HER-Family Ligands Promote Acquired Resistance to Trastuzumab in Gastric Cancer

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Abstract

Despite the clinical benefit of trastuzumab, eventually all HER2-amplified gastric cancer tumors develop drug resistance. We aimed to identify molecular mechanisms of acquired resistance to trastuzumab in gastric cancer by using well-established cell line-based preclinical models, as well as samples from patients with HER2-positive gastric cancer treated with trastuzumab. We studied trastuzumab resistance in NCI-N87 and OE19, two gastric cancer cell lines that overexpress HER2 receptor and are trastuzumab sensitive. Differences at protein, DNA, and RNA levels between the parental and resistant cells were characterized and functional studies were performed. Paired pre- and post-trastuzumab blood and tissue samples from patients with gastric cancer treated with trastuzumab were analyzed. We found that resistant cells were associated with increased activation of MAPK/ERK and PI3K/mTOR pathways driven by SRC activation. Upstream, resistant cells showed increased coexpression of multiple HER-family ligands that allowed for compensatory activation of alternative HER receptors upon HER2 blockade. Simultaneous inhibition of EGFR, HER2, and HER3 by the novel antibody mixture, Pan-HER, effectively reverted trastuzumab resistance in vitro and in vivo. Similarly, an increase in HER-family ligands was observed in serum and tumor from patients with gastric cancer after trastuzumab therapy. We propose that trastuzumab resistance in gastric cancer is mediated by HER-family ligand upregulation that allows a compensatory activation of HER receptors and maintains downstream signaling activation despite trastuzumab therapy. Resistance is reverted by simultaneous inhibition of EGFR, HER2, and HER3, thereby revealing a potential therapeutic strategy to overcome trastuzumab resistance in patients with gastric cancer.

Introduction

Gastric cancer is the fifth most common cancer worldwide and the third leading cause of cancer-related death (1). At diagnosis, most patients present inoperable locally advanced or metastatic disease, with a median overall survival below 1 year (2).

Approximately 20% of gastric cancer tumors, depending on the subtype of the tumor, overexpress the HER2 (2–4). HER2 is a member of the HER-family of receptors and a recognized key therapeutic target in breast and gastric cancer. It is associated with tumor cell proliferation, survival, angiogenesis, migration, and it correlates with a poor outcome and a more aggressive disease (5).

In the ToGA trial, the addition of trastuzumab, a mAb against the HER2 receptor, to chemotherapy improved overall survival (OS) of patients with HER2-positive gastric cancer in 4.2 months (2). This led to the approval of trastuzumab in combination with cisplatin plus fluoropyrimidine as first-line treatment for HER2-positive advanced gastric cancer or gastro-esophageal junction adenocarcinoma (6). Trastuzumab inhibits tumor growth mainly through prevention of HER2 homodimerization and blockade of HER2 downstream signaling (7), inhibition of HER2 extracellular amino-terminal domain (ECD) shedding (8), and induction of antibody-dependent cellular cytotoxicity (ADCC; refs. 9, 10).

Despite the significant survival benefit of trastuzumab in patients with gastric cancer, drug resistance invariably develops (2). Identification of molecular mechanisms of acquired resistance to trastuzumab in gastric cancer is crucial to delineate more effective therapeutic strategies in the clinical setting. Mechanisms of resistance to trastuzumab in gastric cancer are poorly characterized. Preclinical evidence suggests alternative activation of downstream signaling pathways, such as overexpression of FGF 3 (11). Recently, next-generation sequencing analysis revealed secondary alterations in ERBB2 as well as mutations in RAS/PI3K downstream signaling pathways in post-trastuzumab samples from patients with gastro-esophageal junction adenocarcinoma (12). Remarkably, these molecular alterations only
explain trastuzumab resistance in a subset of patients. In this study, we aimed to explore additional mechanisms of acquired resistance to trastuzumab in gastric cancer by using a well-established cell line–based preclinical model, as well as clinical samples from gastric cancer patients treated with trastuzumab.

