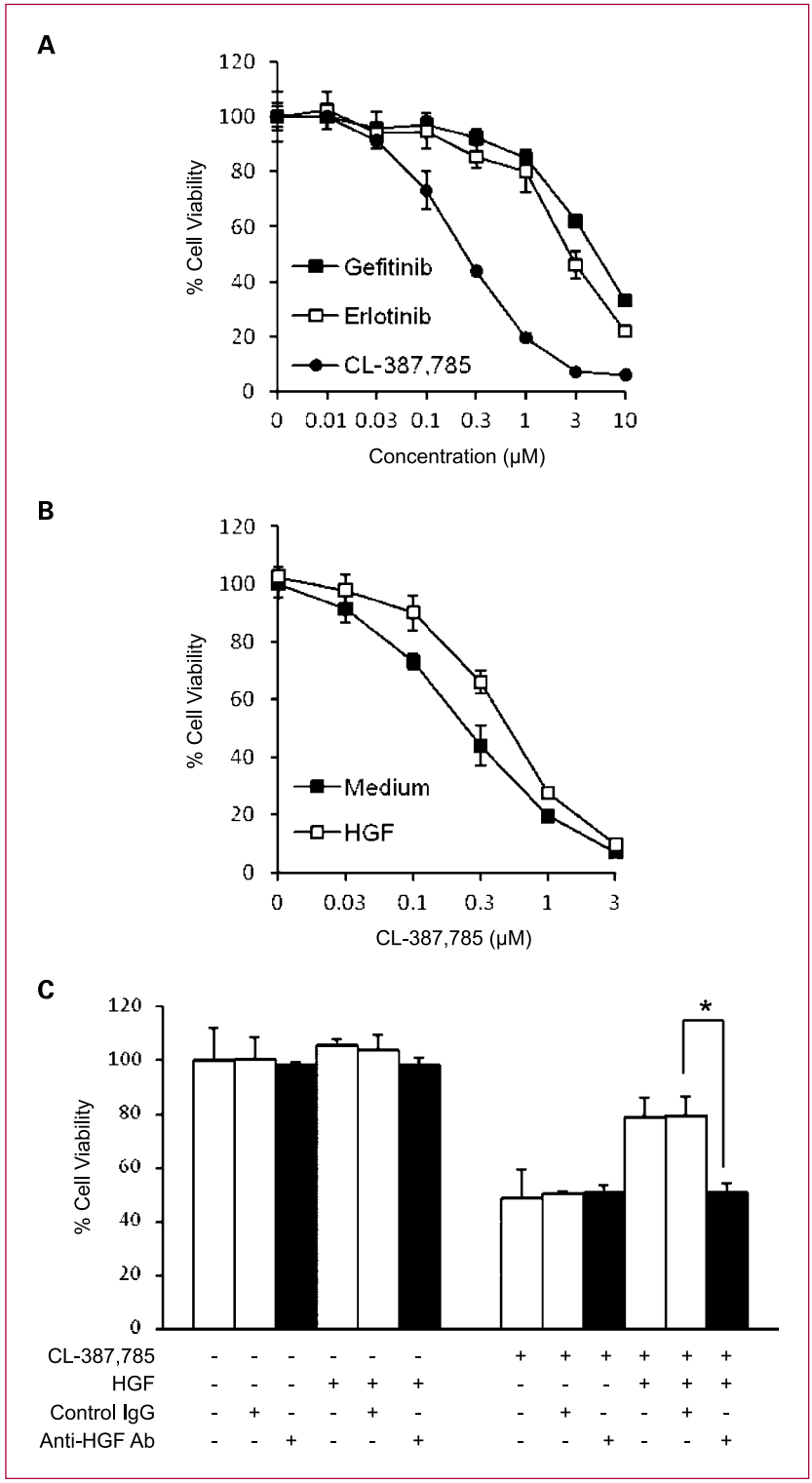

Fig. 1. HGF induced CL-387,785 hyposensitivity of lung adenocarcinoma cells with *EGFR*-L858R/T790M mutations. *A,* H1975 cells were highly sensitive to CL-387,785 (IC_{50} = 300 nmol/L) and resistant to gefitinib and erlotinib. Tumor cells were incubated with increasing concentrations of CL-387,785, gefitinib, or erlotinib and cell growth was determined after 72 h 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 h. The resultant solutions were added to the cultures of tumor cells with or without CL-387,785 (300 nmol/L). Cell growth was determined in the same way as in *A*. *, $P < 0.01$, Mann-Whitney *U* test.

