

MET Activation Mediates Resistance to Lapatinib Inhibition of *HER2*-Amplified Gastric Cancer Cells

Chin-Tung Chen¹, Hyaehwan Kim¹, David Liska¹, Sizhi Gao², James G. Christensen³, and Martin R. Weiser¹

Abstract

HER2 amplification is found in more than 15% of gastric cancers and is associated with poor clinical outcome. Lapatinib, a dual *HER2* and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, has shown promising *in vitro* results in treating *HER2*⁺ cancer cells. However, several studies have shown that activation of alternative receptor tyrosine kinases can mediate resistance to HER-targeted therapy. Here, we investigated whether activated MET can confer resistance to lapatinib inhibition of gastric cancer cells. A panel of gastric cancer cell lines was treated with lapatinib, and we observed that cell proliferation was reduced by 70% and that the degree of *HER2* amplification corresponds to sensitivity to lapatinib. Immunoblotting analysis indicated that phosphorylation of *HER2*, EGFR, MET, AKT, and extracellular signal-regulated kinase was inhibited by lapatinib and presumably led to cell-cycle arrest as observed with flow cytometry. Hepatocyte growth factor (HGF) activation of MET receptors rescued cells from lapatinib-induced growth inhibition by restimulating the downstream pathways and restoring normal cell-cycle progression. This rescue effect could be abrogated by inhibiting MET with PHA-665752 (a highly specific MET inhibitor) or downregulating MET expression with short interfering RNA. No synergy in growth inhibition was observed when cells were treated with a combination of lapatinib and PHA-665752. Repeat studies using insulin-like growth factor 1 and fibroblast growth factor 3 could not uniformly rescue the lapatinib-treated gastric cancer cells. In conclusion, HGF/MET-mediated resistance to lapatinib is a novel mechanism of resistance to *HER2*-targeted agents in gastric cancer cells. Development of inhibitors targeting multiple receptors or common downstream signaling proteins merits further investigation. *Mol Cancer Ther*; 11(3); 660–9. ©2012 AACR.

Introduction

Gastric cancer is the fourth most commonly diagnosed cancer and the second most frequent cause of cancer-related deaths worldwide. Approximately, 989,600 new cases were reported in 2008, with the highest incidence rates in Asia (1). Patients with gastric cancer usually present at a late stage and prognosis is poor: in patients with operable disease, median 5-year survival rate is about 36%, but in patients with advanced or metastatic disease median 5-year survival rate is only 5% to 20%, with a median overall survival of about 1 year (2). Poor patient survival, and lack of a standardized chemotherapy regimen, have prompted interest in the development of

targeted therapies for gastric cancer (3, 4). *HER2/neu*, a member of the human epidermal growth factor receptor (EGFR) family, has attracted particular attention as a potential target because it is amplified and/or overexpressed in 7% to 35% of invasive gastric cancers, and high levels of *HER2* are associated with worse clinical outcome (5–9).

HER2 is a transmembrane receptor tyrosine kinase (RTK) activated through dimerization with members of the EGFR family, leading to a cascade of events involving the downstream signal transduction of Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase/AKT/mTOR pathways. These signaling cascades initiate the rapid cell growth, differentiation, survival, and migration associated with *HER2*⁺ cancer cells (10–13). Overexpression of *HER2* leads to tumor progression by deregulating cell proliferation and apoptosis through enhanced and prolonged signaling of the involved pathways.

Lapatinib (Tykerb, GlaxoSmithKline) is a dual tyrosine kinase inhibitor (TKI) that inhibits phosphorylation of both *HER2* and EGFR, thereby interrupting the downstream signaling pathways such as MAPK and AKT (14–16). Early clinical studies with anti-*HER2* therapy such as lapatinib have shown promising results; however, many of the patients who initially responded

Authors' Affiliations: Departments of ¹Surgery and ²Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York; and ³Department of Cancer Research, Pfizer Global Research and Development, La Jolla Laboratories, La Jolla, California

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Corresponding Author: Martin R. Weiser, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Room C-1075, New York, NY 10065. Phone: 212-639-6698; Fax: 212-794-3198; E-mail: weiser1@mskcc.org

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eventually developed resistance. One of the possible mechanisms of resistance development is the activation of an alternate RTK that restores the signaling pathways (17–19).

MET, a member of the RTK family, is frequently amplified and/or overexpressed in gastric cancer (6, 20–23). MET receptor's only known ligand is the hepatocyte growth factor (HGF), which activates MET upon binding and triggers the signaling of MAPK and AKT, common downstream targets of the EGFR family. Studies by Engelman and others of lung, breast, and colon cancer cells have shown that activations of MET can lessen the inhibitory effects of drugs designed specifically to target members of the EGFR family (24–26). Importantly, a study involving non-small cell lung cancer has shown that MET abrogates the sensitivity of these cells to an analogue of lapatinib (27). Furthermore, our laboratory and others have shown that the reverse, wherein HER kinase activation confers resistance to MET inhibition, is true for some gastric cancer cells (28, 29).

In this study, we show that activated MET can mediate resistance to lapatinib inhibition in *HER2*-amplified gastric cancer cell lines with MET coexpression. We also show that inhibition of MET can abrogate the rescue effects and restore growth inhibition of gastric cancer cells. Our data provide a strong rationale for targeting multiple RTKs with a broad inhibitor or developing a drug that targets common downstream signaling proteins.

Materials and Methods

Cell lines

Human gastric cancer cell lines NCI-N87 and SNU-16 were purchased from American Type Culture Collection. SNU-216 gastric cancer cells were obtained from Korean Cell Line Bank. NCI-N87, SNU-16, and SNU-216 were passaged for fewer than 6 months and their identities were authenticated by short tandem repeat analyses by the respective cell banks. The GTL-16 cell line was a gift from Dr. Silvia Giordano of the Institute for Cancer Research and Treatment at the Torino School of Medicine (Turin, Italy). DiFi, a human colorectal cancer cell line, was provided by Dr. José Baselga of the Vall d'Hebron University Hospital (Barcelona, Spain). Both GTL-16 and DiFi were passaged for fewer than 6 months and their identities were not confirmed by this laboratory when they were received from the respective donors. NCI-N87 cells were grown in RPMI-1640, SNU-216 were grown in RPMI-1640 + 25 mmol/L HEPES + 25 mmol/L sodium bicarbonate, and SNU-16 were grown in RPMI-1640 + 2 mmol/L L-glutamine + 10 mmol/L HEPES + 1 mmol/L sodium pyruvate + 4.5 g/L glucose. GTL-16 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) + high glucose. DiFi cells were grown in DMEM + high glucose supplemented by Ham's F-12. All media were supplemented with 10% fetal calf serum maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Chemicals and growth factors

Lapatinib was purchased from GlaxoSmithKline. PHA-665752 was provided by Pfizer Global Research and Development. Chemical structures of lapatinib and PHA-665752 are shown in Fig. 1A. Human fibroblast growth factor 3 (FGF-3), HGF, and insulin-like growth factor 1 (IGF-1) were purchased from R&D Systems Inc.