Material and Methods

Cellular models and reagents

The human gastric cancer cell line NCI-N87 was purchased from ATCC and the OE19 and OTR6 (OE19 derived trastuzumab resistant) cells were kindly provided by Symphogen A/S (13, 14). All cell lines were Mycoplasma free and authenticity was tested by STR DNA Profiling analysis at the ATCC (October 2014) after resistant cells were generated. The number of passages between thawing and use in the described experiments was 10 or less. Cells were grown as reported previously (15). Trastuzumab (Herceptin, Genentech, Roche) was obtained from the Hospital del Mar pharmacy. Everolimus (RAD001), pimasertib (AS-703026; ref. 16), and saracatinib (AZD0530; ref. 17) were purchased from Selleckchem, and Pan-HER from Symphogen A/S (13). Human recombinant EGF was from Calbiochem (Merck KGaA) and neuregulin (NRG1) from Sigma Aldrich Co (Merck KGaA).

Generation of trastuzumab-resistant cells

NCI-N87 cells were cultured in the presence of 15 µg/mL of trastuzumab until cells were considered resistant (7 months; cell viability reduction ≤20%; refs. 18, 19). Single-cell–derived resistant colonies were isolated from the resistant pools using 8 mm x 8 mm diameter cloning cylinders (Millipore, Merck KGaA) and further propagated for 3 more months in media containing 15 µg/mL trastuzumab (18, 20, 21). Trastuzumab resistance was validated after a cycle of freezing and thawing and after drug withdrawal up to 6 months (22). Parental cells were cultured without trastuzumab to exclude that resistance was due to long time culturing.

Cellular proliferation assay

To analyze the growth inhibitory effect of trastuzumab, alone or in combination with pimasertib, everolimus, or ligands, or perform dose curves, with trastuzumab, Pan-HER, cetuximab, or pertuzumab, 8–12 × 10^4 cells per well in a 24-well plate were treated for 7 days. Cells were stained for 1 hour with crystal violet solution at 0.1%. Quantification was evaluated with ImageJ software. To perform growth curve experiments with lapatinib, we plated 5–7 × 10^3 cells per well in 96-well plate. Cell viability was obtained using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) following the manufacturer’s procedures. IC_{50} values were calculated using CalcuSyn software. Each experiment was performed at least three times and results were plotted as percentage of the control.

Trastuzumab binding by flow cytometry

We measured trastuzumab binding to HER2 receptor as reported previously (15), incubating cells with trastuzumab 100 ng/mL as the primary antibody.

FISH

To assess ERBB2 copy number alterations, we applied the PathVision HER-2 DNA FISH Probe Kit (Abbott Molecular Inc). FISH was performed in fixed material obtained from the cell lines after application of a conventional cytogenetic protocol. About 100 nonoverlapping cells with hybridization signals were examined for each case and amplification was defined as ERBB2/CEP17 ratio ≥ 2.0 (23).

Immunohistochemistry

To study HER2 protein expression, 1 × 10^7 of cells were pelleted, fixed with formalin, and embedded with paraffin (FFPE). HER2 expression was evaluated using HercepTest. Sections of 3 µm were placed on plus charged glass slides. The HER2 antibody used was PATHWAY anti-HER-2/neu (4B5) rabbit monoclonal primary antibody (Ventana Medical Systems, Inc.) and revealed with ultraView Universal 3,3′-diaminobenzidine (DAB) Detection Kit (Ventana Medical Systems, Inc.) as the chromogen and counterstained with hematoxylin. HER2 staining was performed and evaluated in the Pathology Department of Hospital del Mar according to NCCN Guidelines (24).

Protein detection

Whole-cell lysates were subjected to Western blot analyses as reported previously (20). The primary antibodies used were: EGF, HER3, phosphoERK1/2 (T202/Y204), ERK1/2, phosphoAKT (S473), AKT, phosphoS6 (S235/236), S6, phosphoSRC (Y416), and SRC purchased from Cell Signaling Technology; HER2 from BioGenex (clone CB11); α-Tubulin and β-Actin from Sigma-Aldrich Co (Merck KGaA).

