Purpose: Targeting human epidermal growth factor receptor 2 (HER2) therapy is currently considered as the standard treatment for HER2-positive (HER2+ ) advanced gastric cancer. However, as seen in recent clinical trials, most of HER2+ gastric cancer are actually unresponsive to HER2-targeted agents, including lapatinib. The aim of this study is to identify the responsible receptor tyrosine kinases (RTK) potentially conferring lapatinib unresponsiveness in HER2+ gastric cancer and elucidate the molecular mechanism underlying this RTKs-induced resistance.

Experimental Design: A functional RNAi screen targeting human RTKs and related growth factors was used to identify candidate RTKs conferring lapatinib unresponsiveness in HER2+ gastric cancer cells. Independent siRNAs transfection and corresponding ligands supplement were performed to validate the effects of candidate RTKs on lapatinib sensitivity. Cross-talks of pathways involved were analyzed via Western blot analysis. Cell apoptosis and cell motility were detected using FACS system and Transwell assay. Immunohistochemistry was used to analyze protein expression in clinical samples.

Results: MET, HER3, insulin-like growth factor (IGF)-1R, and INSR were identified to mediate lapatinib unresponsiveness in HER2+ gastric cancer cells. Activation of these bypass RTKs attenuated lapatinib-induced apoptosis and suppression of cell motility, mechanistically because of restimulating the shared downstream AKT or ERK signaling, as well as restimulating WNT signaling and epithelial-to-mesenchymal transition (EMT)–like process. Patients’ specimens revealed that these unresponsiveness-conferring RTKs were particularly enriched in the majority of patients with HER2+ gastric cancer.

Conclusions: MET, HER3, IGF1R, and INSR pathways activation represent novel mechanism underlying lapatinib unresponsiveness in HER2+ gastric cancer. Combination strategy may be recommended in treating patients with HER2+ gastric cancer with these pathways activation.

Introduction
Previous studies have demonstrated that the human epidermal growth factor receptor 2 (HER2) signaling pathway is a critical driver of carcinogenesis and tumor progression in approximately 7% to 34% of total patients with gastric cancer (1–3). Targeting HER2 combined with chemotherapy has been the first-line treatment for HER2-positive (HER2+) advanced gastric cancer (1, 4). However, only few patients with HER2+ gastric cancer responded to the HER2-targeting agents. Recently, preliminary results of a phase III trials evaluating the efficacy of lapatinib, a dual tyrosine kinase inhibitor (TKI) targeting both HER2 and epithelial growth factor receptor (EGFR), in conjunction with cytotoxic chemotherapy for HER2+ advanced or metastatic gastric cancer (LOGiC study) were released. Unfortunately, the addition of lapatinib to chemotherapy did not significantly improve OS, the primary endpoint, compared with chemotherapy alone. The median OS was 12.2 versus 10.5 months, respectively (HR, 0.91; 95% CI, 0.73–1.12; \( P = 0.3492 \)). The median PFS was 6.0 versus 5.4 months, response rate 53% versus 40% and duration of response 7.3 versus 5.6 months (5).

Based on the data from LOGiC study, the observed unsatisfactory survival prolongation could be explained by the limited initial response to lapatinib for patients with HER2+...
Translational Relevance

Intrinsic resistance has emerged as one of the major obstacles for clinical use of human epidermal growth factor receptor 2 (HER2)-targeted agents for HER2-positive (HER2⁺) gastric cancer therapy. Elucidating the molecular mechanism of lapatinib unresponsiveness can be critical to identify patients who fail to respond to therapy, and may help design more efficient treatment protocols. Using a functional RNAi screen, our results clearly demonstrate that activation of MET, HER3, insulin-like growth factor 1R (IGF1R), and INSR signaling is responsible for lapatinib unresponsiveness in HER2⁺ gastric cancer. Considering the high prevalence of these activated pathways in HER2⁺ gastric cancer tumors, this may provide crucial explanation to the limited response of lapatinib in HER2⁺ gastric cancer as observed in recently released phase III clinical trials data. We believe patients with HER2⁺ represent heterogeneous individuals with different sensitivities to anti-HER2 agents. These responsible receptor tyrosine kinases could be novel targets for intervention to improve the efficacy of HER2-targeted therapy.

**Materials and Methods**

**Cell lines**

NCI-N87, HGC-27, AGS, and SGC-7901 human gastric cancer tumor cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). MKN-45, MKN-28 gastric cancer cells, and nonmalignant gastric epithelial cell line GES-1 were from 3DBiopharm Biotech Co. Ltd. SKBR3, HCC1954, and BT-474 breast cancer cells were from Breast Cancer Institute of Fudan University (Shanghai, China). SNU-216 cells were from Medical College of Xiamen University (Fuzhou, China). Cell lines were tested and authenticated by short tandem repeat (STR) DNA profiling analysis. Cells were cultured in MEM (HGC-27), F12K medium (AGS), or RPMI 1640 medium containing 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (Invitrogen) at 37°C in a humidified atmosphere with 5% CO₂.

**Drug preparations**

Lapatinib (GW572016; Tykerb) was provided by GlatzSmithKline. NVP-AEW541 was provided by Novartis Pharma. All other TKIs used were purchased from Selecte Chemicals. Recombinant human hepatocyte growth factor (HGF), IGF1, Neuregulin 1, Insulin, fms-Like Tyrosine Kinase 3 ligand, Glial Cell Line-Derived Neurotrophic Factor, and GDNF family receptor alpha 1 (GFRα1) were purchased from PeproTech. Compounds were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and diluted with culture medium to the desired concentration, with a final DMSO concentration <0.2% (v/v). DMSO was also added to control cells in culture.