Quantitative PCR for analysis of gene genomic amplification

Primers and probes for *MET*, *HER2*, *EGFR*, and the single-copy reference gene *RNase P* were obtained from Applied Biosystems. Primer and probe sequence for *MET* were (5'–3'): forward GGAGCCAAAGTCCTTTCATCTGTA, reverse GCAATGGATGATCTGGGAAATAAGGAAT, and FAM-CCGGTTCATCAACTTC. Primer and probe sequence for *HER2* were (5'–3'): forward CCCTGAGCAAAGAGTCACAGATAAA, reverse TGC-CAGGGTCTGAGTCTCT, and FAM-CTGCACTGCGT-TTGTC. Primer and probe sequences for *EGFR* were (5'–3'): forward TTTGGAAAACCTGCAGATCATCAGA, reverse AGTCCGGTTTTATTTGCATCATAGTTAGA and FAM-AAATATGTACTACGAAAATTC. Quantitative PCR assay of genomic DNAs was conducted as previously described (28).

Western blot

Cells were treated with/without growth factors and/or inhibitors in serum-supplemented (10% fetal calf serum) medium. After removal of growth medium, the tissue culture flasks were placed on ice and the cells washed twice with ice-cold Tris-buffered saline (TBS). Cells were then scraped off and placed in ice-cold RIPA lysis buffer (Millipore Corp.) containing protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific). After being shaken for 15 minutes at 4°C, the cells were centrifuged at 20,000 × *g* for 15 minutes and the lysate stored at –80°C until further use. For Western blotting, equal amounts of protein (50 µg) were boiled in Laemmli buffer for 5 minutes, resolved by 10% SDS-PAGE (Invitrogen Corp.), and electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). After blocking nonspecific binding sites with 5% nonfat dry milk in TBS + 0.05% Tween 20 (TBS-T), the membrane was incubated with the respective antibodies overnight at 4°C. After 3 washes with TBS-T, the membrane was incubated for 1 hour at room temperature with a horseradish peroxidase (HRP)-linked secondary antibody, followed by several washes with TBS-T. The immunocomplexes were visualized with the ECL Plus Western Blotting detection system (GE Healthcare).

Antibodies

EGFR, MET, FGF receptor 1, FGF receptor 2, IGF 1 receptor (IGF-1R), and secondary goat antimouse immunoglobulin G (IgG) HRP antibodies were purchased from Santa Cruz Biotechnology Inc. Antibodies against AKT, phospho-AKT (Ser473), phospho-EGFR (Tyr1068),

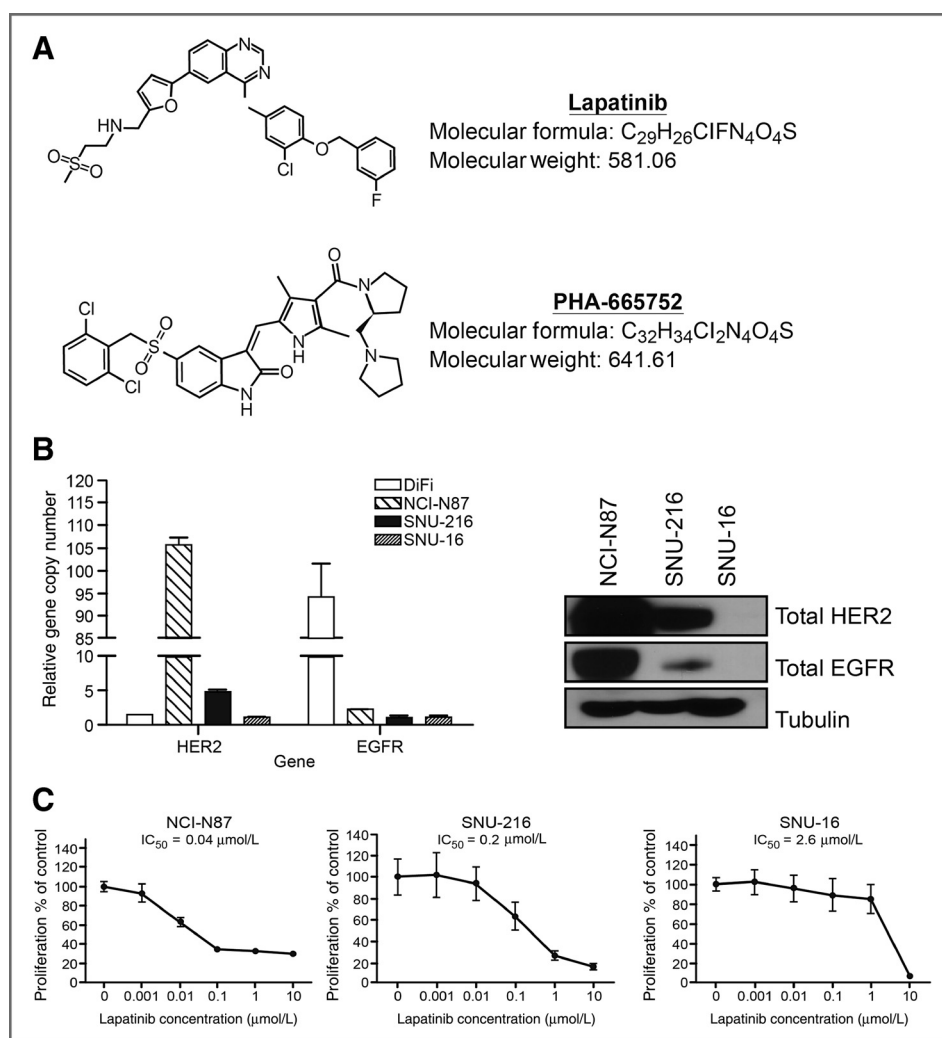


Figure 1. *HER2*-amplified gastric cancer cells are sensitive to lapatinib inhibition. **A**, chemical structures of lapatinib and PHA-665752. **B**, left, DNA from 4 cell lines was subjected to quantitative PCR analysis. Mean *HER2* or *EGFR* copy number is relative to *Rnase P* copy number. Calculations for both *HER2* and *EGFR* were normalized to SNU-16 (arbitrarily set as 1). The DiFi cell line serves as a basis for comparison of *EGFR* amplification. **B**, right, protein lysates were collected from all 3 gastric cancer cell lines and analyzed for total *HER2* and *EGFR* expression. Tubulin serves as loading control. **C**, gastric cancer cell lines were treated with increasing concentrations of lapatinib for 24 hours, followed by determination of cell proliferation as described in Materials and Methods. Data points, average of replicates of 6; bars, SD.

phospho-HER2 (Tyr1221/1222), p44/42 MAPK, and phospho-p44/42 MAPK (Thr202 and Tyr204) phospho-MET (Tyr1234/1235) were purchased from Cell Signaling Technology Inc. Anti-HER2 was obtained from Lab Vision Corp. Anti- α -tubulin was obtained from Calbiochem. Secondary donkey antirabbit IgG HRP was purchased from GE Healthcare.