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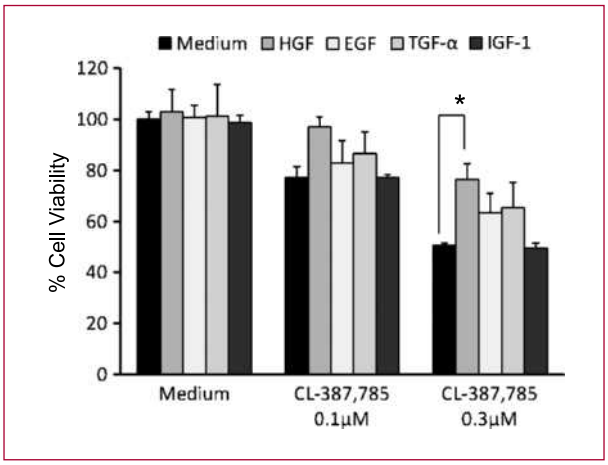

Fig. 2. HGF was most potent in induction of CL-387,785 hyposensitivity of H1975 cells. H1975 cells were incubated with or without CL-387, 785 and/or 50 ng/mL of HGF, EGF, TGF-α, or insulin-like growth factor-I. Cell growth was determined after 72 h of treatment. The percentage of growth is shown relative to untreated controls. *, $P < 0.05$, Mann-Whitney *U* test.

was considered to indicate statistical significance. All statistical analyses were done using StatView version 5.0 (SAS Institute).

Results

HGF induced hyposensitivity to irreversible EGFR-TKI, CL-387,785, in lung cancer cells with EGFR tumor studies. 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 μmol/L), whereas they were resistant to gefitinib and erlotinib, as reported previously (Fig. 1A; refs. 14, 31). HGF (50 ng/mL) alone had no effect on the proliferation of H1975 cells as well as PC-9 and HCC827 cells with activating mutation in *EGFR*. Under these experimental conditions, HGF (50 ng/mL) reduced the degrees of susceptibility of H1975 (IC_{50} = 0.48 μmol/L), PC-9, and HCC827 cells to CL-387,785 (Fig. 1B; Supplementary Fig. 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 insulin-like growth factor-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

