Published in final edited form as:

Cancer Res. 2010 January 1; 70(1): . doi:10.1158/0008-5472.CAN-09-1845.

Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy

Guanglei Zhuang¹, Dana M. Brantley-Sieders², David Vaught¹, Jian Yu⁶, Lu Xie⁶, Sam Wells⁵, Dowdy Jackson⁷, Rebecca Muroaka-Cook¹, Carlos Arteaga^{1,2,4}, and Jin Chen^{1,2,3,4,*}

¹Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN 37232 USA

²Department of Medicine, Division of Rheumatology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232 USA

³Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232 USA

⁴Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, TN 37232 USA

⁵Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232 USA

⁶Shanghai Center for Bioinformation Technology, Shanghai 200235, P. R. China

⁷MedImmune, LLC, Gaithersburg, MD 20878 USA

Abstract

One arising challenge in the treatment of breast cancer is development of therapeutic resistance to trastuzumab, an antibody targeting the human epidermal growth factor receptor-2 (HER2) which is frequently amplified in breast cancers. In this study, we provide evidence that elevated level of the receptor tyrosine kinase EphA2 is an important contributor to trastuzumab resistance. In a screen of a large cohort of human breast cancers, we found that EphA2 overexpression correlated with a decrease in disease-free and overall survival of HER2-overexpressing patients. Trastuzumab-resistant cell lines overexpressed EphA2, whereas inhibiting EphA2 restored sensitivity to trastuzumab treatment in vivo. Notably, trastuzumab treatment could promote EphA2 phosphorylation by activating Src kinase, leading in turn to an amplification of PI3K/Akt and MAPK signaling in resistant cells. Our findings offer mechanistic insights into the basis for trastuzumab resistance and they rationalize strategies to target EphA2 as a tactic to reverse trastuzumab resistance.

Keywords

EphA2; HER2/ErbB2/Neu; breast cancer; tumor resistance; trastuzumab

*Correspondence should be addressed to: Jin Chen, A-4323 MCN, Vanderbilt University School of Medicine, 1161 21st Avenue South, Nashville, TN 37232. jin.chen@vanderbilt.edu. Telephone 615-343-3819.

This study was cosponsored by MedImmune, LLC and Vanderbilt University.

Introduction

Recent advances in the development and application of molecularly targeted therapies for cancer have generated promising new treatments. One such treatment is the recombinant humanized monoclonal anti-HER2 antibody trastuzumab (Herceptin® Genentech). Trastuzumab targets the HER2/ErbB2 oncoprotein (1), a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs). HER2 is overexpressed in 25–30% of human breast cancers and associated with poor patient survival (2). Despite the proven benefit of trastuzumab in treating breast cancer (3–5), not all patients with amplified HER2 respond to trastuzumab. Indeed, only one-third of women with newly diagnosed HER2 positive breast cancer exhibit tumor regression with trastuzumab monotherapy (5). In addition, the majority of patients who achieve an initial response develop trastuzumab resistance within one year (5, 6). Therefore, identifying mechanisms that modulate trastuzumab response and resistance is vital to improve the therapeutic index of this agent.

Eph receptor A2 (EphA2), an Eph family RTK, has been recently linked to breast tumor initiation and metastatic progression (7–9). Experimentally induced overexpression of EphA2 resulted in malignant transformation of non-transformed MCF10A breast epithelial cells and enhanced malignancy of pancreatic carcinoma cells (10, 11). Conversely, siRNA-mediated inhibition of EphA2 expression impaired malignant progression of pancreatic, ovarian and mesothelioma human tumor cell lines, and overexpression of dominant-negative EphA2 constructs suppressed growth and metastasis of 4T1 mouse mammary adenocarcinoma cells *in vivo* (10, 12–14). EphA2-mediated oncogenesis appears to be ligand independent, and EphA2 often signals through crosstalk with other cell surface receptors (15, 16). We recently reported that loss of EphA2 receptor impaired tumor initiation and metastatic progression in MMTV-Neu mice (17). In human and murine breast carcinoma cells, EphA2 forms a complex with HER2, resulting in enhanced activation of Ras-MAPK and RhoA GTPase, and increased cell proliferation and motility. These data indicate that EphA2 promotes breast tumor formation and metastatic progression by amplifying HER2 signaling.