Cell proliferation assay

Cellular proliferation was measured by a commercially available bromo-deoxyuridine (BrdUrd) cell proliferation assay kit (Roche). Briefly, the cells were seeded in sextuples in flat-bottomed 96-well plates at 3,000 to 5,000 cells per well and were allowed to adhere for 24 hours. Thereafter, the cells were treated for 24 hours, as indicated. After incubation with BrdUrd labeling reagent for 2 hours, the cells were fixed, and BrdUrd incorporation into newly synthesized DNA was assessed by incubation with an anti-BrdUrd peroxidase-conjugated antibody for 90 minutes, followed by addition of substrate solution and colorimetric detection at 370 and 492 nm, respectively.

IC₅₀ values were calculated by the GraphPad Prism version 5.0 software (GraphPad Software Inc.).

Cell-cycle analysis

Cells were seeded in 100 mm dishes at a density of 5×10^5 per dish. Twenty-four hours later, the cells were treated with inhibitors, growth factors, or media for 24 hours. Both adherent and floating cells were harvested and stained with ethidium bromide. Quantification for the cell-cycle distribution and the sub-G₁ population was done by flow cytometric analysis.

Cleaved caspase-3 assay

Cells were treated with/without growth factors and/or inhibitors in serum-supplemented (10% fetal calf serum) medium for 12 hours. Lysates were prepared in the same buffer used for Western blotting. One hundred micrograms of protein lysates were used for the PathScan cleaved caspase-3 sandwich ELISA (Cell Signaling Technology Inc.), following the manufacturer's instructions. In brief, extracts were mixed with

sample diluent and incubated in antibody-coated microwell strips. One hundred microliters of cleaved caspase-3 detection antibodies was added to each well. Binding was detected with 100 μ L of HRP-linked streptavidin antibody and 100 μ L of TMB substrate solution. The colored reaction product was measured in a microplate reader at 450 nm.

Statistical analysis

The statistical significance of differences was analyzed by 1-way ANOVA. In cases in which the *P* values for the overall comparisons were less than 0.05, *post hoc* pairwise comparisons were done with the Neuman-Keuls Multiple comparison test. Statistical analyses were completed using GraphPad Prism version 5.0 software.

Short interfering RNA

For short interfering RNA (siRNA) experiments, cells were seeded in sextuples in 96-well plates at 5,000 cells per well in antibiotic-free complete medium and allowed to adhere for 24 hours at 37°C. Thereafter, the cells were transfected with Dharmacon siGenome ON-TARGET plus human MET (sense 5'-GAACUGGUGUCCGGGAUUAUUU-3', antisense 5'-AUAUCCGGGACACCA-GUUCUU-3') siRNA or nontargeting siRNA (#3) according to the manufacturer's instructions (Dharmacon Inc.). After 24 hours, the transfection medium was removed and the cells were treated as indicated. Cell proliferation was determined as outlined above after 24 hours of incubation.

Real-time PCR

Taqman Gene Expression Assays for *MET* and 18s rRNA were purchased from Applied Biosystems. Gene expression was measured with the ABI Prism 7900HT Sequence Detection System from Applied Biosystems. Real-time PCR of cDNA specimens was conducted as previously described (28).

Results

HER2 amplified cells are sensitive to lapatinib inhibition

The gastric cancer cell lines selected for this study, NCI-N87, SNU-216, and SNU-16, displayed varying degrees of *HER2* and *EGFR* gene amplification and protein expression as determined by quantitative PCR and Western blot (Fig. 1B). The NCI-N87 line was highly amplified for the *HER2* gene, the SNU-216 line moderately amplified, and the control cell line SNU-16 was not *HER2* amplified. The degree of *HER2* amplification in NCI-N87 and SNU-216 also corresponds to overexpression of *HER2* proteins in these cells. *EGFR* gene copy number did not differ significantly between the 3 gastric cancer cell lines, although there was significant *EGFR* expression in NCI-N87 compared with SNU-216 and SNU-16.

To determine the sensitivity of the 3 gastric cancer cell lines to a TKI targeting both *HER2* and *EGFR*, each cell line

was exposed to increasing dosages of lapatinib, to measure its effects on cell proliferation (Fig. 1C). NCI-N87, showing the most *HER2* amplification of the 3 cell lines tested, displayed a 70% growth inhibition at the lowest concentration of lapatinib when compared with SNU-216 and SNU-16. In contrast, SNU-216, with a modest amplification of *HER2*, required more than 5-fold the concentration of lapatinib used in NCI-N87 to achieve the same degree of growth inhibition. SNU-16, the control cell line with no *HER2* amplification, was, of the 3 gastric cancer cell lines, the least sensitive to the effects of lapatinib, except at high concentrations.

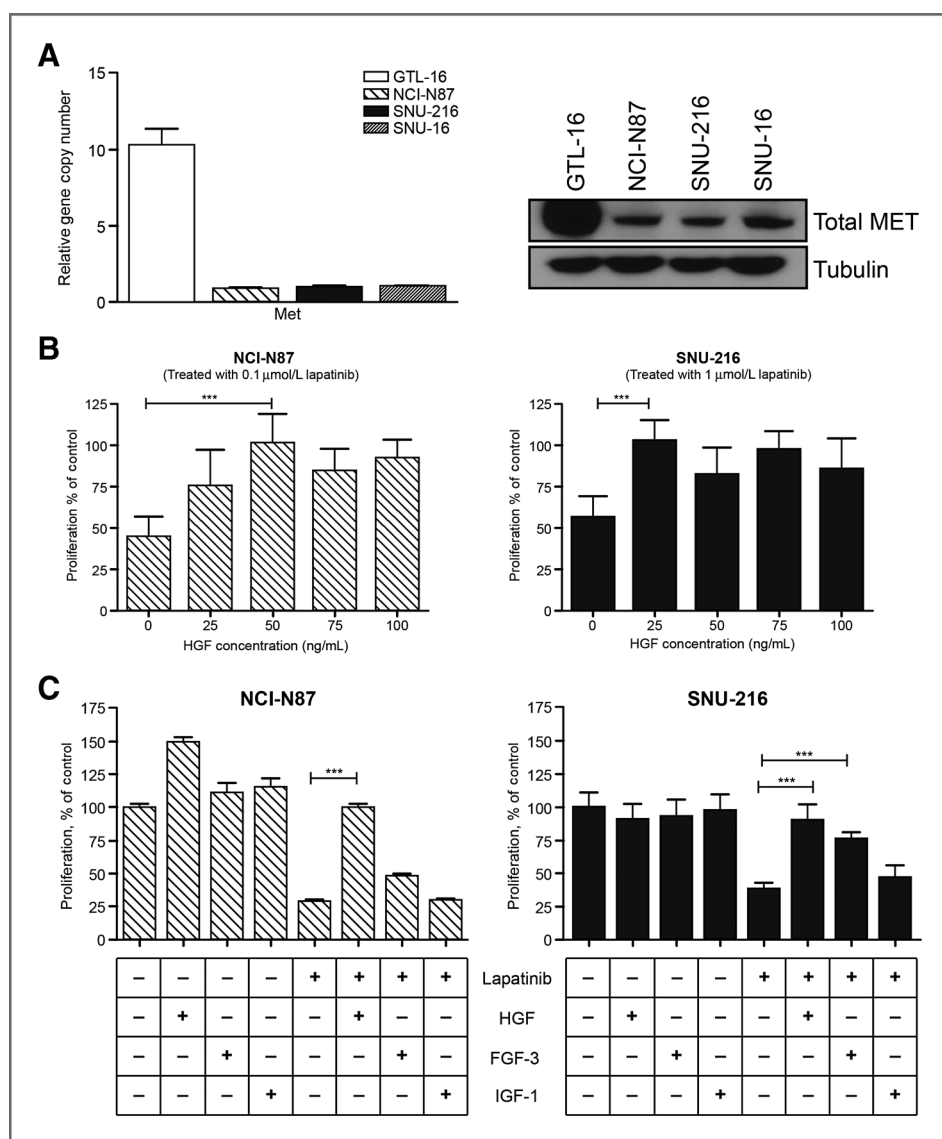
Activation of MET RTK by HGF can rescue HER2⁺ gastric cancer cells from lapatinib inhibition