**shRNA and RNAi screens**

NCI-N87 cells (1,000 per well) were plated in 96-well plates and infected 24 hours later with a retrovirus RNAi library encoding 53 human RTK and 27 related growth factor (3DBiopharm Biotech). Cells in each well were infected with a pool of 4 distinct shRNA targeting different sequences of the same target transcript and a total of 8 independent shRNAs were designed for each gene. Scrambled shRNA controls that do not target any human gene were used as a negative control. Forty-eight hours after initial transduction, half of the plates were treated with lapatinib 0.02 μmol/L and the other half were treated with 0.2% DMSO. Medium containing lapatinib or DMSO control were refreshed every 48 hours. Cells were cultured in drug-free medium and the viability was assessed 7 days after initial lapatinib exposure, using CCK-8 Cell Viability Assay (Dojindo Laboratories) per the manufacturer’s instructions.

**Data analysis: derivation of drug sensitivity and antagonism**

To identify gene targets that increase lapatinib sensitivity or resistance, the sensitivity index (SI) was calculated as

HER2⁺ gastric cancer. On the basis of these findings, individualized lapatinib combination strategy with agents targeting to these enriched resistance-conferring receptors may achieve substantial benefits to patients.
described previously (7), and the individual effects of shRNAs or drug were taken into account. The viability effect of specific shRNA without drug versus the scrambled control shRNA was designated as Rc/Cc. The effect of the drug on the viability of control cells was designated as Cd/Cc. This enabled us to calculate the expected combined effect of shRNA and drug on cell viability (Rc/Cc × Cd/Cc). The observed combined effects of drug and shRNA on cell viability versus untreated scrambled control shRNA-transfected cells was designated as Rd/Cc. Therefore, an index of antagonism or sensitivity for each shRNA was calculated as (Rc/Cc × Cd/Cc) – (Rd/Cc). A positive SI score indicates a sensitizing effect and a negative SI score indicates antagonism to the treatment.

In addition, in order for the SI to usefully predict antagonism or sensitivity, the effect of shRNA combination with lapatinib versus lapatinib alone was also taken into consideration. Thus, an additional criteria that Rd/Cd > 1.1 (for antagonistic shRNAs) and Rd/Cd < 0.9 (for sensitizing shRNAs) was also used for selecting hits in this study (7).

Validation of RNAi screen

Three distinct siRNA species (Qiagen) targeting the same gene were used to revalidate hits from the initial screen. All siRNAs were transfected in 96-well plates using the HiperFect Transfection Reagent (Qiagen) following the manufacturer’s reverse-transfection protocol. Briefly, siRNA (final concentration = 5 nmol/L) was first added to 96-well plates and incubated with HiperFect Transfection Reagent to allow formation of transfection complexes, and 5 × 10⁴ per well N87 cells were then seeded. Scrambled control siRNA (Qiagen) was used as the negative control. At 24 hours following transfection, cells were treated with lapatinib 0.01 to 0.05 μmol/L or 0.2% DMSO alone. Cell viability was assessed 5 days later using CCK-8 Cell Viability Assay. The assay was performed in triplicate. The effect of gene silencing was determined by quantitative PCR.

Cell viability assay and combination data analysis

Assessment of cell viability was performed as follows. Cells were seeded at 5,000 cells per well in 96-well plates and incubated overnight. Cells were then treated with increasing concentrations of the indicated drugs for 72 hours. Treatments at each concentration were carried out in 6 replicate wells and repeated 3 times. Cell viability was determined using the Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer’s instructions. IC₅₀ was determined by using the nonlinear regression model in GraphPad Prism version 5.0 (GraphPad Software).

Combination effects on potency were evaluated using the combination index (CI) as described previously (8, 9), using CalcuSyn version 2.1 (Biosoft), and was derived from the original concept of Chou and Talalay (10). In general, a CI value <0.9, 0.9–1.1, or >1.1 indicates synergy, additivity, or antagonism, respectively. CI/fractional effect curves represent the CI versus the fraction of cells unaffected/killed by drugs in combination.

Immunoprecipitation

Cells were lysed in buffer containing 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and protease and phosphatase inhibitor cocktails. HER2 was immunoprecipitated from total protein extracts (200 μg) overnight, washed in PBS-0.1% Tween 20 buffer, and immunoblotted to detect EGFR, HER3, MET, IGFIR, and INSR. Irrelevant IgG served as a negative control.

Immunoblotting analysis

Cell analyses by Western blot analysis were cultured in full serum. Cells were lysed in radioimmunoprecipitation assay buffer supplemented with complete protease inhibitor cocktail (Roche). Protein concentrations were determined using the BCA Protein Assay Kit (Bioyotime). Antibodies against HER2, p-HER2, EGFR, p-EGFR, p-HER3, MET (25H2), p-MET (Tyr1234/1235), IGFIR β receptor, p-IGFIR β receptor (Tyr1133/1136), AKT, p-AKT (Ser473), p42/44 MAP kinase, p-p42/44 MAP kinase (Thr202/Tyr204), PTEN, E-cadherin, vimentin, SNAIL, ZEB1, β-catenin, and p-GSK3β were purchased from Cell Signaling Technology. Antibody against HER3 was purchased from Thermo Fisher Scientific. Antibody against β-actin was purchased from The Jackson Laboratory. Blots were probed with indicated primary antibodies, then incubated with the horseradish peroxidase–conjugated secondary antibody and detected by enhanced chemiluminescence reagent (Pierce).

