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 HER2-positive gastric cancer patients 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 gastric cancer patients 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 co-expression 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 gastric cancer patients after trastuzumab therapy. We propose that trastuzumab resistance in gastric cancer is mediated by HER-family ligands upregulation that allow a compensatory activation of HER receptors and maintain 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 gastric cancer patients.

INTRODUCTION

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

Approximately 20% of gastric cancer tumors, depending on the subtype of the tumor, overexpress the human epidermal growth factor receptor-2 (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 monoclonal antibody (mAb) against the HER2 receptor- to chemotherapy improved overall survival (OS) of HER2-positive gastric cancer patients 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) (9,10).

Despite the significant survival benefit of trastuzumab in gastric cancer patients, 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 fibroblast growth factor 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 gastro-esophageal junction adenocarcinoma patients (12). Remarkably, these molecular alterations only explain trastuzumab resistance in a subset of patients. In the current 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 American Type Culture Collection (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 autenticity 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 ten or less. Cells were grown as previously reported (15). Trastuzumab (Herceptin, Genentech, Roche) was obtained from the Hospital del Mar pharmacy. Everolimus (RAD001), pimasertib (AS-703026) (16), and saracatinib (AZD0530) (17) were purchased from Sellechckem, and Pan-HER from Symphogen A/S (13). Human recombinant epidermal growth factor (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 15µg/mL of trastuzumab until cells were considered resistant (7 months; cell viability reduction $\leq$20%) (18,19). Single-cell derived resistant clones were isolated from the resistant pools using 8mm x 8mm diameter cloning cylinders (Millipore, Merck KGaA) and further propagated for three more months in media containing 15µg/mL trastuzumab (18,20,21). Trastuzumab resistance was validated after a cycle of freezing and thawing and after drug withdrawal up to six 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 x 10^3 cells per well in a 24-well plate were treated for seven 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 x 10^3 cells per well in 96-well plate. Cell viability was obtained using CellTitler 96® AQueous One Solution Cell Proliferation

Assay (MTS) following manufacturer's procedures. IC50 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 previously reported (15), incubating cells with trastuzumab 100ng/mL as the primary antibody.

Fluorescence in situ hybridization (FISH)

To assess *ERBB2* copy number alterations we applied the PathVysion 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 non-overlapping cells with hybridization signals were examined for each case and amplification was defined as ERBB2/CEP17 ratio ≥2.0 (23).

Immunocytochemistry (ICC)

To study HER2 protein expression, $1x10^7$ of cells were pelleted, fixed with formalin, and embedded with paraffin (FFPE). HER2 expression was evaluated using HercepTest™. Sections of 3μM 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 previously reported (20). The primary antibodies used were: EGFR, HER3, phosphoERK1/2 (T202/Y204), ERK1/2, phosphoAKT (S473), AKT, phosphoS6 (S235/236), S6, phosphoSRC (Y416), and SRC purchased from Cell Signalling 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 (Chemiluminescent Readout) #7982 of Cell Signaling Technology following 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 (CRG) in the 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.*

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells in basal conditions and treated with trastuzumab 15μg/mL and Pan-HER 10μg/mL for 24 hours. cDNA was synthetized 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 I Master (Roche) in the LightCycler® 480 Real Time PCR-System device (Roche). The primers used were: *EGF* Fw: *5'*-CGTGTCGTGAAGGTTTTATG-3' and *EGF* Rv: *5'*-GTTCTTTAGATCAACTTCACC-3'; *AREG* Fw*: 5'*-GCATGATTGACAGTAGTTTATC-3' and *AREG* Rv*:* 5'-TTCTAAGCTGGACTGTAATAAC-3'; *TGFA* Fw: 5'-TGTGTCTGCCATTCTGGGTA-3' and *TGFA* Rv 5'-GACCTGGCAGCAGTGTATCA-3'; *HBEGF* Fw*:* 5'-GGAGAATGCAAATATGTGAAG-3' and *HBEGF* Rv*:* 5'-TTCCACTGGGAGGCTCAG-3'; *NRG1* Fw: 5'-CAGCCCAAGAGCCTGTTAAG-3' and *NRG1* Rv: 5'-ACTGCTCTGGGAGCTTGTGT-3' *ATP5E* Fw: 5'-GATCTGGGAGTATCGGATG-3' and *ATP5E* Rv: 5'-CCGGCGTCTTGGCGATTC-3'. Gene expression was calculated as 2 to the power of - $\Delta\Delta C_t$, where $\Delta\Delta C_t = (Ct_{Gene} - Ct_{ATP5E \text{ or } ACTIN})_{\text{Assay}}$.