Figure 2A shows that *MET* was not amplified in NCI-N87, SNU-216, and SNU-16, and that all 3 gastric cancer cell lines expressed the *MET* receptor. To determine whether *MET* activation can rescue the *HER2*-amplified gastric cancer cell lines from lapatinib-induced growth inhibition, NCI-N87 and SNU-216 were treated with lapatinib and increasing concentrations of the *MET* ligand HGF for 24 hours. The results showed that, at concentrations of 50 ng/mL (NCI-N87) and 25 ng/mL (SNU-216) HGF, the growth inhibitory effects of lapatinib were reversed (Fig. 2B).

We then assessed other growth factors for their ability to rescue gastric cancer cells from lapatinib. Receptors for FGF-3 and IGF-1 are present on NCI-N87 and SNU-216 (Supplementary Fig. S1A), and cell proliferation assays with these growth factors (50 ng/mL for NCI-N87 and 25 ng/mL for SNU-216) and lapatinib (0.1 μ M for NCI-N87 and 1 μ M for SNU-216) were then carried out. As seen in Fig. 2C, only HGF showed a significant abrogation of lapatinib inhibition in NCI-N87, whereas both FGF-3 and HGF were able to rescue inhibited SNU-216. A 2-fold increase in the concentrations of FGF-3 and IGF-1 did not alter the results (Supplementary Fig. S1B). Addition of FGF-3 to lapatinib-treated gastric cancer cells could not restore MAPK signaling to the same degree as HGF while IGF-1 has no noticeable effects on the phosphorylation of MAPK (Supplementary Fig. S1C). IGF-1 and FGF-3 could not restore AKT phosphorylation in NCI-N87 as effectively as HGF, and both FGF-3 and IGF-1 modestly restore AKT phosphorylation in SNU-216.

MET confers resistance to lapatinib inhibition by restoring MAPK and AKT signaling

HER2, *EGFR*, and *MET* receptors, as well as MAPK and AKT, were dephosphorylated in lapatinib-treated gastric cancer cells (Fig. 3A). The degradation of the MAPK signaling pathway presumably induced growth inhibition in both gastric cancer cell lines via *G*₁ cell-cycle arrest, showed by an increased proportion of cells in the *G*₁ phase as measured by flow cytometry (Figs. 3B and 4A). Correspondingly, the percentage of cells in S phase decreased from 21% to 6% in NCI-N87 cells and from 35% to 20% in SNU-216. We observed an increase in the number of cells



in the sub- G_1 population and cleaved caspase-3 proteins, indicating that lapatinib induced apoptosis in NCI-N87 as well (Figs. 4B and C).

The addition of HGF to lapatinib-treated gastric cancer cells phosphorylated MET RTK and restored MAPK and AKT signaling (Fig. 3A). The reactivated MAPK pathway may mediate the escape from lapatinib-induced growth inhibition by allowing G_1 -arrested NCI-N87 and SNU-216 cells to exit G_1 and enter normal cell-cycle progression (Figs. 3B and 4A). The population of apoptotic cells in NCI-N87 also decreased when AKT was rephosphorylated by MET RTK (Fig. 4C).

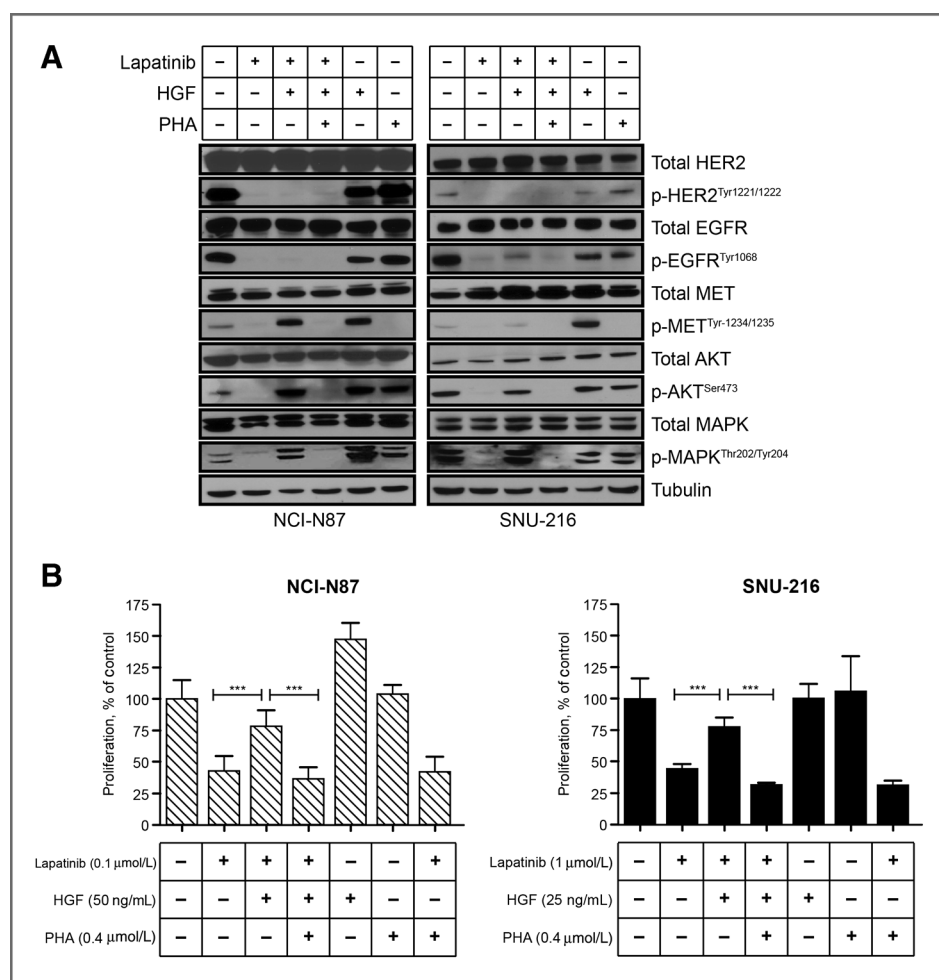
Addition of PHA-665752 can suppress the rescue effects of MET RTK

NCI-N87 and SNU-216 cells were treated with a highly selective MET tyrosine kinase inhibitor, PHA-665752 and

showed no significant growth inhibition (Fig. 3B). When the gastric cancer cells were treated with a combination of lapatinib, HGF, and PHA-665752, MET-mediated resistance to lapatinib inhibition was completely abrogated and growth inhibition was restored (Fig. 3B). Figure 3A shows that the addition of PHA-665752 was able to prevent activation of the MET RTK by blocking phosphorylation of the MET tyrosine kinase domain (Tyr1234/1235). Inactivation of MET, HER2, and EGFR by lapatinib and PHA-665752 were able to abolish the downstream phosphorylation of MAPK and AKT, seen in untreated and in HGF-treated cells. We noted restoration of G_1 arrest in both gastric cancer cell lines, characterized by an increase in the G_1 population (62% to 84% in NCI-N87 and 45% to 61% in SNU-216; Fig. 4A). Apoptotic cells also increased in NCI-N87, as shown by a return to the lapatinib-treated level (Fig. 4B and C).