Phospho-receptor tyrosine kinase array

We screened the activity of different receptor tyrosine kinase (RTK) and signaling nodes using PathScan RTK Signaling Antibody Array Kit (ChemiluminescentReadout) catalog no. 7982 of Cell Signaling Technology following the manufacturer’s procedures. Image acquisition was done with the Typhoon Scanner Control v5.0 software and quantified with the ImageQuant TL v7.0. The digitalization part was done at Centre for Genomic Regulation in the Parc de Recerca Biomédica de Barcelona (PRBB).

Gene expression microarray

RNA isolation and gene expression microarray were performed using the Human Gene 2.0 ST Array GeneChip (Affymetrix) as detailed in Supplementary Material and Methods.

qRT-PCR

Total RNA was extracted from cells in basal conditions and treated with trastuzumab 15 µg/mL and Pan-HER 10 µg/mL for 24 hours. cDNA was synthesized using random primers and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Samples from three independent RNA extractions were amplified using specific primers and LightCycler 480 SYBR Green 1 Master (Roche) in the LightCycler 480 Real Time PCR-System device (Roche). The primers used were: EGF Forward: 5’-CGTTGTCGG-GAAGGTITATG-3’ and EGF Reverse: 5’-GTCTTTGATG-CAACCTCACC-3’; AREG Forward: 5’-GCATGATGGACAGATGTATCC-3’ and AREG Reverse: 5’-TTCAAGTGCCGCAGTCTAA-3’; TGFα Forward: 5’-TGTTGCCTGCTTGGTGAGTAC-3’; TGFα Reverse: 5’-GACCTGTCAGGTCTGATCA-3’; HBEGF Forward: 5’-GGGAAATGGCAAATATGTGAAG-3’ and HBEGF Reverse: 5’-TTCCACTGGGAGGTCTCAG-3’; NRG1 Forward: 5’-CACCCCAAG-GACCTGTTGA-3’ and NRG1 Reverse: 5’-ATGCTGTGGGGCAGTTTGT-3’ and ATP5E Forward: 5’-GCATGGGAGATTGTCCTG-3’ and ATP5E Reverse: 5’-CGGCCCTTTGCGGAGATC-3’.
Gene expression was calculated as 2 to the power of −ΔΔCt, where ΔΔCt = (CtGene − CtACTIN) − (CtReference − CtACTIN) Assay.

**ELISA analysis**

Cell culture medium from 1–5 × 10^6 cells plated in 100 mm culture dish with 10 mL of medium for 72 hours was concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore, Merck KGaA) following the manufacturer’s procedures. Ligands’ levels in the medium were measured using the Human EGF DuoSet #DY236, Human Amphiregulin DuoSet #DY262, and Human TGF-alpha DuoSet #DY239 ELISA (R&D Systems) according to the manufacturer’s procedures.

**Subcutaneous tumorigenesis**

Five-week-old male Fox Chase SCID Beige mice were purchased from Charles River Laboratories and hosted in the pathogen-free animal facility at the PRBB. Animal treatments were done according to institution-approved protocols. A total of 5 × 10^6 cells were resuspended in sterile PBS with 50% of Matrigel (BD Biosciences) and subcutaneously injected into the flank of the mice. Tumor volume was determined twice a week from caliper measurements of tumor length (L) and width (W) according to the formula (L × W^2 × 0.1416)/6. When tumor volume reached 200–300 mm^3, mice were randomized to three groups with 10 mice in each one. IgG isotype control, trastuzumab (20 mg/kg), and Pan-HER (60 mg/kg) were administrated intraperitoneally three times a week until significant effect on tumor growth was observed of the treatment versus control or until tumor volume was too large, for ethical reasons (30 days for parental and 85 days for resistant cells). Mice were euthanized and part of the tumors was FFPE for IHC studies.