Colony-formation assay and growth curves

Colony-formation assay and growth curves were conducted as previously described (11). Briefly, NCI-N87 cells were seeded at 2 × 10⁴ cells per well in triplicate in 12-well plates and cultured in the absence and the presence of the drug or drug combination for 2 weeks. After fixing the cells with 4% paraformaldehyde, cell numbers were quantified by staining the cells with 0.1% crystal violet (Sigma-Aldrich), extracting dye with 10% acetic acid, and determining the optical density at 600 nm.

Detection of apoptosis

Cell apoptosis was detected using the Annexin V–FITC Apoptosis Detection Kit (Invitrogen) according to the manufacturer’s instructions (12). Briefly, NCI-N87 tumor cells (3 × 10⁶/mL) were cultured in 6-well plates and treated with lapatinib, lapatinib plus HGF/IGF1, or lapatinib plus HGF/IGF1 and PF-04217903/NVP-AEW541 for 48 hours. Both floating and adherent cells were harvested and stained with Annexin V and propidium iodide (PI) and analyzed on a Cytomics FC 500 flow cytometer (Beckman Coulter) using CXP software (Beckman Coulter).

In vitro migration assays

Cell migration was analyzed by a BD Falcon Cell Culture Insert System (BD Biosciences) with 8-μm pores. For motility assays, 1 × 10⁵ SNU-216 cells were seeded into upper uncoated inserts, treated with lapatinib 0.05 μmol/L, or HGF 50 ng/mL, IGFI 100 ng/mL, NRG1 50 ng/mL, insulin...

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1,000 ng/mL. Cells were seeded in serum-free medium and translocated to 10% serum media for 30 hours. After removal of the nonmigrated cells, the remaining cells were fixed, stained, and analyzed by inverted microscopy. Migrated cell numbers were obtained by counting 6 random fields at ×200 magnification from each group of three independent experiments.

**Growth inhibition assay in vivo**

Six-week-old male athymic nude mice were purchased from the Shanghai Slac Laboratory Animal Co. Ltd., and randomized into 1 of 4 groups (n = 8/group). Tumors were established in male nude mice by subcutaneous implantation of NCI-N87 tumor fragments (~ 2 × 2 × 2 mm) obtained from donor mice. Treatment started at 7 days after implantation (day 0) with either lapatinib (30 mg/kg, orally daily for 24 days), AEW-541 (30 mg/kg, orally daily for 24 days) or sterile PBS (orally daily for 24 days) or the combination of lapatinib and AEW-541 at the doses above. Tumor volumes were determined from caliper measurements of tumor length (L) and width (W) according to the formula L \( \frac{W}{2} \). Both tumor size and body weight were measured twice per week.

**Patients and specimens**

A total of 156 specimens of primary gastric adenocarcinomas were obtained by surgical resection between 2007 and 2010 at Shanghai Cancer Hospital, Fudan University (Shanghai, China). Samples were acquired with informed consent, under the protocol approved by Shanghai Cancer Hospital research ethics committee. Paraffin blocks were included for all samples. Tissue microarrays were constructed in collaboration with Shanghai Biochip Co. Ltd., as described previously (13). All samples from patients with gastric cancer were reviewed histologically after hematoxylin and eosin staining. Representative cores were taken from paraffin blocks, away from necrotic and hemorrhagic regions.

Primary antibodies used for immunohistochemical assays were commercially available: antibodies against p-HER3, EGFR, p-EGFR, p-MET (Y1234/1235), and PTEN were from Cell Signaling Technology. Antibodies against p-IGFIR receptor (Y1161) and IGF1 receptor were from Abcam. An anti–HER2/neu (48S) rabbit primary antibody and an anti–Total c-MET (SP44) rabbit monoclonal primary antibody were from Roche. Antibody against HER3 was from Thermo Fisher Scientific. Immunohistochemistry (IHC) of paraffin sections was carried out using a 2-step protocol (MaxVision HRP-Polymer Detection System) according to the manufacturer’s instructions. Briefly, paraffin sections were deparaffinized and then rehydrated. After microwave antigen retrieval, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide. Nonspecific binding sites were blocked with PBS containing 10% normal goat serum. After staining with primary antibodies and peroxidase polymer-conjugated secondary antibody, sections were incubated with diaminobenzidine solution and counterstained with hematoxylin. Negative control slides without the primary antibodies were included for all samples.

**Scoring system of immunostaining**

Slides were independently evaluated by 2 investigators who were blinded to the clinical information. For HER2, EGFR, MET, and IGFIR staining, only tumor cell membrane staining was considered (14). Immunostaining was scored according to a semiquantitative 4-grade scale as follows: 0, no staining; 1+, incomplete circumferential staining; 2+, complete circumferential staining; and 3+, complete strong circumferential staining. HER3, p-HER3, p-EGFR, p-MET, and p-IGFIR samples were scored as 0, absent; 1+, weak; 2+, moderate; and 3+, strong, with consideration of both the proportion and the intensity of the staining pattern in tumor cytoplasm, membrane, or nucleus. Tumors with >10% of stained tumor cells were regarded as positive for each marker. Samples with a score of 1+, 2+, or 3+ were considered positive for all the markers (14–18) except HER2, for which scores of 0 and 1+ were considered to be negative according to the criteria recommended by Hofmann and colleagues.
(1, 3). For PTEN staining, scoring was performed as previously described (19). The surrounding normal epithelium served as an internal control and tumor immunoreactivity was scored accordingly: 0, no immunoreaction; 1+, reduced intensity of immunoreaction compared with normal epithelium, and 2+, intensity equal to normal epithelium. Samples with a score of 0 or 1+ were considered negative and 2+ were positive.