Figure 2. HGF activation of MET RTK confers resistance to lapatinib-induced growth inhibition. A, left, DNA from 4 cell lines was subjected to quantitative PCR analysis for *MET*. Average *MET* copy number is relative to *Rnase P* copy number. Calculations for *MET* were normalized to SNU-16 (arbitrarily set as 1). Cell line GTL-16 serves as a basis for comparison for *MET* amplification. A, right, total MET protein expressions in GTL-16, NCI-N87, SNU-216, and SNU-16 were analyzed by Western Blotting. Tubulin serves as loading control. B, *HER2*-amplified cell lines NCI-N87 and SNU-216 were treated with lapatinib (0.1 $\mu\text{mol/L}$ for NCI-N87, 1 $\mu\text{mol/L}$ for SNU-216) and increasing dosages of HGF for 24 hours. Cell proliferation was analyzed by BrdUrd colorimetric assay. All cell proliferation data points were average of replicates of 6; bars, SD; *, $P < 0.0001$. C, NCI-N87 and SNU-216 cells were treated with or without lapatinib at the concentrations of 0.1 $\mu\text{mol/L}$ and 1 $\mu\text{mol/L}$, respectively. In addition, some treatment groups were supplemented with HGF, FGF-3, or IGF-1 at the concentrations of either 50 ng/mL for NCI-N87 or 25 ng/mL for SNU-216. Both gastric cancer cells were treated for 24 hours before cell proliferation was analyzed by BrdUrd colorimetric assay. All cell proliferation data points were average of replicates of 6; bars, SD; *, $P < 0.0001$.

Figure 3. Lapatinib inhibition of downstream signaling pathways can be restored by activated MET RTK, conferring resistance to growth inhibition. PHA-665752 can be used to abrogate this rescue effect by inhibiting MET activation. **A**, NCI-N87 and SNU-216 were treated with or without the inhibitors lapatinib (0.1 $\mu\text{mol/L}$ for NCI-N87 and 1 $\mu\text{mol/L}$ for SNU-216) and PHA-665752 (0.4 $\mu\text{mol/L}$) for 12 hours. After 12 hours, cells were also treated with or without HGF (50 ng/mL for NCI-N87 and 25 ng/mL for SNU-216) for 15 minutes before whole-cell lysates were collected and analyzed by Western blotting. **B**, both gastric cancer cell lines were treated with a different combination of lapatinib (0.1 $\mu\text{mol/L}$ for NCI-N87 and 1 $\mu\text{mol/L}$ for SNU-216), PHA-665752 (0.4 $\mu\text{mol/L}$), and/or HGF (50 ng/mL for NCI-N87 and 25 ng/mL for SNU-216) for 24 hours. Determination of cell proliferation data points were average of replicates of 6; bars, SD; *, $P < 0.0001$.



RNA interference silencing of *MET* restores lapatinib-induced growth inhibition

To confirm that MET RTK is responsible for lapatinib resistance, both gastric cancer cell lines were transfected with either nonsilencing siRNA, MET-targeting siRNA, or no siRNA, respectively. In Fig. 5A, cells transfected with nonsilencing siRNA showed no significant decrease in *MET* mRNA expression compared with cells not transfected with any siRNA. In contrast, *MET* mRNA was significantly downregulated (~85%–90%) in both NCI-N87 and SNU-216 following transfection with MET siRNA. When both transfected groups in NCI-N87 and SNU-216 were additionally treated with lapatinib and HGF, MET-mediated resistance to lapatinib-induced growth inhibition was lost (Fig. 5B).

Discussion

Molecular therapies targeting specific RTKs responsible for cell proliferation, survival, and migration have become more attractive as therapeutic strategies in gastric cancer, in which, despite modern surgery and chemotherapy, survival remains poor (2–4). HER2 is of particular

interest as a drug target because it has been shown to be amplified and/or overexpressed in a subset of gastric cancers (5–9). Knockdowns of HER2 protein expression also results in decreased cell viability in *HER2*⁺ gastric cancer cell lines and reduced tumor growth *in vivo* (30, 31). Lapatinib, a dual TKI that targets both HER2 and EGFR, is one of the drugs currently being assessed in clinical trials for potential use in gastric cancer therapy. Lapatinib, which has shown promising *in vitro* results in the treatment of *HER2*⁺ cancer cells, is already approved for the treatment of specific subsets of *HER2*⁺ breast cancer patients. However, numerous studies have revealed a particular weakness in the therapeutic strategies targeting single receptors such as HER2, wherein drug resistance can be conferred through the activation of compensatory/overlapping survival pathways (32–35).

We hypothesized that MET may confer resistance to HER2-directed therapy in gastric cancer. The MET RTK has been implicated as a mediator of resistance to therapies targeting members of the HER family of RTKs in breast, colon, and lung cancer cells (24–27). Furthermore, HER2 and MET are coexpressed in 12% of unselected gastric cancer but 24% of the intestinal subtype (36).