In this report, we investigated the role of EphA2 in regulation of breast cancer sensitivity to trastuzumab. We found that high EphA2 levels enhanced both intrinsic and acquired trastuzumab resistance. Elevated EphA2 in resistant cells appears to be activated by trastuzumab treatment-induced Src kinase, and activated EphA2 amplifies signaling through the PI3 kinase/Akt and MAP kinase pathways in resistant cells. In addition, microarray analysis of a large cohort of human breast cancer specimens revealed that high levels of EphA2 expression in HER2 positive patients predict poor prognosis. Thus, these results provide new mechanistic insights into the molecular basis of anti-HER2 resistance, and targeting EphA2 could represent an appealing therapeutic strategy to increase the efficacy of HER2-based treatments in breast cancer.

Materials and Methods

Survival analysis

The van der Vijver database, with microarray profiles of 295 human breast tumors and associated clinical data, was obtained from Rosetta Inpharmatics (<http://www.rii.com/publications/2002/nejm.html>). The first 25% patients that exhibit higher HER2 expression were defined as HER2 positive, as described (18–20). The HER2+ patients were further stratified into two groups based on the expression levels of EphA2. Kaplan-Meier analyses were computed using R survival package. Statistical differences were determined by log-rank tests.

Cell culture

The MMTV-Neu tumor derived cell line (21), parental MCF10A and MCF10A cells stably overexpressing HER2 were maintained as described previously (17). Parental and trastuzumab-resistant SK-BR-3 and BT474 cells were generously provided by Francisco Esteva (UT MD Anderson Cancer Center, TX) and Carlos Arteaga (Vanderbilt University, TN) (22, 23), respectively. Three-dimensional spheroid cultures were established on Matrigel as described (24). Cultures were maintained for 8 days prior to photodocumentation. Digital images were analyzed and the percentage of Ki67 positive cells was quantified using LSM Image Browser (Zeiss) software. Results were derived from 10 colonies in two independent experiments. Statistical differences among groups were determined by Student's *t* test.

Mice and in vivo tumor studies

3–4 week old athymic nude female mice were implanted with 1.5 mg, 60-day release 17 β -estradiol pellets subcutaneously. The next day, trastuzumab-resistant BT-474 cells [1.5×10^7 ; HR5] were resuspended in 100 μ l PBS/100 μ l growth-factor reduced Matrigel and injected into the number 4 inguinal mammary gland fat pad as previously described (22). Tumor engraftment and growth was verified by palpation and tumor volume was measured by a caliper. Two weeks after transplantation, the mice were treated with control IgG (10 mg/kg) (clone R347, MedImmune, LLC), anti-EphA2 antibody (10 mg/kg) (clone 3F2-3M, MedImmune, LLC), trastuzumab (20 mg/kg), or a combination of anti-EphA2 antibody and trastuzumab, by twice weekly intraperitoneal injection. Tumors were harvested two weeks after treatment and data were derived from 10 independent animals per treatment group in two independent experiments.

Histological analyses

Tumors were sectioned by the Vanderbilt University Immunohistochemistry Core Facility. Immunohistochemical staining for EphA2, PCNA and CD31 was performed as described previously (25). Proliferation or apoptosis was quantified by calculating the average percentage of PCNA or TUNEL positive nuclei relative to total nuclei (4 random fields of at least 4 independent tumor samples).

FRET Analysis of Src biosensor

The MCF7 cells expressing HER2 were transfected with Src biosensor (generously provided by Yingxiao Wang, University of Illinois) and serum starved for 48 hours before being treated with trastuzumab (10 μ g/ml). Images and FRET analysis were performed on an LSM 510 META confocal microscope (Zeiss) using a 40X/1.3 NAPlan-Neofluar objective lens and 458nm laser excitation for CFP and FRET. Emission from CFP versus YFP/FRET was discriminated using appropriate bandpass emission filters (BP 475-525 for CFP and LP560 for YFP/FRET). The fluorescence intensity of CFP and YFP images were measured using the Zeiss Image Examiner software before being quantified and analyzed by Prism 5 (GraphPad). Quantification was based on 20 cells/time point in two independent experiments. Statistical differences were analyzed using Student's *t* test.