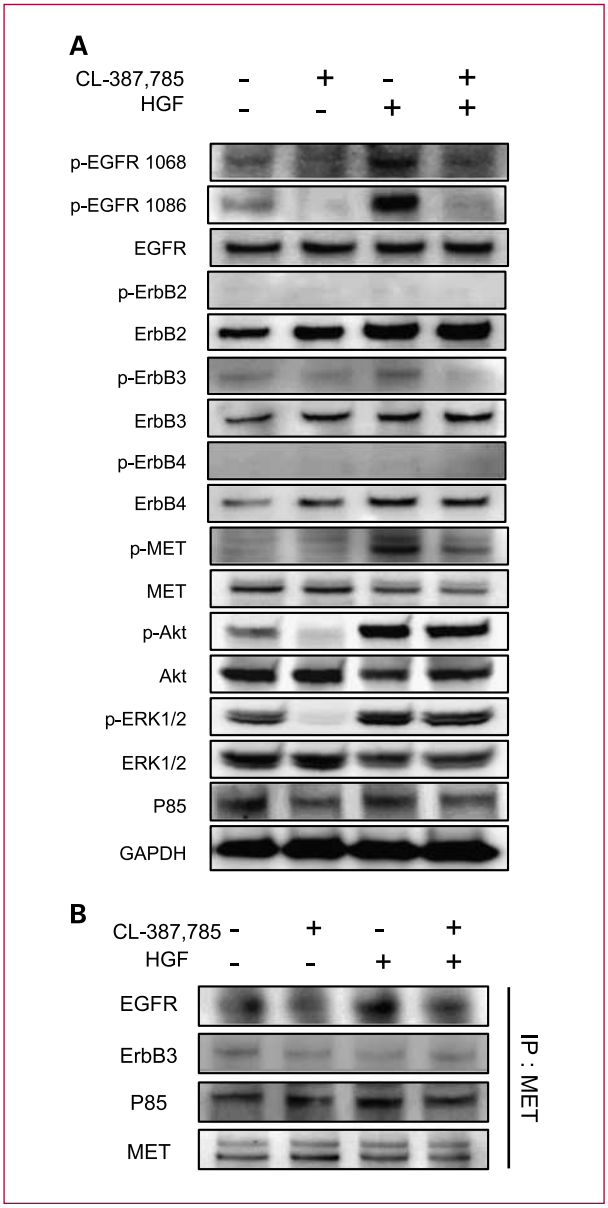

Fig. 3. HGF induces CL-387,785 hyposensitivity of lung adenocarcinoma cells with *EGFR*-T790M mutation by restoring phosphorylation of Akt and ERK1/2 but not EGFR and ErbB3. *A*, CL-387,785 inhibited the phosphorylation of EGFR but did not affect phosphorylation of Akt and ERK1/2 in the presence of HGF. Tumor cells were treated with or without CL-387,785 (300 nmol/L) and/or HGF (50 ng/mL) for 1 h. 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.

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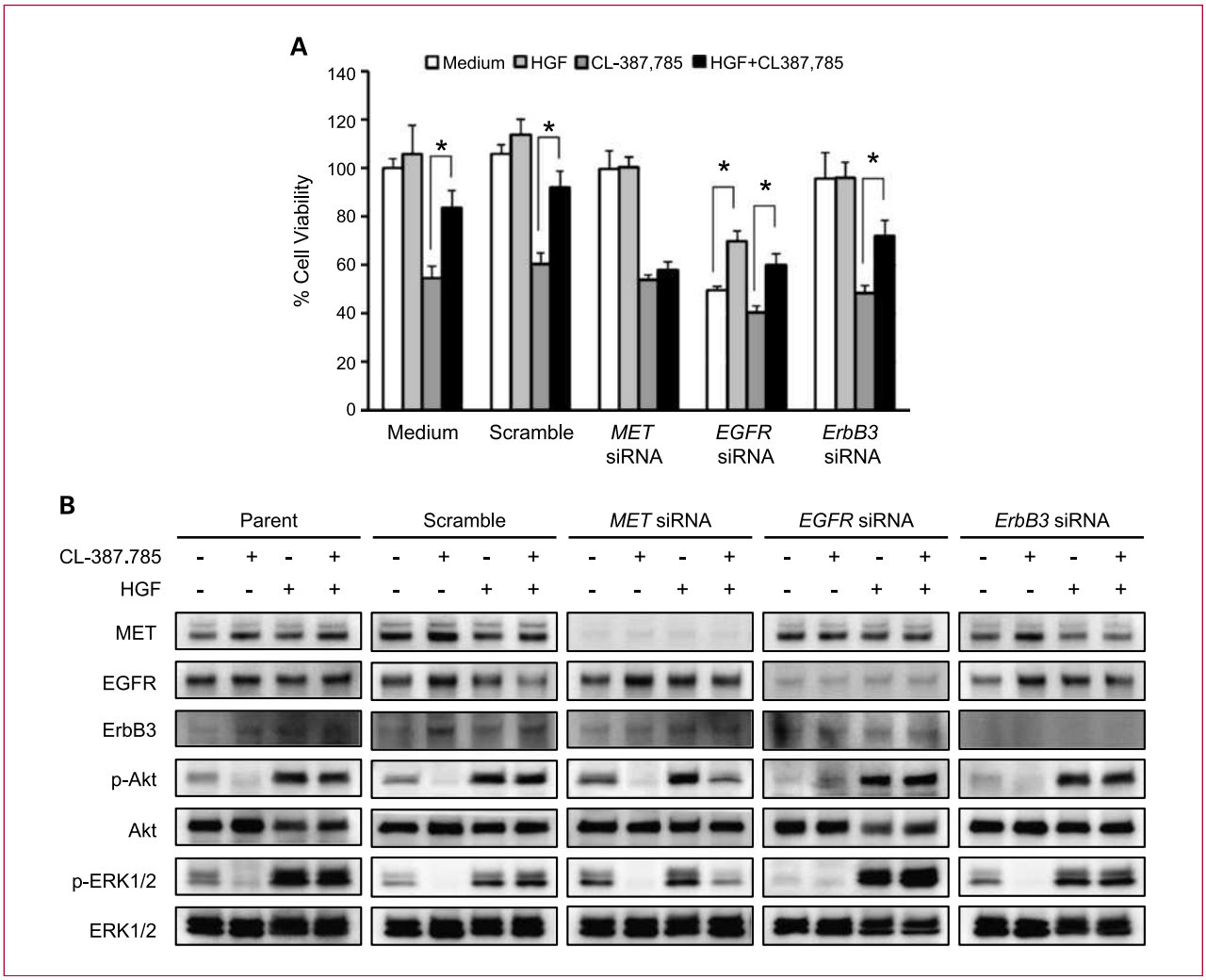