Statistical analysis

Statistical analyses were performed with SPSS 18.0 software (SPSS) or with GraphPad Prism version 5.0 (GraphPad Software). Quantitative variables were analyzed by the Student t test or one-way ANOVA with Bonferroni posttest. The Pearson χ² test or Fisher exact test was used to compare qualitative variables. Differences between cell viability and growth curves were analyzed by two-way ANOVA followed by the Bonferroni multiple comparison test. A two-tailed value of <0.05 was considered statistically significant.

Results

RNAi screening for pathways responsible for lapatinib unresponsiveness

To identify a HER2 "addicted" cell line as the model for RNAi screening, intrinsic lapatinib sensitivity of 6 human gastric cancer cell lines (HGC-27, SGC-7901, AGS, NCI-N87, MKN-45, and MKN-28) were determined. Results indicated that HER2+ NCI-N87 cells were more sensitive to lapatinib in these cell lines, with a half-maximum inhibitory concentration (IC₅₀) at 0.05 μmol/L, compared with all other HER2-negative (HER2−) gastric cancer cell lines, with IC₅₀ equal to or more than 10 μmol/L (Supplementary Fig. S1A and S1B). Lapatinib treatment inhibited HER2 and EGFR activation in HER2+ NCI-N87 cells and blocked downstream signaling through the MAPK and AKT pathways in a dose-dependent manner (Supplementary Fig. S1C). Thus, HER2 "addicted" and lapatinib responsive NCI-N87 cell line represents an appropriate model for further investigation on lapatinib drug sensitivity.

To identify RTKs whose knockdown sensitizes tumor cells to lapatinib treatment, NCI-N87 gastric cancer cells were transfected with a shRNA library targeting 53 human RTK genes, 27 related growth factors, and scrambled control shRNA. As described previously (7), SI was generated for each individual gene to identify proteins whose knockdown induce a drug-sensitive or antagonistic phenotype. A positive SI score indicates a sensitizing effect, whereas a negative SI score indicates antagonism to the treatment. All shRNA hits that changed lapatinib sensitivity and their SIs are shown in Fig. 1A; SI > 0.0474 (upper quartile) was considered to promote a strong drug-sensitizing phenotype. The top 10 potent lapatinib sensitivity-promoting candidates are listed in Supplementary Table S1.

A phenotype caused by at least 2 distinct siRNA species is usually required to avoid off-target effects (20). To validate the specificity of the effects observed in shRNA screening, 6 of the most interesting RTK genes (MET, IGF1R, HER3, INSR, FLT3, and RET) from the most potent lapatinib-sensitizing candidates and 2 related ligands (HGF and IGF1) were re-assayed using 3 additional siRNAs (Qiagen). Silencing of 7 target hits (MET, IGF1R, HGF, IGF1, HER3, INSR, and FLT3) via independent siRNAs significantly enhanced the effectiveness of lapatinib treatment (P < 0.05; Fig. 1B), and thus were considered to be "on-target." Downregulation of each gene was confirmed by polymerase chain reaction (PCR) or Western blot analysis (Supplementary Fig. S2).

To confirm the hypothesis that activation of these RTKs contributes to lapatinib resistance, NCI-N87 cells were treated with lapatinib (0–0.1 μmol/L) for 2 weeks, accompanied by the corresponding ligands or selective inhibitors for RTKs. As shown in Fig. 1C, ligand induced activation of MET, IGF1R, HER3, and INSR with HGF, IGF1, NRG1, and insulin resulted in a significantly increased number of lapatinib-resistant colonies (P < 0.05). Adding the corresponding TKI (PF-04217903, AEW-541, AZD8931, and GSK1904529A) to the cells reversed the ligand-induced resistance (P < 0.05). Notably, as there was no commercial available highly selective HER3 inhibitor and a multitarget inhibitor AZD8931 that targets EGFR, HER2, and HER3 was used to inhibiting HER3 in this study. Activation of FLT3 or RET by their corresponding ligands, FLT3 ligand or GDNF plus GFRα1, failed to induce lapatinib resistance in NCI-N87 cells. Ligands and TKIs' effects on the corresponding receptors were confirmed by Western blot analysis (Supplementary Fig. S3). Consistently, FLT3 and RET protein were hardly expressed in this line (Supplementary Fig. S2) and no response to their ligand were observed (Supplementary Fig. S3). In summary, MET, HER3, IGF1R, and INSR are potential RTKs, whose blockade sensitizes lapatinib whereas their activation confer lapatinib resistance in HER2+ NCI-N87 cells.