ELISA analysis

Cell culture medium from 1,5 x 10^6 cells plated in 100-mm culture dish with 10mL of medium for 72 hours was concentrated using Amicon® Ultra-4 Centrifugal Filter Units (Millipore, Merck KGaA) following 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 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. Five x 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 x W^2 x 3,1416) / 6. When tumor volume reached 200-300mm^3 mice were randomized to three groups with 10 mice in each one. IgG isotype control, trastuzumab (20mg/kg), and Pan-HER (60mg/kg) were administered 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 immunohistochemical studies.

Immunohistochemistry (IHC)

Paraffin sections were used for IHC analysis as described in *Immunocytochemistry (ICC)* section. Tumor sections were stained with hematoxylin-eosin (H&E) to assess histological changes. To evaluate proliferation and apoptosis, slices were incubated with the antibodies Ki-67 (clone MIB-1, Dako, Agilent Technologies) mouse monoclonal antibody 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. Antigen–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 was presented as the percentage of positive tumor cells.

Patient samples

Blood samples and tumor specimens from HER2-positive gastric cancer and gastro-esophageal junction adenocarcinoma patients 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 #A31881 (ThermoFisher SCIENTIFIC) and *EGF* expression analysis was performed as described in *Quantitative real time polymerase chain reaction (qRT-PCR)*.

Statistical analysis

Statistical analyses were carried out using one-way ANOVA tests followed by *post hoc* Tukey adjustment or Student's *t*-test. Prism 5.0 software (GraphPad®) was used for the statistical analyses. Data shown is mean ± standard deviation (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 1B). 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 morphological 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, NRAS, BRAF*, and *PIK3CA* (listed in *Supplementary Material and Methods*). We did not identify the emergence of mutations in these genes in resistant compared to 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 to 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. Immunohistochemistry showed HER2 3+ immunoscore in both parental and resistant cells, although a small reduction in protein staining was observed in resistant compared to parental cells that was confirmed by Western blot analysis (Fig. 1B, 2A, 3E, and 4B).

The HER2 inhibitor lapatinib did not affect the viability of resistant cells compared to parental cells (Fig. 1C and 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 HER2 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, 2B, and 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 pharmacological 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 Fig. 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 to 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 and 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 and Supplementary Fig. S3), which was correlated with effective suppression of HER2 downstream effectors (Supplementary Fig. S4C). Of note, since *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 or 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 to parental cells. Following trastuzumab therapy, a trend towards an increase in total *EGFR*, *ERBB3*, and *ERBB4* was observed compared to 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 to parental cells (Fig. 3A and 3B). Additionally, increased levels of AREG and TGFα were detected in the cell culture medium of resistant cells compared to 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 ligands 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 and 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 non-overlapping epitopes (13). Pan-HER significantly

reduced cell viability of parental cells even in the presence of ligands (Fig. 3D and 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)

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 trastuzumab-resistant cells and this was correlated with a successful depletion of EGFR, HER2, and HER3 as well as an inhibition of the downstream effectors (Fig. 4 and Supplementary Fig. S7). To study whether triple blockade of EGFR, HER2, and HER3 was necessary to overcome trastuzumab resistance, we treated resistant cells with specific EGFR (cetuximab) or HER2/HER3 (pertuzumab) targeted therapies. Neither cetuximab nor pertuzumab were able to revert trastuzumab resistance (Fig. 4A and Supplementary Fig. S7). Such results, together with the simultaneous upregulation of several HER-family ligands (Fig. 3B and 3C) confirmed that compensatory ligand-induced co-activation of alternative HER family members is involved in driving resistance to trastuzumab. Therefore, complete and simultaneous blockade of all HER family members is necessary to revert trastuzumab resistance in our model.