**IHC**

Paraffin sections were used for IHC analysis as described in Immunocytochemistry section. Tumor sections were stained with hematoxylin and eosin (H & E) to assess histologic changes. To evaluate proliferation and apoptosis, slices were incubated with the antibodies Ki-67 (clone MIB-1, Dako, Agilent Technologies) mouse mAb at 1:100, anti-phosphorylated (Ser10) Histone 3 (Cell Signaling Technology) rabbit polyclonal antibody at 1:100, and active caspase-3 (clone ASP175 (5A1), Cell Signaling Technology) rabbit monoclonal antibody at 1:100. Antibody–antibody reaction was detected by incubation with an anti-mouse/rabbit Ig–dextran polymer coupled with peroxidase (Flex+, Dako, Agilent Technologies). Sections were then visualized with DAB as chromogen and counterstained with hematoxylin. H & E and IHC staining was performed and evaluated in the Pathology Department of Hospital del Mar. Data were presented as the percentage of positive tumor cells.

**Patient samples**

Blood and tumor specimens from patients with HER2-positive gastric cancer and gastro-esophageal junction adenocarcinoma that received trastuzumab and chemotherapy treatment were collected at diagnosis (pre-trastuzumab) and after trastuzumab therapy (post-trastuzumab) at Hospital del Mar. Ligand levels of serum samples were measured by ELISA as described in ELISA analysis. In FFPE tumor tissue samples, RNA was extracted using the MagMAX FFPE DNA/RNA Ultra Kit catalog no. A31881 (Thermo Fisher Scientific) and EGF expression analysis was performed as described in qRT-PCR section.

**Statistical analysis**

Statistical analyses were carried out using one-way ANOVA tests followed by post hoc Tukey adjustment or Student t test. Prism 5.0 software (GraphPad) was used for the statistical analyses. Data shown is mean ± SD of three independent replicates.

**Results**

**Generation and characterization of trastuzumab-resistant gastric cancer cells**

To identify potential mechanisms of acquired resistance to trastuzumab, we performed an extensive screening on gastric cancer cell lines that could be used to generate resistance to trastuzumab and mimic the clinical setting. The requirements were: gastric cancer adenocarcinoma, HER2 receptor amplification and overexpression, and high sensitivity to trastuzumab. Only two cell lines (NCI-N87 and OE19) fulfilled these requirements. NCI-N87 cells were selected to generate single-cell trastuzumab-resistant derivatives and characterize the resistance (Fig. 1A and B). Then results were confirmed in the OTR6 cells (OE19-derived trastuzumab resistant). Differences in cell morphology were observed between parental and resistant cells, showing the latter a flatter morphologic appearance (Fig. 1B). To explore potential mechanisms of resistance, we performed next-generation sequencing to analyze hotspot regions of a panel of genes commonly altered in cancer and related to HER2 signaling, including ERBB2, EGFR, KRAS, N-RAS, BRAF, and PIK3CA (listed in Supplementary Material and Methods). We did not identify the emergence of mutations in these genes in resistant compared with parental cells.

**Trastuzumab-resistant cells grow independently of HER2**

To exclude the role of HER2 in the resistant phenotype, we determined whether there were substantial differences in HER2 receptor amplification, expression, and activation in resistant compared with parental cells. Trastuzumab-resistant cells maintained ERBB2 gene amplification similar to parental cells, as assessed by FISH analysis (Fig. 1B). Moreover, genomic analysis did not detect point mutations in ERBB2 gene. IHC showed HER2 3+ immunoscore in both parental and resistant cells, although a small reduction in protein staining was observed in resistant compared with parental cells that was confirmed by Western blot analysis (Figs. 1B, 2A, 3E, and 4B). The HER2 inhibitor lapatinib did not affect the viability of resistant cells compared with parental cells (Fig. 1C; Supplementary Fig. S1), suggesting that resistant cells were no longer dependent on HER2 for their growth and survival. Although we had excluded the role of a secondary mutation in ERBB2 gene as a mechanism of resistance, it remained possible that reduced drug-receptor binding affinity was involved in resistance (21, 25). To test whether trastuzumab was actively recognizing the cell surface of HER2, we performed flow cytometry analysis which revealed that trastuzumab was binding to 100% of the cells in both the parental and resistant cells (Fig. 1D).