RTK activation promotes lapatinib resistance in a panel of HER2-addicted cancer cell lines

To verify the role of these RTKs in conferring lapatinib unresponsiveness in other HER2+ cell lines, additional 4 previously defined HER2-amplified cancer cell lines (SNU-216 gastric cancer cell line and BT-474, SKBR3, and HCC1954 breast cancer cell lines; ref. 6) were collected and tested for sensitivity to lapatinib. The mean IC₅₀ value of BT-474, NCI-N87, SKBR3, SNU-216, and HCC1954 cells was 0.04, 0.05, 0.11, 0.19, and 0.81 μmol/L, respectively. As expected, compared with the other 4 cell lines, HCC1954 demonstrated obvious p-MET and p-IGF1R activation (Fig. 2A), which may contributed to its significant intrinsic unresponsiveness to lapatinib. Indeed, inhibiting MET or IGF1R activation by PF-04217903 or AEW-541 significantly restored lapatinib effectiveness on proliferation in HCC1954 cells, which were associated with their further blocking the downstream AKT and ERK signaling (Fig. 2B).

To confirm the causal relationship between ligand-induced activation of the 4 RTKs and lapatinib resistance, BT-474, SKBR3, SNU-216, and HCC1954 cells were also
Figure 2. RTKs activation promotes lapatinib unresponsiveness in a panel of HER2-addicted cancer cell lines. A, HCC1954 cells show intrinsic unresponsiveness to lapatinib and more RTKs activation. Cell proliferation assay showing the intrinsic sensitivities to lapatinib in 5 indicated HER2-positive cell lines (72 hours). Data points indicate average of replicates of 6; bars indicate SEM. Immunoblots showing the baseline activation status of the indicated RTKs. B, MET or IGF1R inhibition restores lapatinib sensitivity in HCC1954 cells. Colony-formation assays show that addition of PF-04217903 or AEW-541 increases lapatinib sensitivity in HCC1954 cells. *, P < 0.05 compared with the lapatinib-treated control group. Immunoblots show that combination of PF-04217903 or AEW-541 with lapatinib leads to MET or IGF1R inhibition, and further inhibits AKT and ERK signaling when compared with lapatinib alone. C, ligand-induced lapatinib resistance in HER2-amplified cancer cell lines. Colony-formation assays show that NRG1-, HGF-, IGF1-, and insulin-induced activation of HER3, MET, IGF1R, and INSR cause lapatinib resistance in SNU-216 gastric cancer cells. NRG1-induced HER3 activation causes lapatinib resistance in BT-474 and SKBR3 cells. NRG1- and HGF-induced HER3 and MET activation cause lapatinib resistance in HCC1954 cells. *, P < 0.05 compared with the lapatinib-treated control group. D, ligand-induced lapatinib resistance correlates with baseline overexpression of certain RTK proteins, not with the distribution of heterodimers with HER2. Left: immunoblots showing the baseline expression status of the indicated RTKs. Right: co-immunoprecipitation and immunoblots showing the baseline distribution of heterodimers between the indicated RTK and HER2 receptor. HER2 was immunoprecipitated from whole cell lysates and immunoblotted for the indicated RTK. Irrelevant IgG serves as a negative control.
Multiple RTKs Confer Lapatinib Unresponsiveness in HER2+ GC

Consider that progression disease and treatment failure are often caused by tumor invasion and metastasis, rather than tumor growth in many cases, we assessed the potential effect of bypass RTKs in lapatinib-induced suppression of tumor motility, which is the first step in the cascade of tumor invasion and metastasis. Transwell analysis demonstrated that SNU-216 cells migration was significantly suppressed by lapatinib at a concentration of 0.05 μmol/L, which had little effect on cell viability (<10%). However, either HGF, IGF1, or NRG1 cotreatment significantly rescued SNU-216 cells from lapatinib-induced suppression of motility (Fig. 3B and Supplementary Fig. S4B). These data confirmed that activation of bypass RTKs also attenuate lapatinib-induced suppression of cell migration and may potentially facilitate tumor invasion and metastasis. Western blot analysis explored the molecular mechanism underlying RTK-induced lapatinib unresponsiveness. As shown in Fig. 3C, adding HGF to lapatinib treatment in MET-overexpressed HER2+ NCI-N87 cells not only activated MET, but also led to restimulation of downstream effectors AKT and ERK1/2 even in the presence of lapatinib, although phosphorylation of HER2 and EGFR continued to be suppressed. Furthermore, addition of PF-04217903 to lapatinib/HGF treatment inhibited HGF-induced MET phosphorylation and AKT, ERK1/2 activation, thus partly restoring lapatinib-induced blockade of downstream signaling. These results were confirmed in another MET-overexpressed HER2+ SNU-216 cells, but not in MET-low-expressed HER2+ SKBR3 cells. Adding HGF to lapatinib treatment in SKBR3 cells did not activate MET, AKT, or ERK1/2, yet could not lead to lapatinib resistance (Fig. 3C).

Together, these data demonstrate that in MET and HER2 co-overexpressed gastric cancer cells, MET activation confers lapatinib resistance through restimulating the MAPK and AKT signaling pathways. Similarly, IGF1R activation induces lapatinib resistance by restoration of AKT signaling in HER2 and IGF1R co-overexpressed gastric cancer cells (Fig. 3D). HER3 activation induces lapatinib resistance by restoration of AKT signaling in HER2 and HER3 co-overexpressed gastric cancer cells (Supplementary Fig. S4C).