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 to the parental cells (Supplementary Fig. S8A). Additionally, *NRG1, AREG,* and *EGF* ligands were overexpressed in OTR6 resistant cells (Supplementary Fig. S8B), and 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 S9B). At a molecular level, Pan-HER had a more profound effect in reducing EGFR, HER2,

HER3, and downstream effectors phosphorylation compared to 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 to 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 three 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 to control or trastuzumab treated tumors (Fig. 5C and Supplementary Table S1A). Immunohistochemistry analysis showed lower staining percentage of the proliferative markers Ki-67 and phosphorylated (S10) Histone H3 (pHist H3) in Pan-HER treated tumors compared to control or trastuzumab group (Fig. 5C and 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 and Supplementary Table S1B). Altogether, histological analysis confirmed a higher Pan-HER antitumor efficacy compared to control and trastuzumab effect.

HER-family ligands are increased in clinical samples from gastric cancer patients 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 five HER2-positive gastric cancer patients. Clinical characteristics of the patients are summarized in Supplementary Table S2. Of note, re-biopsy and collection of post-trastuzumab samples was limited due patient fragility and difficulty in tumor access. An increase in median EGF, AREG, and TGFα concentration was observed in post-trastuzumab compared to pre-trastuzumab serum samples (Fig. 6A and

Supplementary Fig. S11A). In tissue samples, *EGF* expression was 2.7 times higher in post-trastuzumab tumor biopsy compared to pre-treatment tumor sample (Fig. 6B and Supplementary Fig. S11B). Other markers could not be characterized due to insufficient tumor sample availability.

DISCUSSION

The approval of trastuzumab for HER2-positive gastric cancer patients 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 / PIK3K 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 gastric cancer patients. Preclinical models have shown a potential involvement in EGF ligand upregulation in trastuzumab resistance in gastric cancer patients (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.

Co-expression of multiple receptor tyrosine-kinase (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 *et al,* 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 the present study we show that Pan-HER more effectively inhibits cell viability compared to 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 to 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 re-implanted 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 three 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 *et al* 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 antibody-dependent cytotoxicity (ADCC) and enhanced complement-dependent cytotoxicity (CDC) activation similar to trastuzumab (13). 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 reverting resistance 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 1) the need to dynamically evaluate levels of HER ligands before, during, and after trastuzumab therapy and 2) the need to evaluate Pan-HER as a potential therapeutic strategy to overcome trastuzumab resistance in clinical trials.

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FIGURE LEGENDS

Figure 1. Proliferation of trastuzumab-resistant cells is independent of HER2. A) Cell viability analyzed by staining the cells with crystal violet after treating the cells with trastuzumab (15µg/mL) during seven days. ****p*<0.001. **B)** Representative images of parental and resistant cells under light microscopy (upper images). *ERBB2* amplification by FISH: *ERBB2* gene in red and centromere of chromosome 17 (CEP17) in green (intermediate images). HER2 overexpression by IHC (lower images). **C)** Dose curve of lapatinib by MTS assay after 72 hours. IC50 values of lapatinib: IC50 parental cells 0.26µM (SD 0.09), IC50 resistant cells not reached. ****p*<0.001. Molecular effects in Supplementary Fig. S1. **D)** Histogram representing mean fluorescence intensity of trastuzumab-PE (Tz-PE) expressing cells corresponding to 10.000 events. Parental and resistant cells (in dark and light grey respectively) were incubated with trastuzumab (100ng/mL) and a goat anti-human IgG conjugated with phycoeritrin (PE) as secondary antibody.

Figure 2. Resistance to trastuzumab is driven by activation of SRC and downstream MAPK and PIK3 signaling. A) Biochemical analysis of HER2 and downstream effectors in basal conditions and following trastuzumab therapy. Cells were cultured in the presence of trastuzumab (15µg/mL) for 24 hours. Immunoblots were performed using antibodies to the indicated proteins. **B)** Cell lysates of parental and resistant cells in basal conditions were incubated with a phospho-array. Antibodies against RTK and signaling nodes are spotted in duplicates. Red squares indicate the most relevant changes in intensity. Quantification of the phospho-array in Supplementary Fig. S2. **C)** Cell viability analyzed by staining the cells with crystal violet after treating the cells with the indicated drugs during seven days. ***p*<0.01 and ****p*<0.001. Representative images in Supplementary Fig. S3, and molecular effects in Supplementary Fig. S4.