Results

Overexpression of EphA2 in HER2-positive patients predicts poor prognosis

Because our previous investigations in mouse models suggest that cooperation between HER2 and EphA2 may promote mammary tumor formation, we sought to determine if EphA2 could be an effective therapeutic target for HER2-positive breast cancer patients. To analyze the impact of EphA2 overexpression on the prognosis of HER2-positive breast

cancer patients, we examined previously published microarray data for a panel of 295 breast cancer samples (26). Seventy-four HER2-positive samples were examined for EphA2 mRNA expression. The resulting Kaplan-Meier analysis of survival data revealed that high levels of EphA2 expression correlated with a decrease in overall (Figure 1A) and recurrence-free survival (Figure 1B) in HER2-positive breast cancer patients. These data indicate that EphA2 overexpression in HER2 positive patients may predict poor prognosis, and elevated EphA2 may enable breast cancer cells to resist anti-HER2 treatment.

EphA2 overexpression confers cellular intrinsic resistance to trastuzumab

To investigate whether EphA2 overexpression is sufficient to confer resistance to trastuzumab, we transduced a constitutively activated (CA-EphA2) or a kinase dead (KD-EphA2) form of human EphA2 into MCF10A.HER2 cells (27) (Supplemental Figure 1A). MCF10A.HER2 cells formed large acinar-like structure with a filled lumen and were sensitive to trastuzumab treatment (Figure 2A)(28). Introduction of CA-EphA2 into in MCF10A.HER2 cells further enhanced cell proliferation, but this increased cell growth in MCF10A.HER2 cells expressing CA-EphA2 was refractory to trastuzumab (Figure 2A&B). In contrast, expression of catalytically inactive KD-EphA2 in MCF10A.HER2 cells decreased basal rates of proliferation, which were further decreased upon treatment with trastuzumab (Figure 2A&B). These data are consistent with previous data demonstrating cooperation between HER2 and EphA2 to drive cellular proliferation (17), and further suggest that EphA2 kinase activity is able to promote trastuzumab resistance in HER2-overexpressing breast cells.

Interestingly, MCF10A.HER2 cells express elevated levels of EphA2 protein relative to those in parental MCF10A cells (Supplemental Figure 1B). To determine if inhibition of EphA2 increases innate sensitivity to trastuzumab, MCF10A.HER2 cells were treated with an anti-human EphA2 antibody, a ligand-mimetic activating antibody that specifically binds to EphA2 and induces receptor internalization and degradation (Supplemental Figure 1B). While the anti-EphA2 antibody had no effect on non-transformed MCF10A cells that express low levels of EphA2, the antibody significantly inhibited cell growth in MCF10A.HER2 cells. More importantly, a combination of anti-EphA2 antibody and trastuzumab inhibited cell growth with greater potency than either antibody alone (Figure 2C&D). Taken together, these data suggest that EphA2 overexpression is one mechanism of intrinsic resistance to trastuzumab.

As an independent approach to determine whether EphA2 expression levels correlate with trastuzumab resistance, we overexpressed HER2 in a panel of human breast cancer cell lines that express EphA2 protein at low or high levels (Supplemental Figure 2A). BT-474 and SK-BR-3 cells that express high levels of endogenous HER2 but low levels of EphA2 were growth inhibited in response to trastuzumab, so were MCF7 and T47D that overexpress HER2. (Supplemental Figure 2B). In contrast, HBL100, MDA-468, MDA-231, and BT-549 expressed high levels of EphA2, and were resistant to the growth inhibitory effects of trastuzumab. These data are consistent with a correlation between EphA2 expression and trastuzumab response in HER2-overexpressing human breast cancer cells.