To further confirm the involvement of IGF1R signaling in lapatinib resistance, we constructed a constitutively active IGF1R vector. NCI-N87 cells were stably infected with an empty lentiviral vector, CD8-IGFR with a constitutive activity.
of IGF1R (13) or CD8-IGFR-YF with an IGF1R kinase-inactive mutation (provided by Dr. L. Xiong; ref. 14). As expected, CD8-IGFR-N87 cells, but not CD8-IGFR-YF-N87 cells, have a constitutive autophosphorylation activity of the IGF1R receptor and resulted in a significant increase in the emergence of lapatinib-resistant colonies (Supplementary Fig. SSA and SSB). Consistently, constitutive activation of IGF1R led to AKT activation even in the presence of lapatinib (Supplementary Fig. SSB), again suggesting a role of the IGF1R–AKT axis in IGF1R signaling-induced lapatinib resistance.

To further clarify the mechanism underlying RTKs attenuating lapatinib-induced suppression of cell migration, we assessed their effects on key components of WNT pathway and EMT properties. Surprisingly, we found that both MET and IGF1R activation reversed lapatinib-induced p-GSK3β and β-catenin inhibition, and restored expression of EMT-inducing transcription factors (EMT-TF), SNAIL and ZEB1, and led to vimentin re-expression (Fig. 3C and D). These data indicated that RTKs-induced WNT signaling activation and EMT-like properties may also be involved in conferring lapatinib resistance.

Synergistic effects of AEW541 and lapatinib in vitro and in vivo

To determine whether there is in vitro synergistic antitumor effect between lapatinib and IGF1R inhibitor NVP-AEW541, multiple drug analyses were conducted in IGF1R-overexpressed HER2+ NCI-N87 cells. Addition of AEW541 to lapatinib resulted in a significant increase in cell growth toxicity when compared with either drug alone (P < 0.0001; Fig. 4A). The CI value at the IC50 was 0.88 ± 0.29. The CI/fractional effect curve showed that the synergistic effects between these 2 agents were getting stronger as the concentration increased (Fig. 4A). Interestingly, greater synergistic effects were detected when the IGF1R pathway was activated by exogenous IGF1, with the CI value at the IC50 increasing to 0.58 ± 0.16 (Fig. 4B). These data support the hypothesis that a combination strategy targeting both HER2 and IGF1R receptors in IGF1R-overexpressed HER2+ gastric cancer cells could be more effective, especially when the 2 pathways are both activated.

In vivo study further confirmed the efficacy of this combination strategy in xenografts model. Addition of AEW541 to lapatinib was found to significantly impair the growth of subcutaneously implanted NCI-N87 tumors when compared with either drug alone (Fig. 4C). No significant differences were observed in animal body weight (Fig. 4D).

HER3, MET, and IGF1R signaling pathways are frequently activated in HER2+ gastric cancer tumors

RNAi screening and in vitro experiments have demonstrated that lapatinib intrinsic resistance in HER2+ gastric cancer was associated with HER3, MET, and IGF1R activation or expression status. To further validate the clinical significance of these findings, we analyzed the expression and activation profile of these pathways in 156 primary gastric cancer surgical samples. HER2, EGFR, HER3, MET, and IGF1R expression and phosphorylation status (p-HER2, p-EGFR, p-HER3, p-MET, and p-IGF1R), as well as PTEN expression, were assessed by IHC. The clinical pathologic characteristics of the patients and the IHC results are summarized in Supplementary Table S3. Typical examples of positive staining for each receptor are shown in Fig. 5A. Of the 156 primary gastric cancer samples, 154 were suitable for IHC assessment. HER2 overexpression was found in 27 tumors (17.5%).

There are significantly more EGFR+, HER3+, and MET+ cases in patients with HER2+ tumors than in those with HER2− tumors (EGFR, 96.3% vs. 63.8%, P < 0.001; HER3, 96.3% vs. 58.3%, P < 0.001; MET, 81.5% vs. 36.2%, P < 0.001; Table 1). Similarly, EGFR, HER3, MET, and IGF1R activation are also significantly more frequent in HER2+ tumors than HER2− tumors (p-EGFR, 92.6% vs. 34.6%, P < 0.001; p-HER3, 81.5% vs. 39.4%, P < 0.001; p-MET, 70.4% vs. 25.2%, P < 0.001; p-IGF1R, 77.8% vs. 51.2%, P = 0.011; Table 1). However, PTEN expression loss tumors were found in only 3.7% of patients with HER2+ gastric cancer and were significantly less common than in HER2− tumors (46.5%, P < 0.0001; Table 1). Semiquantitative analysis of IHC staining for the expression of these receptors or their activated forms demonstrated similar observations (Fig. 5B). The mean scores for EGFR, p-EGFR, MET, p-MET, and p-IGF1R were significantly higher in HER2+ patients than in HER2− patients (P < 0.0001). Notably, 17 of 27 (63.0%) HER2+ patients had at least one receptor activated. These data revealed that the intrinsic resistance-conferring RTKs were particularly enriched in the majority of HER2+ gastric cancer tumors, indicating the potential clinical application of these findings.

Discussion

According to recently released data from phase II and phase III trials (5, 21), more than 80% of HER2+ gastric cancer tumors are actually unresponsive to lapatinib. However, because of the lack of systematic study, the mechanisms conferring intrinsic resistance to lapatinib are poorly defined in HER2+ gastric cancer. Unlike in HER2+ breast cancers, in which disorders in the PI3K pathway are considered to be the major cause of intrinsic and acquired resistance to anti-HER2 agents (11, 19), the PI3K pathway seems less related with lapatinib resistance in gastric cancer, because according to our data or others’ (22), PI3KCA mutation was previously reported to occur in only 4% to 11% of gastric cancer tumors (23, 24). Although in HER2+ BC, PTEN loss, and PIK3CA mutation were previously reported to occur in 19% and 42%, respectively (25).