**Resistance to trastuzumab is associated with SRC-mediated activation of MAPK/ERK and PI3K/AKT pathways**

To explore the role of HER2 cell signaling in resistance, we characterized HER2 downstream effectors in parental and resistant cells. Resistant cells had an increase in basal levels of SRC, ERK 1/2, and S6 phosphorylation, which was confirmed by a
phospho-array analysis (Fig. 2A and B; Supplementary Fig. S2). As expected, trastuzumab treatment reduced phosphorylation of HER2 downstream proteins SRC, AKT, ERK 1/2, and S6 in parental cells. However, HER2 downstream effectors persisted activated in the resistant cells (Fig. 2A).

To establish whether the MAPK/ERK and PI3K/mTOR pathways played a role in maintaining survival and cell growth in the resistant cells, functional pharmacologic studies were performed with the MEK inhibitor pimasertib and the mTOR inhibitor everolimus. Each drug effectively targeted ERK and S6, respectively, and partially inhibited cell viability of resistant cells. Of note, the addition of trastuzumab had no additional effect, supporting the limited role of HER2 in driving resistance (Fig. 2C; Supplementary Figs. S3, S4A and S4B). Simultaneous inhibition of both ERK and PIK3 pathways by the combination of pimasertib and everolimus resulted in higher cell viability inhibition compared with each drug alone, suggesting that both MAPK/ERK and PIK3/mTOR pathways were critical in maintaining cell growth and survival in the resistant cells (Fig. 2C; Supplementary Fig. S3).

To assess whether persistent activation of MAPK/ERK and PI3K/mTOR pathways in resistant cells was driven by the common upstream activator SRC, we treated resistant cells with the small molecule SRC kinase inhibitor saracatinib. Saracatinib...
significantly decreased cell viability in the resistant cells (Fig. 2C; Supplementary Fig. S3), which was correlated with effective suppression of HER2 downstream effectors (Supplementary Fig. S4C). Of note, because SRC mutations have been shown to drive lapatinib resistance in a gastric cancer preclinical model (26), we sequenced the whole SRC gene. SRC mutations were neither detected in parental nor resistant cells.

Taken together, these results strongly suggested that sustained activation of both MAPK/ERK and PI3K/mTOR pathways mediated by SRC was critical to the maintenance of cell survival in resistant cells.

Multiple HER-family ligands are overexpressed in trastuzumab-resistant cells

To explore upstream mechanisms of SRC-mediated MAPK/ERK and PI3K/mTOR pathways activation in the trastuzumab-resistant cells, we analyzed expression of HER-family receptors and related ligands. Gene expression array showed no significant differences in basal expression of total HER receptors in resistant compared with parental cells. Following trastuzumab therapy, a trend toward an increase in total EGFR, ERBB3, and ERBB4 was observed compared with parental lines (Fig. 3A). Strikingly, HER-family ligands including epidermal growth factor (EGF), amphiregulin (AREG), transforming growth factor alpha (TGFA), heparin-binding EGF-like growth factor (HBEGF), and neuregulin 1 (NRG1) were significantly overexpressed in resistant compared with parental cells (Fig. 3A and B). In addition, increased levels of AREG and TGFA were detected in the cell culture medium of resistant compared with parental cells (Fig. 3C). NRG1 and EGF could not be assessed in medium because of a very low limit of detection. Hence, we hypothesized that simultaneous overexpression of several HER-family ligands allowed for a compensatory effect to maintain the activation of downstream signaling pathways upon HER2 blockade in the resistant cells.