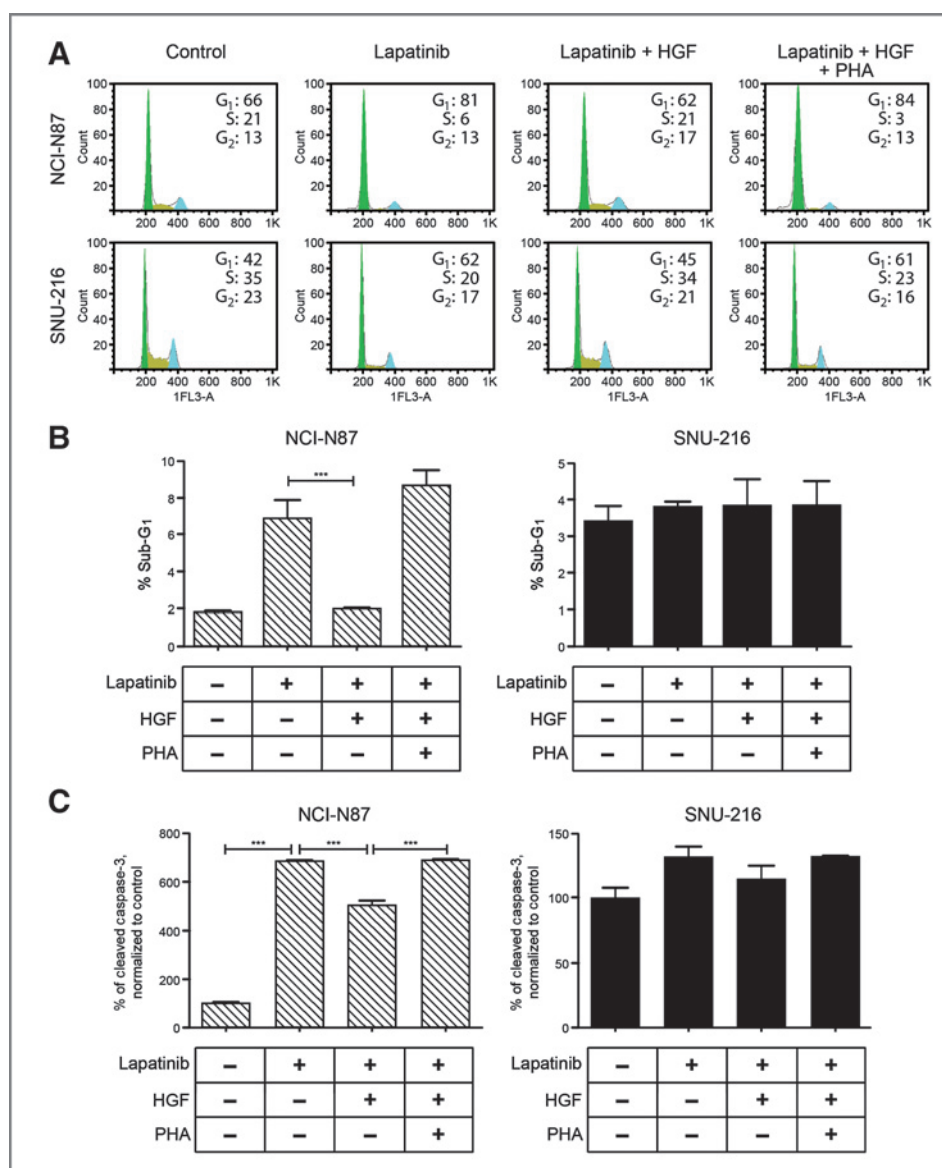


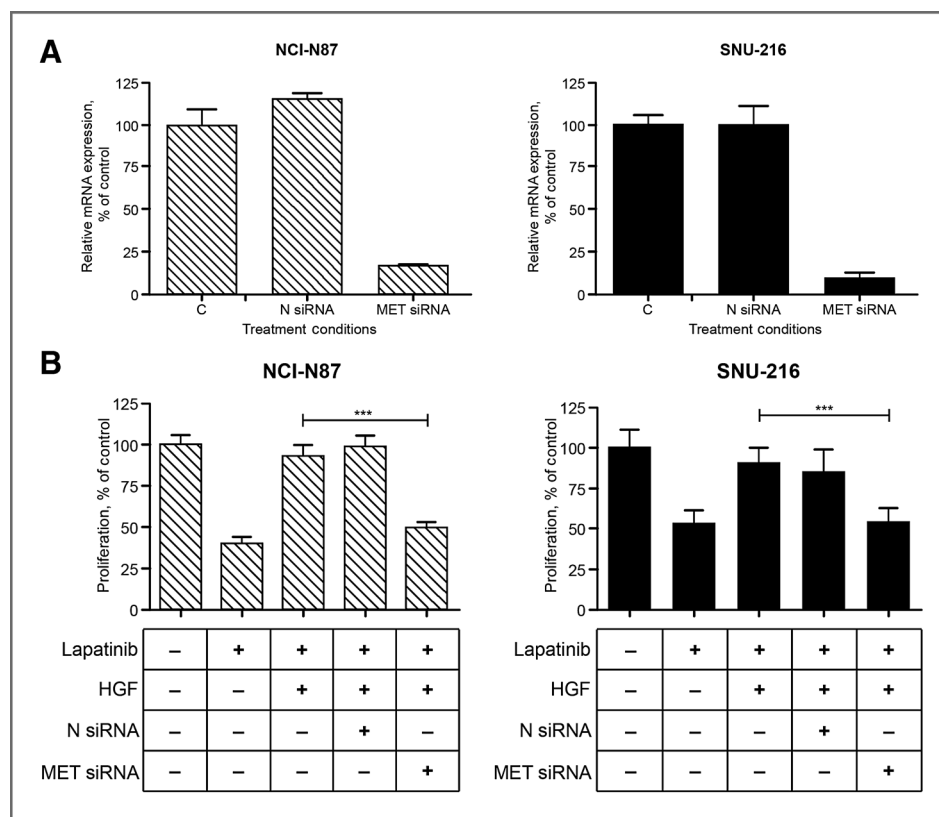
Figure 4. Lapatinib-induced growth inhibition, due to G₁ arrest and apoptosis, can be abrogated by MET. Addition of PHA-665752 can overcome MET-mediated rescue of gastric cancer cells from lapatinib inhibition. A and B, NCI-N87 and SNU-216 were treated for 24 hours with lapatinib (0.1 μ mol/L for NCI-N87 and 1 μ mol/L for SNU-216), \pm HGF (50 ng/mL for NCI-N87 and 25 ng/mL for SNU-216), and with or without PHA-665752 (0.4 μ mol/L). Flow cytometric determination of the cell-cycle distribution was as described in Materials and Methods. Representative results of 3 independent experiments. C, NCI-N87 and SNU-216 were treated with or without lapatinib (0.1 μ mol/L for NCI-N87 and 1 μ mol/L for SNU-216), PHA-665752 (0.4 μ mol/L), and/or HGF (50 ng/mL for NCI-N87 and 25 ng/mL for SNU-216) for 12 hours before whole-cell lysates were collected. Protein lysates (100 μ g) were analyzed for amount of cleaved caspase-3 as described in Materials and Methods. Columns, average of triplicates; bars, SD; *, P < 0.0001.

Our results show that the *HER2*⁺ gastric cancer cell lines NCI-N87 and SNU-216 are more sensitive to lapatinib-induced growth inhibition than a non-*HER2* amplified cell line (SNU-16). Growth inhibition in the gastric cancer cells, induced by decreased MAPK signaling, is likely mediated through G₁ cell-cycle arrest. Despite suppression of AKT signaling in both gastric cancer cell lines, we noted apoptotic events only in NCI-N87 and not in SNU-216. Kim and colleagues, who made similar discoveries, have suggested that the *HER2* gene amplification ratio may be an important factor in determining susceptibility to lapatinib-induced apoptosis, and/or that there are different mechanisms of action for lapatinib in various cell lines (15).