Fig. 4. Specific downregulation of *MET*, but not *EGFR* and *ErbB3*, reversed CL-387,785 hyposensitivity and phosphorylation of Akt and ERK1/2 induced by HGF. *A*, siRNA specific for *MET*, but not for *EGFR* or *ErbB3*, reversed CL-387,785 hyposensitivity by HGF. Control, *MET*-specific, *EGFR*-specific, or *ErbB3*-specific siRNAs were introduced into H1975 cells. The growth of cells with or without CL-387,785 (300 nmol/L) and/or HGF (50 ng/mL) was measured by MTT assay. *, $P < 0.01$, Mann-Whitney *U* test. *B*, downregulation 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 h, the cells were treated with or without CL-387,785 (300 nmol/L) and/or HGF (50 ng/mL) for 1 h, and cell extracts were prepared and immunoblotted with the indicated antibodies.

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 ERK1/2). In contrast, in the presence of EGFR inhibition, MET may show reduced association with inactivated EGFR. Therefore, HGF may

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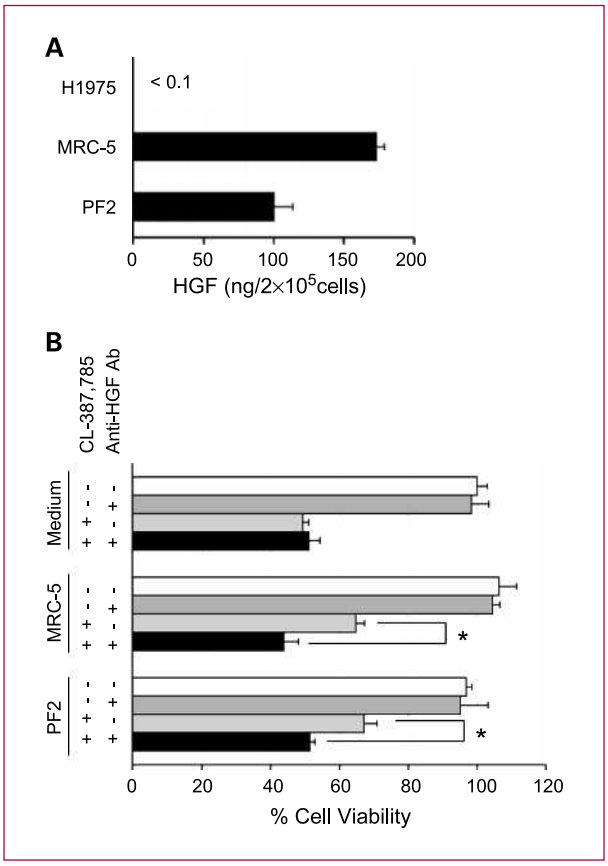

Fig. 5. Fibroblast-derived HGF induces CL-387,785 hyposensitivity in lung cancer cells with *EGFR*-T790M mutations. *A,* HGF production by lung cancer cell line (H1975), human embryonic lung fibroblasts (MRC-5), and primary cultured fibroblasts from the tumor of lung cancer patient 2 (PF2). The cells were incubated in medium for 48 h, the culture supernatants were harvested, and their HGF concentrations were determined by ELISA. *B,* coculture with fibroblasts induced CL-387,785 hyposensitivity in lung cancer cells. The lung cancer H1975 cell line was cocultured 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 nmol/L) for 72 h, and lung cancer cell growth was determined after 72 h of treatment by MTT assay. *, $P < 0.01$, Mann-Whitney *U* test.

stimulate Akt and ERK1/2 signaling predominantly via MET under these conditions.