HER-family ligand overexpression protects from trastuzumab inhibition

To explore whether HER-family ligands were able to induce trastuzumab resistance, we exposed the parental cells to high concentrations of ligands. Incubation of parental cells with EGF or NRG1 stimulated cell growth (Fig. 3D). The addition of either ligand reduced the inhibitory effect of trastuzumab (Fig. 3D; Supplementary Fig. S5). Accordingly, biochemical analysis revealed an activation of HER downstream effectors following ligand stimulation, which was not completely abrogated by the addition of trastuzumab (Fig. 3E).
To evaluate whether simultaneous complete blockade of all members of the HER-family of receptors could prevent ligand-induced downstream activation, we used Pan-HER: a novel antibody mixture comprised of synergistic pairs of antibodies targeting EGFR, HER2, and HER3 in nonoverlapping epitopes (13). Pan-HER significantly reduced cell viability of parental cells even in the presence of ligands (Fig. 3D; Supplementary Fig. S5) and this was correlated with effective inhibition of EGFR, HER2, HER3, and downstream signaling specifically pAKT/pS6/pERK for EGF and pAKT/pS6 for NRG1 (Fig. 3E).

Simultaneous inhibition of EGFR, HER2, and HER3 by Pan-HER overcomes acquired resistance to trastuzumab in vitro

Because Pan-HER was blocking HER-family downstream activation even in the presence of ligands, we aimed to explore whether Pan-HER could revert trastuzumab acquired resistance in our model. Notably, Pan-HER significantly reduced cell viability of parental cells even in the presence of ligands (Fig. 3D; Supplementary Fig. S5) and this was correlated with effective inhibition of EGFR, HER2, HER3, and downstream signaling in biochemical analysis specifically pAKT/pS6/pERK for EGF and pAKT/pS6 for NRG1 (Fig. 3E).

Mechanisms of resistance to trastuzumab are confirmed in another preclinical model

Remarkably, results were confirmed in the OTR6-resistant cells derived from the trastuzumab-sensitive HER2-amplified OE19 cell line. Trastuzumab was less effective in reducing ERK 1/2 and S6 phosphorylation in OTR6-resistant cells compared with the parental cells (Supplementary Fig. S8A). In addition, NRG1, AREG, and EGF ligands were overexpressed in OTR6-resistant cells (Supplementary Fig. S8B). A high increase in NRG expression was observed upon trastuzumab treatment in OE19 parental cells (Supplementary Fig. S8C). Similar to NCI-N87 cells, EGF and NRG1 stimulation reduced trastuzumab growth inhibitory rate in OE19 parental cells, and simultaneous blockade of all HER-family of receptors by Pan-HER was able to prevent ligand-induced downstream activation (Supplementary Fig. S9A and C).
At a molecular level, Pan-HER had a more profound effect in reducing EGFR, HER2, HER3, and downstream effectors phosphorylation compared with trastuzumab, even in the presence of ligands (Supplementary Fig. S9C). Moreover, Pan-HER significantly reduced cell viability of OTR6 trastuzumab-resistant cells and this was correlated with a successful depletion of EGFR, HER2, and HER3 and inhibition of the downstream effectors (Supplementary Fig. S10).

**Triple inhibition of EGFR, HER2, and HER3 by Pan-HER overcomes trastuzumab resistance in vivo**

To expand our studies to *in vivo* models, the Pan-HER antibody mixture was tested in parental and trastuzumab-resistant–derived xenografts. As expected, in parental xenografts both trastuzumab and Pan-HER significantly reduced tumor growth compared with the control group (Fig. 5A). In resistant xenografts, resistance to trastuzumab was observed after 1.5 months of treatment, while no tumor growth was observed under treatment with Pan-HER during 3 months (Fig. 5B). H & E staining of parental and resistant xenografts showed that Pan-HER–treated tumors were smaller with less percentage of ischemic necrosis and more hyalinized fibrosis compared with control or trastuzumab-treated tumors (Fig. 5C; Supplementary Fig. S10). IHC analysis showed lower staining percentage of the proliferative markers Ki-67 and phosphorylated (S10) Histone H3 (pHist H3) in Pan-HER–treated tumors compared with control or trastuzumab group (Fig. 5C; Supplementary Table S1B). Staining of the apoptosis marker cleaved caspase 3 (c-casp 3) did not totally reflect Pan-HER superiority, probably because Pan-HER–induced apoptosis occurred previous to mice sacrifice (Fig. 5C; Supplementary Table S1B). Altogether, histologic analysis confirmed a higher Pan-HER antitumor efficacy compared with control and trastuzumab effect.