Interestingly, we noted dephosphorylation of MET receptors in the NCI-N87 and SNU-216 lines treated with

lapatinib (Fig. 3A). A similar phenomenon was also observed in a NSCLC cell line, in which an analogue of lapatinib disrupted the physical complex between MET and *HER2* receptors and inhibited MET activity (27). These findings are consistent with several studies that support cross-activation between MET and EGFR receptors (24, 37, 38). In our gastric cancer cell lines, the mechanism of baseline MET phosphorylation remains unclear. We cannot clearly implicate direct cross-talk through *HER2*/MET heterodimers as coimmunoprecipitation experiments only showed heterodimerization of *HER2* and EGFR but not MET and *HER2*/EGFR (data not shown; ref. 27). Other intermediaries or indirect cross-talk may be important as has been shown with the intracellular Src kinase (39, 40). Alternatively, lapatinib may have off-target effects on MET; however, this has not been shown

Figure 5. Downregulation of MET RTK expression will restore lapatinib-induced growth inhibition. A, both gastric cancer cell lines were transfected with either no siRNA (C), nonsilencing siRNA (N siRNA) or MET-silencing siRNA for 24 hours. Transfected cells were harvested and real-time PCR was used to determine relative *MET* mRNA expression. Columns, average of triplicates. B, transfected cells were also treated with or without lapatinib (0.1 μ mol/L for NCI-N87 and 1 μ mol/L for SNU-216) and \pm HGF (50 ng/mL for NCI-N87 and 25 ng/mL for SNU-216) for 24 hours before determination of cell proliferation by BrdUrd assay. Columns, average of replicates of 6; bars, SD; *, $P < 0.0001$.



in a model using A549 NSCLC cells in which MET activation is not significantly affected by treatment with lapatinib (41).

We found that MET is coexpressed with HER2 and EGFR in our gastric cancer cell lines, and HGF-mediated activation of MET can rescue NCI-N87 and SNU-216 from lapatinib-induced growth inhibition. Smaller concentrations of HGF were required to confer resistance in SNU-216 than in NCI-N87, most likely due to reduced sensitivity to lapatinib in SNU-216 (Fig. 2B). Phosphorylation of MET reversed the growth inhibitory effects of lapatinib through reactivation of the downstream signaling pathways MAPK and AKT. Presumably, the restored signaling of MAPK and AKT pathways allowed the gastric cancer cells to exit G_1 arrest and to enter normal cell-cycle progression, and decreased the frequency of apoptotic events in NCI-N87.

We investigated other RTKs including IGF-R1 and FGFR because IGF-1R can promote resistance to anti-EGFR treatment and FGF-3 is amplified/overexpressed in selected gastric cancers (42, 43). Coculture of lapatinib-treated gastric cancer cell lines with IGF-1 or FGF-3 showed that only HGF significantly rescued NCI-N87 cells from lapatinib-induced growth inhibition while FGF-3 conferred resistance to lapatinib similar to HGF in SNU-216. Increasing the concentrations of FGF-3 and IGF-1 showed no significant change in proliferation of lapatinib-treated gastric cancer cell lines. Immunoblot analysis of both cell lines showed that neither FGF-3 nor

IGF-1 could restore MAPK signaling as strongly as HGF. It is possible that such rescue by FGF-3 in SNU-216 is possible because of the cell line's reduced sensitivity to lapatinib when compared with NCI-N87.

Recently, Liu and colleagues examined a panel of cancer cells with *HER1/HER2* amplification coupled with MET overexpression and reported synergy in growth inhibition, when cells are treated with dual inhibitors (44). They conclude that these receptors cooperate in promoting cell proliferation and survival and that simultaneous inhibition is necessary to achieve maximal clinical effect. In our study, synergy was not observed in NCI-N87 and SNU-216 treated with dual inhibitors, despite *HER2* amplification and MET overexpression (Fig. 3B). Inhibition of HER2 was sufficient to induce significant growth arrest without requiring combinatorial treatment with a MET inhibitor, suggesting that dual inhibition is not necessary until resistance develops. This indicates that genotype alone may not predict response and that lineage may be important.

Our findings are consistent with those of Engelman and colleagues, who found that "oncogenically-addicted" cells do not require dual therapy until resistance develops with activation of a compensatory RTK pathway (45). They argue that dual therapy should not be initially used to avoid increased toxicity and that intermittent, short duration multidrug therapy could prevent the development of resistance (46).

Our findings support the hypothesis that, in gastric cancers that coexpress HER2, EGFR, and MET, lapatinib-

induced growth inhibition are abrogated through the activation of MET RTK, which restores shared downstream signaling pathways such as MAPK and AKT. Importantly, PHA-665752, a highly selective MET tyrosine kinase inhibitor, prevents MET-mediated mechanisms of resistance to lapatinib inhibition. PHA-665752 resensitizes NCI-N87 and SNU-216 to the effects of lapatinib by inhibiting MET phosphorylation and suppressing MAPK and AKT signal transduction. We also confirmed our finding that downregulation of MET expression with siRNA can bypass the rescue effects of MET and restore growth inhibition of the gastric cancer cells by lapatinib. In addition, we associated degree of *HER2* amplification with response to lapatinib. Further experiments showed that cells less sensitive to lapatinib-induced growth inhibition are more susceptible to the rescue effects of other growth factors. This suggests that quantitative analysis of *HER2* amplification might better predict response to lapatinib.