We hypothesize that lapatinib intrinsic resistance are more likely to be associated with activation of alternative RTK pathways and try to identify these responsible RTKs. Through screening 53 human RTKs and 27 related growth factors, we tested and validated 8 candidates and finally identified MET, HER3, IGF1R, and INSR as key pathways...
modulating intrinsic resistance to lapatinib in HER2⁺ gastric cancer cell lines. These results were further verified in a panel of HER2⁺ cell lines, depending on the expression and activation status of a certain RTK.

Notably, HER3 was identified as a key mediator of lapatinib resistance in all tested HER2⁺ cell lines. This finding was echoed by the report of trastuzumab study, in which the trastuzumab resistance in HER2-amplified breast cancer cells was considered to be related to HER3 expression and activation (26, 27). Furthermore, we found that almost all (96.3%) HER2⁺ gastric cancer tumors from 154 specimens have HER3 expression and most of them (81.5%) are

Figure 4. Synergistic effects of combination treatment of lapatinib with AEW-541 in vitro and in vivo. A and B, combination usage of NVP-AEW541 with lapatinib significantly increases cell growth toxicity in NCI-N87 cells, regardless of stimulation by IGF1 or not. Cells were treated with increasing concentrations of the indicated drugs for 72 hours. Data points indicate average of replicates of 6 and bars indicate SEM. CI/fractional effect curves showed synergistic antiproliferative effects in drug combination, especially when the IGF1R pathway was stimulated with IGF1 50 ng/mL. Multiple drug analyses were conducted by calculating CI values. In general, CI values <0.9, 0.9–1.1, or >1.1 indicate synergy, additivity, or antagonism, respectively. C and D, antitumor activity of lapatinib and AEW-541 on N87-bearing xenografts. Tumors were established in male nude mice by subcutaneous implantation of NCI-N87 tumor fragments obtained from donor mice. Mice were randomized into one of 4 groups (n = 8/group). Treatment started at 7 days after implantation (day 0) with either lapatinib (30 mg/kg, orally daily for 24 days), AEW-541 (30 mg/kg, orally daily for 24 days), or sterile PBS (orally daily for 24 days) or the combination of lapatinib and AEW-541 at the doses above. Tumor volumes were determined from caliper measurements of tumor length (L) and width (W) according to the formula LW²/2. Both tumor size and body weight were measured twice per week. Data are mean ± SEM; *, P < 0.05, combination group versus lapatinib group.
activated, indicating pivotal HER3 activities on lapatinib resistance in gastric cancer.

Previously, the MET pathway was reported to mediate lapatinib resistance in HER2⁺ gastric cancer cells (28, 29). This was verified in this study, but via a different research method. Compared with the traditional resistant clones generation method, we believe functional RNAi screening have some advantages in finding all possible mechanisms by avoiding the possibility of resistant clone loss in the long selection process, and thus is an unbiased approach for resistance research. By this unbiased screening approach, we found multiple RTKs modulating lapatinib unresponsiveness, some of them are seldom reported before.

To our knowledge, IGF1R and INSR pathways are at first time reported conferring lapatinib unresponsiveness in HER2⁺ gastric cancer. In this study, lapatinib effectiveness was found significantly enhanced by independent siRNAs or TKIs targeting IGF1R and INSR. Although both IGF1-induced IGFR activation or insulin-induced INSR activation and infected constitutive IGFR activation resulted in a significant increase in the emergence of lapatinib-resistant colonies using long-term colony-formation assay. Because of lacking commercial available highly selective INSR ligand and TKI, only IGFR was further investigated in this study. A large proportion of HER2⁺ gastric cancer tumors (77.8%) have IGF1R activation, indicating the potential clinical application of combination strategy to target both receptors. Indeed, we found synergistic effect between IGF1R inhibitor and lapatinib in IGF1R-overexpressed HER2⁺ gastric cancer cells both in vitro and in vivo.

Cross-talk between signaling pathways, that is, switching to an alternative bypass survival signaling when one pathway is blocked, is considered to be driven resistance to targeted therapy (30). There could be cross-talk in multiple levels between the HER2/EGFR and MET or IGF1R pathways: first, receptors of these pathways form homodimers or heterodimers with each other and lead to transactivation with each other (31, 32); second, they share key downstream ERK or AKT signaling nodes. We found RTK activation-induced lapatinib resistance was more likely to be correlated with baseline overexpression of certain RTKs, rather than with the distribution of RTK-HER2 heterodimers in these HER2⁺ cells, especially in the case of HER3, MET, and IGF1R receptors. Our data demonstrated that stimulated RTKs conferred lapatinib resistance by activating downstream ERK or AKT signaling directly, rather than through heterodimers transactivation.

Importantly, we also found cross-talk between these RTKs signaling with the WNT pathway, which is well known of its critical role in regulating EMT process and maintaining stem cell–like properties (33, 34). In this study, both MET and IGF1R activation facilitated cell migration by attenuating lapatinib-induced p-GSK3β and β-catenin inhibition, and restored expression of EMT-inducing transcription factors, and finally restimulating EMT-like process. Recent studies have demonstrated that EMT plays a critical role not only in tumor metastasis but also in tumor recurrence and resistance and that it is tightly linked with the biology of cancer stem–like cells or cancer-initiating cells (35). Our findings are important because, they indicate that WNT-induced EMT process activated by alternative RTKs may also be involved in conferring lapatinib resistance and are seldom reported before. Indeed, we found WNT signaling activation via WNT1 supplementation or transfected β-catenin overexpression could both induced EMT process and lead to...
lapatinib resistance, and WNT pathway inhibitor could reverse the resistance (unpublished data).