**HER-family ligands are increased in clinical samples from patients with gastric cancer treated with trastuzumab**

To explore whether HER-family ligands were involved in trastuzumab resistance in patients, we analyzed pre-trastuzumab and post-trastuzumab paired samples from 5 patients with HER2-positive gastric cancer. Clinical characteristics of the patients are summarized in Supplementary Table S2. Of note, rebiopsy and collection of post-trastuzumab samples was limited because of patient fragility and difficulty in tumor access. An increase in median EGF, AREG, and TGFα concentration was observed in post-trastuzumab compared with pre-trastuzumab serum samples (Fig. 6A; Supplementary Fig. S11A). In tissue samples, EGF expression was 2.7 times higher in post-trastuzumab tumor biopsy compared with pre-treatment tumor.
sample (Fig. 6B; Supplementary Fig. S11B). Other markers could not be characterized because of insufficient tumor sample availability.

**Discussion**

The approval of trastuzumab for patients with HER2-positive gastric cancer represented a breakthrough in the treatment of this disease. Unfortunately, responses are transient and resistance to trastuzumab invariably emerges. To study the molecular mechanisms underlying trastuzumab resistance, we studied two trastuzumab-resistant cell lines and as proof-of-concept confirmed the findings in patients’ samples. Our preclinical results showed that acquired resistance to trastuzumab was driven by mRNA increase of multiple HER-family ligands that allowed compensatory activation of MAPK/PI3K downstream signaling in the presence of trastuzumab. The novel antibody mixture Pan-HER effectively reverted trastuzumab resistance both in vitro and in vivo. Accordingly, analysis of clinical samples showed an increase in HER-family ligands levels after treatment with trastuzumab.

To our knowledge, there is limited evidence on the role of HER-family ligands in innate and acquired resistance to trastuzumab in patients with gastric cancer. Preclinical models have shown a potential involvement in EGF ligand upregulation in trastuzumab resistance in patients with gastric cancer (27, 28). Similarly, in breast cancer cell lines, ligand-induced activation of HER receptors has been linked to trastuzumab resistance (29). Also a potentially broad role of widely expressed receptor-tyrosine kinase ligands has been shown in innate and acquired resistance to small-molecule tyrosine-kinase inhibitors (30). For the first time in gastric cancer, our findings are supported by proof-of-concept data from a small group of patients treated with
trastuzumab, who showed an increase in HER-family ligands in serum and tumor tissue after trastuzumab-based therapy.