This study illustrates the potential limitations of molecular therapies targeting a dominant RTK and serves as another example of acquired drug resistance mediated by activation of secondary RTK restoring downstream signaling pathways (32–35). In developing more effective and durable treatment strategies, multitargeted therapy should be considered.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;61:69–90.
- Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER-2 positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomized controlled trial. *Lancet* 2010;376:687–97.
- Ohtsu A. Chemotherapy for metastatic gastric cancer: past, present, and future. *J Gastroenterol* 2008;43:256–64.
- Arkenau H. Gastric cancer in the era of molecularly targeted agents: current drug development strategies. *J Cancer Res Clin Oncol* 2009;135:855–66.
- Resende C, Ristimaki A, Machado JC. Genetic and epigenetic alteration in gastric carcinogenesis. *Helicobacter* 2010;15:34–9.
- Nakajim M, Sawada H, Yamada Y, Watanabe A, Tatsumi M, Yamashita J, et al. The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. *Cancer* 1999;85:1894–902.
- Park D, Yun JW, Park JH, Oh SJ, Kim HJ, Cho YK, et al. HER-2/neu amplification is an independent prognostic factor in gastric cancer. *Dig Dis Sci* 2006;51:1371–9.
- Zhang XL, Yang YS, Xu DP, Qu JH, Guo MZ, Gong Y, et al. Comparative study on overexpression of HER2/neu and HER3 in gastric cancer. *World J Surg* 2009;33:2112–8.
- Vita FD, Giuliani F, Silvestris N, Catalano G, Ciardiello F, Orditura M. Human epidermal growth factor receptor 2 (HER2) in gastric cancer: a new therapeutic target. *Cancer Treat Rev* 2010;36:S11–5.
- Ménard S, Pupa SM, Campiglio M, Tagliabue E. Biologic and therapeutic role of HER2 in cancer. *Oncogene* 2003;22:6570–8.
- Ménard S, Casalini P, Campiglio M, Pupa SM, Tagliabue E. Role of HER2/neu in tumor progression and therapy. *Cell Mol Life Sci* 2004;61:2965–78.
- Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. *Curr Opin Cell Biol* 2009;21:177–84.
- Baselga J, Swain SM. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nat Rev Cancer* 2009;9:463–75.
- Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, et al. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 2002;21:6255–63.
- Kim JW, Kim HP, Im SA, Kang S, Hur HS, Yook YK, et al. The growth inhibitory effect of lapatinib, a dual inhibitor of EGFR and HER2 tyrosine kinase, in gastric cancer cell lines. *Cancer Lett* 2008;272:296–306.
- Wainberg ZA, Anghel A, Desai AJ, Ayala R, Luo T, Safran B, et al. Lapatinib, a dual EGFR and HER2 kinase inhibitor, selectively inhibits HER2-amplified human gastric cancer cells and is synergistic with trastuzumab in vitro and in vivo. *Clin Cancer Res* 2010;16:1509–19.
- Xia W, Bacus S, Hegde P, Husain I, Strum J, Liu L, et al. A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. *Proc Natl Acad Sci U S A* 2006;103:7795–800.
- Chen FL, Xia W, Spector NL. Acquired resistance to small molecule ErbB2 tyrosine kinase inhibitors. *Clin Cancer Res* 2008;14:6730–4.
- Liu L, Greger J, Shi H, Liu Y, Greshock J, Annan R, et al. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. *Cancer Res* 2009;69:6871–8.
- Hara T, Ooi A, Kobayashi M, Mai M, Yanagihara K, Nakanishi I. Amplification of c-myc, K-sam, and c-met in gastric cancers: detection by fluorescence *in situ* hybridization. *Lab Invest* 1998;78:1143–53.
- Maulik G, Shrikhande A, Kijima T, Ma PC, Morrison PT, Salgia R. Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. *Cytokine Growth Factor Rev* 2002;13:41–59.
- Inoue T, Kataoka H, Goto K, Nagaike K, Igami K, Naka D. Activation of c-Met (hepatocyte growth factor receptor) in human gastric cancer tissue. *Cancer Sci* 2004;95:803–8.
- Janjigian YY, Tang LH, Coit DG, Kelsen DP, Francone TD, Weiser MR, et al. MET expression and amplification in patients with localized gastric cancer. *Cancer Epidemiol Biomarkers Prev* 2011;20:1021–7.
- Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park J, et al. Met amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039–43.
- Shattuck DL, Miller JK, Carraway KL 3rd, Sweeney C. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. *Cancer Res* 2008;68:1471–7.
- Liska D, Chen CT, Bachleitner-Hofmann T, Christensen JG, Weiser MR. HGF rescues colorectal cancer cells from EGFR inhibition via MET activation. *Clin Cancer Res* 2011;17:472–82.
- Agarwal S, Zerillo C, Kolmakova J, Christensen JG, Harris LN, Rimm DL, et al. Association of constitutively activated hepatocyte growth factor receptor (Met) with resistance to a dual EGFR/Her2 inhibitor in non-small-cell lung cancer cells. *Br J Cancer* 2009;100:941–9.

28. Bachleitner-Hofmann T, Sun MY, Chen CT, Tang L, Song L, Zeng Z, et al. HER kinase activation confers resistance to MET tyrosine kinase inhibition in MET oncogene-addicted gastric cancer cells. *Mol Cancer Ther* 2008;7:3499–508.
29. Corso S, Ghiso E, Cepero V, Sierra JR, Migliore C, Bertotti A, et al. Activation of HER family members in gastric carcinoma cells mediates resistance to MET inhibition. *Mol Cancer* 2010;9:121.
30. Arrington AK, Dahlberg PS, Davydova J, Vickers SM, Yamamoto M. ERBB2 suppression decreases cell growth via apoptosis in gastrointestinal adenocarcinomas. *Surgery* 2009;146:213–9.
31. Arrington AK, Davydova J, Vickers SM, Yamamoto M. Anti-ERBB2 shRNA suppress both cell growth and tumor growth in ERBB2-over-expressing upper gastrointestinal adenocarcinomas. *J Gastrointest Surg* 2009;13:1754–61.
32. Workman P. The opportunities and challenges of personalized genome-based molecular therapies for cancer: targets, technologies, and molecular chaperones. *Cancer Chemother Pharmacol* 2003;52: S45–56.
33. Becker JC, Müller-Tidow C, Serve H, Domschke W, Pohle T. Role of receptor tyrosine kinases in gastric cancer: new targets for a selective therapy. *World J Gastroenterol* 2006;12:3297–305.
34. Stommel JM, Kimmelman AC, Ying H, Nabioullin R, Ponugoti AH, Wiedemeyer R, et al. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. *Science* 2007; 318:287–90.
35. Garrett JT, Arteaga CL. Resistance to HER2-directed antibodies and tyrosine kinase inhibitors. *Cancer Biol Ther* 2011;11:793–800.
36. Kubicka S, Claas C, Staab S, Khnel F, Zender L, Trautwein C, et al. p53 mutation pattern and expression of c-erbB2 and c-met in gastric cancer. *Dig Dis Sci* 2002;47:114–21.
37. Radaeva S, Ferreira-Gonzalez A, Sirica AE. Overexpression of C-NEU and C-MET during rat liver cholangiocarcinogenesis: a link between biliary intestinal metaplasia and mucin-producing cholangiocarcinoma. *Hepatology* 1999;29:1453–62.
38. Jo M, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, Strom SC. Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. *J Biol Chem* 2000;275:8806–11.
39. Mueller KL, Hunter LA, Ethier SP, Boerner JL. Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells. *Cancer Res* 2008;68: 3314–22.
40. Kataoka Y, Mukohara T, Tomioka H, Funakoshi Y, Kiyota N, Fujiwara Y, et al. Foretinib (GSK1363089), a multi-kinase inhibitor of MET and VEGFRs, inhibits growth of gastric cancer cell lines by blocking inter-receptor tyrosine kinase networks. *Invest New Drugs* 2011. [Jan 25, 2012].
41. Olausson KA, Commo F, Tailler M, Lacroix L, Vitale I, Raza SQ, et al. Synergistic proapoptotic effects of the two tyrosine kinase inhibitors pazopanib and lapatinib on multiple carcinoma cell lines. *Oncogene* 2009;28:4249–60.
42. Katoh M, Terada M. Oncogenes and tumor suppressor genes. In: Nishi M, et al. editors. *Gastric cancer*. Tokyo: Springer-Verlag; 1993. p. 196–208.
43. Jones HE, Gee J, Hutcheson IR, Knowlden JM, Barrow D, Nicholson RI. Growth factor receptor interplay and resistance in cancer. *Endoc Relat Cancer* 2006;Suppl 1:S45–51.
44. Liu L, Shi H, Liu Y, Anderson A, Peterson J, Greger J, et al. Synergistic effects of foretinib with HER-targeted agents in MET and HER1- or HER2-coactivated tumor cells. *Mol Cancer Ther* 2011;10:518–30.
45. Rexer BN, Engelman JA, Arteaga CL. Overcoming resistance to tyrosine kinase inhibitors: lessons learned from cancer cells treated with EGFR antagonists. *Cell Cycle* 2009;8:18–22.
46. Kaiser J. Combining targeted drugs to stop resistant tumors. *Science* 2011;331:1542–5.