Figure 3. Resistant cells overexpress multiple HER family ligands and HER family ligands overexpression protects from trastuzumab inhibition. A) Heatmap representation of 15 selected genes expression. Columns represent the mean of the intensities in the groups of triplicates parental control, parental trastuzumab, resistant control, and resistant trastuzumab. Cells were cultured in the presence of trastuzumab (15µg/mL) for 48 hours. Columns are clustered with ward.D2 method and rows are clustered with average method using in both cases correlation distances. Color intensity means degree of gene expression modulation. **B)** Analysis of indicated gene

expression by qRT-PCR. Gene expression levels were normalized to *ATP5E* as the housekeeping gene. Resistant cells data was normalized to the respective parental cells expression level (set at 1, dotted line). ***p<0.001. **C)** Levels of AREG and TGFα protein in cell culture medium of resistant cells normalized to the respective parental levels (set at 1, dotted line) measured by ELISA. *p<0.05. **D)** Cell viability analyzed by staining the cells with crystal violet after treating the cells with trastuzumab (15µg/mL), Pan-HER (10µg/mL), EGF (1nM), or NRG1 (5nM) during seven days. *p<0.05 and ***p<0.001. Representative images in Supplementary Fig. S5. **E)** Biochemical analysis of HER family receptors and downstream effectors following ligand stimulation. Cells were cultured in the presence of trastuzumab (15µg/mL) or Pan-HER (10µg/mL) for 24 hours, followed by 15minutes with EGF (1nM) or NRG1 (5nM). Immunoblots were performed using antibodies to the indicated proteins.

Figure 4. Simultaneous inhibition of EGFR, HER2 and HER3 is necessary to inhibit cell growth in trastuzumab-resistant cell lines. A) Cell viability analyzed by staining resistant cells with crystal violet. Cells were treated with trastuzumab, cetuximab, pertuzumab, and Pan-HER dose curves during seven days. IC50 values of resistant cells: IC50 Pan-HER 26.20µg/mL (SD 0.96), IC50 trastuzumab, cetuximab, or pertuzumab not reached. **p<0.01 and ***p<0.001. Representative images in Supplementary Fig. S7. **B)** Biochemical analysis of HER family receptors and downstream effectors following trastuzumab and Pan-HER treatment. Cells were cultured in the presence of trastuzumab (15µg/mL) or Pan-HER (10µg/mL) for 24 hours. Immunoblots were performed using antibodies to the indicated proteins.

Figure 5. Pan-HER *in vivo* efficacy on parental and resistant xenograft models. A, B) Effects of trastuzumab and Pan-HER on gastric cancer tumor xenografts. Mice were injected subcutaneously in the dorsal flank with parental or resistant cells. Once tumor reached an average tumor size of 200-300mm^3, mice were treated intraperitoneally three times a week with: IgG isotype control, trastuzumab (20mg/kg), or Pan-HER (60mg/kg). Each group consisted of ten mice. Tumor volumes were normalized individually to the volume at the first day of treatment. Pan-HER significantly reduced tumor volume compared to trastuzumab in both xenografts. *p<0.05, **p<0.01, and ***p<0.001. **C)** Representative images of H&E staining and immunostaining for Ki-67, pHist H3, or c-casp 3 of parental and resistant xenograft tumor sections. Scale bar is 5mm in H&E and 200µm in IHC images. Red arrows point representative positive cells in IHC images.

Figure 6. HER-family ligands increase after trastuzumab treatment in clinical samples from HER2-positive gastric cancer patients. A) Scatter plot of EGF, AREG, and TGFα serum concentration from paired pre- and post-trastuzumab samples from HER2-positive gastric cancer patients (n=5) measured by ELISA. **B)** *EGF* mRNA levels from FFPE tumor tissue from paired pre-trastuzumab and post-trastuzumab samples (n=3) by qRT-PCR. ***p*<0.01.