Coexpression of multiple RTK and compensatory downstream signaling activation has been shown to limit the efficacy of single-target drugs in other cancer types (29, 31–33). Of note, in breast cancer models, long-term treatment with trastuzumab leads to overexpression of EGFR and HER3, which circumvents HER2 inhibition (31). This suggests that inhibition of multiple RTKs is potentially necessary to reach a complete abrogation of redundant downstream signaling activation. According to the results in Jacobsen and colleagues, simultaneously targeting of EGFR, HER2, and HER3 was necessary to prevent trastuzumab resistance induced by EGF and NRG1 ligand stimulation and by the compensatory upregulation of HER-family of receptors in several cell lines (13). Upon receptor binding, Pan-HER induces internalization and degradation of EGFR, HER2, and HER3, preventing ligand binding to the receptors. Furthermore, in this study we show that Pan-HER more effectively inhibits cell viability compared with trastuzumab alone in parental cells, suggesting the increased benefit of triple inhibition of RTKs even in HER2-addicted cells. This concept is reinforced in the NCI-N87 trastuzumab-resistant cells, where single RTK mAbs, cetuximab, trastuzumab, or pertuzumab, or dual RTK inhibitor, lapatinib, had a limited effect compared with the more powerful inhibitory effect of Pan-HER. Similar to our data, in breast cancer xenografts with trastuzumab resistance, Pan-HER, but not trastuzumab emtansine (T-DM1) or the combination of trastuzumab with pertuzumab or lapatinib, was able to arrest tumor growth (33). In our model, tumors derived from trastuzumab-resistant cells did not completely recapitulate the in vitro resistant phenotype after being inoculated subcutaneously in mice. However, after 1.5 months of treatment, these tumors were able to grow acquiring resistance to trastuzumab. Differences between tumor cells behavior in 2D cultures in vitro and in vivo exist even in terms of proliferation rate (34). Now it is accepted that there are a number of situations where a particular in vitro phenotype can only be reproduced in solid tumors when cells have grown as 3D multicellular tumor spheroids (35, 36). Moreover, resistance can even vary when resistant xenografts are reimplanted into untreated mice (37). In our resistant xenograft, trastuzumab resistance is not the result of a permanent genetic change in the tumor cells, but rather is mediated by HER-family ligands upregulation. For this reason, resistance could be affected by reversible changes that likely to occur in the tumor and/or its microenvironment. Therefore, it might explain the delayed trastuzumab resistance observed. Notably, no tumor growth was observed under treatment with Pan-HER during 3 months indicating the role of HER-family ligands and receptors in trastuzumab resistance.

In line with our results of downstream signaling activation under HER-family ligand stimulation, Wilson and colleagues observed that EGF preferentially activated the MAPK/ERK pathway, whereas NRG1 mainly mediated PI3K/mTOR pathway activation (30). This suggests that dual activation of ERK1/2 and S6 in our gastric cancer trastuzumab-resistant model may potentially be caused by a combination of EGF, NRG1, or other HER ligands upregulation. Moreover, it supports the concept that extensive redundancy activation of RTKs signaling is observed in cancer cells. RTKs downstream activation including activation of the PI3K/mTOR signaling pathway or the common-node SRC has been linked to both preclinical and clinical resistance to HER2-targeted therapy in different cancer types (12, 26, 38–44). Similarly, in our gastric cancer trastuzumab-resistant model,
MAPK/ERK and PI3K/mTOR activation was mediated by increased SRC phosphorylation, as a common node downstream of RTKs ligand-induced activation. Drug inhibition of SRC (or concomitant inhibition of ERK and PI3K) would therefore be a good therapeutic strategy to revert trastuzumab resistance, as shown in our preclinical cell culture model. However, small molecule inhibitors do not trigger the immune system, whereas Pan-HER induces ADCC and enhanced complement-dependent cytotoxicity activation similar to trastuzumab. Therefore, Pan-HER would be a more powerful clinical therapeutic strategy to overcome trastuzumab resistance taking into consideration the essential role of the immune system in cancer therapy. Pan-HER is a promising candidate not only in reversion to trastuzumab but also as a targeted therapy against EGFR, HER2, or HER3. Clinical trials with Pan-HER are ongoing.

Taken together, the data presented herein suggests that ligand-induced, redundant activation of HER-family of receptors is a potential mechanism of resistance to trastuzumab in gastric cancer, supported by proof-of-concept evidence in a small cohort of patients. Simultaneous inhibition of all members of the HER family of receptors is therefore necessary to revert or prevent trastuzumab resistance. Potential clinical implications of our findings are (i) the need to dynamically evaluate levels of HER ligands before, during, and after trastuzumab therapy and (ii) the need to evaluate Pan-HER as a potential therapeutic strategy to overcome trastuzumab resistance in clinical trials.

Disclosure of Potential Conflicts of Interest

B. Bellossio has received speakers bureau honoraria from Roche, Amgen, Pfizer, and Novartis, and has consultant/advisory board relationships with Roche, Pfizer, and Amgen. C. Montagut reports receiving a commercial research grant from Symphogen, has received speakers bureau honoraria from Roche and Symphogen, and has an advisory/consultancy agreement with Symphogen. No potential conflicts of interest were disclosed by the other authors.

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