EphA2 elevation contributes to acquired trastuzumab resistance

Genome-wide profiling of gene expression showed that EphA2 and HER2 are not always co-expressed in human breast cancer. We reasoned that upon prolonged trastuzumab treatment, a subset of HER2 positive tumors that initially express low levels of EphA2 and respond to trastuzumab may increase EphA2 expression, leading to a decrease in trastuzumab sensitivity. To test this possibility, we analyzed EphA2 expression in two independent trastuzumab-resistant human breast cancer cell lines, SK-BR-3 and BT-474,

which were derived from *in vitro* or *in vivo* selection for acquired resistance to trastuzumab, respectively (22, 23). As shown in Figure 3A, EphA2 levels were considerably higher in two independently-derived trastuzumab-resistant clones from each cell line, relative to their trastuzumab-sensitive parental cells. To test whether this EphA2 overexpression is required to maintain trastuzumab resistance, we treated the parental and the trastuzumab-resistant cells with anti-EphA2 antibody, in the presence or absence of trastuzumab. As expected, sensitive SK-BR-3 and BT-474 cells were growth inhibited by trastuzumab whereas resistant cells were not. Anti-EphA2 antibody alone did not significantly affect cell growth in SK-BR-3 or BT-474 cells. However, EphA2 inhibition restored cellular sensitivity to trastuzumab in each resistant cell line, as shown in both 2-dimensional cell culture (Figure 3B) and 3-dimensional Matrigel culture (Figure 3C). These data suggest that EphA2 is upregulated in treatment-induced trastuzumab-resistant cells and that high levels of EphA2 in resistant cells contribute to acquired trastuzumab resistance.

Targeting EphA2 inhibits trastuzumab-resistant tumor growth *in vivo*

Having demonstrated the combinatorial activity of anti-EphA2 antibody and trastuzumab for growth inhibition of trastuzumab-resistant cells *in vitro*, we next investigated the therapeutic potential of an anti-EphA2 antibody for the treatment of trastuzumab-resistant tumor growth *in vivo* in an orthotopic xenograft model. Trastuzumab-resistant BT-474 cells were injected into the mammary fat pad of female athymic nude mice. Two weeks after transplantation when tumor volume reached approximately 200 mm³, mice were treated with either control IgG or anti-human EphA2 antibody (10mg/kg), in the presence or absence of trastuzumab (20mg/kg). Consistent with a prior report (22), resistant BT-474 tumors did not respond to trastuzumab treatment as compared to IgG-treated tumors. Anti-EphA2 antibody treatment moderately reduced tumor size, relative to controls. In contrast, co-administration of anti-EphA2 antibody with trastuzumab markedly reduced tumor volume (Figure 4A&B).

To examine cellular changes within treated tumors, we analyzed cell proliferation and apoptosis in tissue sections by staining for proliferating cell nuclear antigen (PCNA) and by TUNEL assay, respectively. Quantitation of PCNA+ nuclei revealed a nearly 2-fold decrease in PCNA staining in tumors treated with the combination of anti-EphA2 antibody versus tumors treated with control IgG ($P<0.05$; Figure 4C). In contrast, treatment with anti-EphA2 antibody alone, or with trastuzumab alone, did not significantly alter the proportion of PCNA+ cells as compared to IgG-treated tumors. Similarly, apoptosis was increased approximately 6-fold in tumors treated with a combination of anti-EphA2 antibody and trastuzumab ($P<0.01$; Figure 4C), but was unaltered in tumors treated with either antibody alone. Taken together, these data suggest that targeting EphA2 may be effective for the suppression of trastuzumab-resistant breast tumor growth in the presence of trastuzumab.