Specific downregulation 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 downregulated *MET*, *EGFR*, and *ErbB3* expression, respectively, with specific siRNAs using H1975 cells. Downregulation 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. Downregulation 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, downregulation 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; Supplementary Fig. S2). To further investigate whether the susceptibility of H1975 cells to CL-387,785 could be affected by crosstalk to stromal fibroblasts, we cocultured the H1975 cells with MRC-5 cells or PF2 using Transwell systems. Whereas H1975 cells were highly sensitive to CL-387,785, exogenously added HGF induced CL-387,785 hyposensitivity of H1975 cells as mentioned above. Coculture 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; Supplementary Fig. S3). These

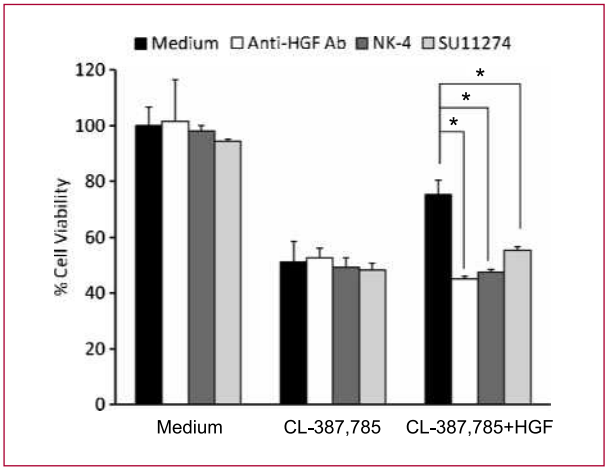

Fig. 6. Anti-HGF antibody, NK4, or SU11274 abrogated HGF-induced CL-387,785 hyposensitivity in lung cancer cells with *EGFR*-T790M mutation. H1975 cells were treated for 72 h with or without CL-387,785 (300 nmol/L) and/or HGF (50 ng/mL) in the presence or absence of anti-HGF neutralizing antibody (2 μg/mL), NK4 (300 nmol/L), or SU11274 (1 μmol/L). Cell growth was determined by MTT assay. *, $P < 0.01$, Mann-Whitney *U* test.

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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 TKI. The MET-TKI, SU11274, moderately reversed the HGF-induced CL-387,785 hyposensitivity at the nontoxic concentration of 1 μmol/L. Both anti-HGF neutralizing antibody (2 μg/mL) and HGF antagonist, NK4 (300 nmol/L), completely abrogated the CL-387,785 hyposensitivity at nontoxic concentrations (Fig. 6), suggesting the promising potential of these compounds to overcome HGF-induced resistance.

Discussion

In the present study, we showed 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 coculture 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 showed 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. showed 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 *EGFR*-T790M–mediated erlotinib resistance (37).

To overcome T790M mutation-mediated resistance, several agents, such as irreversible EGFR-TKIs, anti-EGFR antibody (38), and heat shock protein 90 inhibitors (39), have been developed and evaluated with regard to their efficacy in preclinical and clinical trials. Of the irreversible EGFR-TKIs, PF00299804, HKI-272, EKB-569, and BIBW2992 are currently in clinical trials, whereas commercially available CL-387,785 is not (19, 40). Yoshimura et al. reported promising results showing that EKB-569 caused partial responses in two 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 and thus show antitumor activity against several cell lines, including H1975 (38). However, although these agents show favorable responses in tumors with T790M mutation, it is clear that resistance can also develop against this class of inhibitors. We showed that HGF could induce hyposensitivity to irreversible EGFR-TKI. In addition, our preliminary experiments indicated that although 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, mitogen-activated protein kinase/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

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progression of TKI-naive 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 coculture with HGF-producing fibroblasts, suggesting the possible involvement of microenvironments in resistance to irreversible EGFR-TKIs. Moreover, we showed 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*.

Disclosure of Potential Conflicts of Interest

S. Yano: honoraria, AstraZeneca, Chugai Pharmaceuticals. The other authors disclosed no potential conflicts of interest.

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