Recently, Wilson and colleagues reported that most kinase 'addicted' human cancer cells can be rescued from kinase inhibitors by exposing them to one or more RTK ligands, highlighting the extensive redundancy of RTK-transduced signaling in cancer cells and the potential role of widely expressed RTK ligands in innate and acquired resistance (6). As alternative RTKs activation is considered to be a widespread resistance mechanism to targeted therapy, identifying the responsible RTKs becomes particularly important to overcome drug resistance. Using a functional RNAi screen, our study clearly demonstrates that activation of MET, HER3, and IGF1R/INSR signaling is responsible for lapatinib unresponsiveness in HER2\textsuperscript{+} gastric cancer. Considering the high prevalence of these pathways activation in HER2\textsuperscript{+} gastric cancer tumors, this may largely explain the limited response of HER2-targeted agents in gastric cancer, as observed in previous phase II and phase III clinical trials, and cotargeting EGFR/HER2 with responsible bypass receptors may significantly improve the efficacy of HER2-targeted agents.

In conclusion, this study highlights the power of "synthetic lethality" RNAi screens to identify potential RTKs conferring unresponsiveness and/or combination drug targets for lapatinib therapy. Our results confirmed the hypothesis that alternative activation of bypass pathways could be a novel mechanism conferring anti-HER2 resistance in gastric cancer. We believe that HER2\textsuperscript{+} patients represent heterogeneous individuals with different sensitivities to anti-HER2 agents. HER2 monotherapy is not enough for those patients with activation of these pathways. This study may be helpful to implementation of combination targeting therapy on patients with HER2\textsuperscript{+} gastric cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z. Zhang, J. Wang, D. Ji, Z. Wu, L. Liu, Q. Fang, J. Li

Development of methodology: R. Liu, R. Geng, L. Xiong, Q. Fang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Zhang, C. Wang, Z. Wu, L. Liu, D. Zhu, J. Chang

Table 1. Association of bypass signaling activation and HER2 expression status

<table>
<thead>
<tr>
<th>No. of patients (%)</th>
<th>HER2 IHC status</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (0–1\textsuperscript{+})</td>
<td>Positive (2\textsuperscript{+}–3\textsuperscript{+})</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>Absent</td>
<td>46 (36.2)</td>
<td>1 (3.7)</td>
<td>47 (30.5)</td>
<td>&lt;0.0001\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>81 (63.8)</td>
<td>26 (96.3)</td>
<td>107 (69.5)</td>
<td></td>
</tr>
<tr>
<td>p-EGFR</td>
<td>Absent</td>
<td>83 (65.4)</td>
<td>2 (7.4)</td>
<td>85 (55.2)</td>
<td>&lt;0.0001\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>44 (34.6)</td>
<td>25 (92.6)</td>
<td>69 (44.8)</td>
<td></td>
</tr>
<tr>
<td>HER3</td>
<td>Absent</td>
<td>53 (41.7)</td>
<td>1 (3.7)</td>
<td>54 (35.1)</td>
<td>&lt;0.0001\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>74 (58.3)</td>
<td>26 (96.3)</td>
<td>100 (64.9)</td>
<td></td>
</tr>
<tr>
<td>p-HER3</td>
<td>Absent</td>
<td>77 (60.6)</td>
<td>5 (18.5)</td>
<td>82 (53.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>50 (39.4)</td>
<td>22 (81.5)</td>
<td>72 (46.8)</td>
<td></td>
</tr>
<tr>
<td>MET</td>
<td>Absent</td>
<td>81 (63.8)</td>
<td>5 (18.5)</td>
<td>86 (55.8)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>46 (36.2)</td>
<td>22 (81.5)</td>
<td>68 (44.2)</td>
<td></td>
</tr>
<tr>
<td>p-MET</td>
<td>Absent</td>
<td>95 (74.8)</td>
<td>8 (29.6)</td>
<td>103 (66.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>32 (25.2)</td>
<td>19 (70.4)</td>
<td>51 (33.1)</td>
<td></td>
</tr>
<tr>
<td>IGFR</td>
<td>Absent</td>
<td>28 (22.0)</td>
<td>6 (22.2)</td>
<td>34 (22.1)</td>
<td>0.984</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>99 (78.0)</td>
<td>21 (77.8)</td>
<td>120 (77.9)</td>
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</tr>
<tr>
<td>p-IGFR</td>
<td>Absent</td>
<td>62 (48.8)</td>
<td>6 (22.2)</td>
<td>68 (44.2)</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>65 (51.2)</td>
<td>21 (77.8)</td>
<td>86 (55.8)</td>
<td></td>
</tr>
<tr>
<td>PTEN</td>
<td>Negative</td>
<td>59 (46.5)</td>
<td>1 (3.7)</td>
<td>60 (39.0)</td>
<td>&lt;0.0001\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>68 (53.5)</td>
<td>26 (96.3)</td>
<td>94 (61.0)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Twenty-five percent of all cells had an expected count <5; the Fisher exact test. The Pearson $\chi^2$ test for all other analyses.

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