EphA2 regulates breast cancer cell sensitivity to trastuzumab by modulation of Akt and MAPK activities

Breast cancer resistance to HER2 inhibitors could arise through multiple mechanisms, including activation of alternative growth factor receptor or enhancing downstream signaling pathways. We investigated potential mechanisms by which EphA2 contributes to trastuzumab resistance in HER2-overexpressing breast cancer. We found that elimination of EphA2 by siRNA knockdown or anti-EphA2 antibody reduced phospho-Akt and phospho-Erk levels in trastuzumab-resistant cells (Figure 5A&B), suggesting that EphA2 expression and activity are required to maintain signaling through the PI3 kinase/Akt and MAP kinase signaling pathways.

To determine whether PI3K-Akt and Ras-MAPK signaling pathways play a causal role in trastuzumab resistance, we treated SK-BR-3 cells with a PI3K inhibitor LY294002 (Figure

5C) or a MEK inhibitor U0126 (Figure 5D) and analyzed cell growth in the presence or absence of trastuzumab. In sensitive cells, cell growth is inhibited by trastuzumab, and addition of LY294002 or U0126 did not further impact cell growth significantly. However, while resistant cells do not respond to trastuzumab, they are exquisitely sensitive to the MEK inhibitor (Figure 5D). In fact, resistant cells are more sensitive to U0126 than trastuzumab-sensitive cells, suggesting that trastuzumab-resistant cells are dependent on MAPK signaling. In addition, either PI3K inhibitor or MEK inhibitor significantly restored trastuzumab sensitivity in resistant cells. Together, our data suggest that anti-EphA2 antibody therapy reverses trastuzumab resistance by inhibiting the activation of both Akt and MAPK.

Chronic trastuzumab treatment activates EphA2 through Src kinase

To investigate how EphA2 is activated in trastuzumab-resistant cells, we examined the involvement of Src kinase, because prior studies showed that the Src directly interacts with HER2 and is activated in HER2-overexpressing cancer cells (29, 30). Co-expression of HER2 and EphA2 in COS7 cells was sufficient to induce tyrosine phosphorylation of EphA2, and this process was inhibited by a Src inhibitor PP2. In addition, constitutively activated v-Src induced phosphorylation of EphA2 independently of HER2 (Supplemental Figure 3A), suggesting that HER2 may modulate EphA2 activity through Src. We next investigated whether Src can be activated by trastuzumab. A previous study suggested that short exposure to trastuzumab rapidly inhibits Src kinase activity (31). However, we found that longer treatment of SK-BR-3 cells with trastuzumab increased Src phosphorylation at Y416, an indicator of Src activation (Supplemental Figure 3B). To further determine whether prolonged trastuzumab treatment can activate Src kinase, we used a Src biosensor that enables the visualization of Src activity in live cells with high spatiotemporal resolution by fluorescence resonance energy transfer (FRET) technology (32, 33). Trastuzumab induced a 15–25% reduction in Src activity within 1 hour in MCF7.HER2 cells transfected with the Src biosensor, but the decrease in Src activity gradually recovered with prolonged trastuzumab incubation (Figure 6A). After 24 hours of treatment, Src activity increased by 35% in MCF7.HER2 relative to control cells (Figure 6B), while EphA2 levels were not changed (Supplemental Figure 3C). These data support the existence of a switch from trastuzumab-induced Src inhibition to activation, which could modulate EphA2 activity in resistant cells. Indeed, EphA2 and Src were highly phosphorylated in trastuzumab-resistant cells. Src inhibitors, PP2 (Figure 6C) or dasatinib (data not shown), inhibited activities of both Src and EphA2.

To determine whether Src kinase contributes to trastuzumab resistance, we treated SK-BR-3 cells with trastuzumab, dasatinib, or a combination of trastuzumab and dasatinib, and assessed cell viability. Dasatinib inhibited cell growth in both sensitive and resistant cells. While resistant cells did not respond to trastuzumab, dasatinib partially restored trastuzumab sensitivity in resistant cells (Figure 6D). Together, these results provide a clear link between activation of Src and EphA2 in trastuzumab resistance.

Discussion

In this report, we described a novel mechanism by which HER2+ breast cancers acquire resistance to trastuzumab. The receptor tyrosine kinase EphA2 was found to correlate with a poor prognosis in patients with HER2-overexpressing breast cancers, and had a greater negative impact on patient survival in HER2-overexpressing breast cancers as compared to other breast cancers. We found that overexpression of EphA2 in HER2+ breast cancer cells was sufficient to confer innate resistance to trastuzumab. Furthermore, antibody-mediated EphA2 inhibition enhanced tumor response to trastuzumab both in cell culture and *in vivo*.

These data suggest that therapeutic inhibition of EphA2 may represent a strategy for improving the clinical response of trastuzumab.

What is the mechanism by which elevated EphA2 confers tumor cell resistance to trastuzumab? Resistance to anti-ERBB2/HER2 agents could arise through multiple mechanisms, including altered receptor-antibody interaction, activation of alternative growth factor receptor signaling pathways and deregulation of downstream signaling pathways (34, 35). The most common downstream signaling pathway that contributes to trastuzumab resistance is the PI3K-Akt pathway. Persistent activation of PI3K-Akt signaling in resistant cells could result from multiple mechanisms such as oncogenic mutations of PI3K (36), loss of PTEN (31), or upregulation of insulin-like growth factor-I receptor (IGF-IR) and EGFR activity (22, 37). In this case, targeting EphA2 inhibited the PI3K-Akt pathway in trastuzumab resistant cells (Figure 5). In addition to regulating Akt activity, we discovered that EphA2 also modulates phospho-Erk levels in resistant cells. Increased EphA2 expression in resistant cells enhanced phospho-Erk levels, and targeting EphA2 with siRNA or anti-EphA2 antibody inhibited Erk activity (Figure 5). These data, together with reports from other laboratories (38, 39), suggest that the development of trastuzumab resistance may involve simultaneous activation of multiple parallel signaling cascades including PI3K/Akt and MAPK pathways (40–42). Indeed, a MEK inhibitor that suppresses phospho-Erk significantly decreased the viability of resistant cells (Figure 5D). Suppression of MAPK activity by EphA2 antibody was also observed in MCF10A 3D culture (data not shown), as well as in MMTV-Neu cells (Supplemental Figure 4A), where Erk phosphorylation recovered after prolonged treatment with gefitinib, a dual inhibitor of EGFR and ErbB2/Neu (43). The combination of anti-EphA2 antibody and gefitinib completely abrogated MAPK activity and inhibited tumor growth *in vivo* (Supplemental Figure 4). Together, these data suggest that modulation of both Akt and MAPK signaling is a primary mechanism through which EphA2 contributes to trastuzumab resistance.

How is EphA2 receptor activated in trastuzumab-resistant cells? We have previously shown that EphA2 forms a complex with HER2/ErbB2 and can be phosphorylated in the presence of HER2/ErbB2 (17). However, we failed to detect direct EphA2 tyrosine phosphorylation by HER2 in an *in vitro* kinase assay (data not shown), indicating the possibility of involvement of another kinase. One candidate is the non-receptor tyrosine kinase Src, as Src directly interacts with HER2 and is activated in HER2-overexpressing cancer cells (29, 30). Indeed, Src is sufficient to activate EphA2 and is required for the phosphorylation of EphA2 by HER2 (Supplemental Figure 3A). Although trastuzumab reportedly inhibits Src activity within short time frame (31), we observed increased Src activity in cells upon prolonged exposure to trastuzumab (Supplemental Figure 3B). Using a FRET-based Src reporter to monitor Src activity in live cells, we found that short term exposure to trastuzumab inhibits Src kinase activity, consistent with the previous report (31). However, prolonged treatment resulted in increased Src activity (Figure 6A&B). These results were supported by biochemical studies, in which Src phosphorylation at Y416 was increased in prolonged trastuzumab treatment. The mechanism of switch between trastuzumab-induced Src inhibition and activation is unclear. We speculate that continuous exposure to trastuzumab may cluster HER2 at the plasma membrane and recruit Src into HER2/EphA2 complex, resulting in activation of Src and phosphorylation of EphA2 receptor.

Our findings that EphA2 co-expresses with HER2 and confers trastuzumab resistance in HER2-positive breast cancers could directly impact the clinical management of these patients. We propose that individuals with EphA2 and HER2 positive breast cancer might benefit from pharmacological inhibition of EphA2 in combination with anti-HER2 therapies. EphA2 expression may also be used as a prognostic marker to predict trastuzumab resistance and treatment outcome. In patients who initially are negative for EphA2 but subsequently

developed resistance to trastuzumab, elevated EphA2 could be one of the mechanisms that confer tumor resistance to HER2 inhibitors. Targeting EphA2 may represent a novel strategy to overcome trastuzumab resistance. In summary, our studies provide new mechanistic insight into the molecular basis of trastuzumab resistance. These studies provide basis for rational design of combination therapies to overcome tumor resistance to trastuzumab.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Francisco Esteva (UT MD Anderson Cancer Center, Houston, TX), Tony Hunter (Salk Institute, La Jolla, CA), and Yingxiao Wang (University of Illinois, Urbana-Champaign, IL) for providing trastuzumab-resistant SK-BR-3 breast cancer cell lines, retroviral vectors containing constitutively activated and kinase dead EphA2, and Src FRET biosensor, respectively. We acknowledge the Vanderbilt imaging and histology core facility for assisting in image requisition and section of tumor tissues. We also thank Drs. Hal Moses, Al Reynolds, and Vivian Siegel for critical reading of the manuscript. This work was supported by NIH grants CA95004 and CA114301 (J. Chen), CA1179151 (D. Brantley-Sieders), and Department of Defense predoctoral fellowships, W81XWH-08-1-029 and W81XWH-08-0250 (to D. Vaught and G. Zhuang, respectively).

References

1. Carter P, Presta L, Gorman CM, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci U S A*. 1992; 89:4285–9. [PubMed: 1350088]
2. Hudis CA. Trastuzumab--mechanism of action and use in clinical practice. *N Engl J Med*. 2007; 357:39–51. [PubMed: 17611206]
3. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol*. 1996; 14:737–44. [PubMed: 8622019]
4. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol*. 1999; 17:2639–48. [PubMed: 10561337]
5. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol*. 2002; 20:719–26. [PubMed: 11821453]
6. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001; 344:783–92. [PubMed: 11248153]
7. Vaught D, Brantley-Sieders DM, Chen J. Eph receptors in breast cancer: roles in tumor promotion and tumor suppression. *Breast Cancer Res*. 2008; 10:217. [PubMed: 19144211]
8. Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. *Cell*. 2008; 133:38–52. [PubMed: 18394988]
9. Wykosky J, Debinski W. The EphA2 receptor and ephrinA1 ligand in solid tumors: function and therapeutic targeting. *Mol Cancer Res*. 2008; 6:1795–806. [PubMed: 19074825]
10. Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. EphA2: a determinant of malignant cellular behavior and a potential therapeutic target in pancreatic adenocarcinoma. *Oncogene*. 2004; 23:1448–56. [PubMed: 14973554]
11. Zelinski DP, Zantek ND, Stewart JC, Irizarry AR, Kinch MS. EphA2 overexpression causes tumorigenesis of mammary epithelial cells. *Cancer Res*. 2001; 61:2301–6. [PubMed: 11280802]
12. Fang WB, Brantley-Sieders DM, Parker MA, Reith AD, Chen J. A kinase-dependent role for EphA2 receptor in promoting tumor growth and metastasis. *Oncogene*. 2005; 24:7859–68. [PubMed: 16103880]

13. Landen CN Jr, Chavez-Reyes A, Bucana C, et al. Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. *Cancer Res.* 2005; 65:6910–8. [PubMed: 16061675]
14. Nasreen N, Mohammed KA, Antony VB. Silencing the receptor EphA2 suppresses the growth and haptotaxis of malignant mesothelioma cells. *Cancer.* 2006; 107:2425–35. [PubMed: 17041885]
15. Chen J, Zhuang G, Frieden L, Debinski W. Eph receptors and Ephrins in cancer: common themes and controversies. *Cancer Res.* 2008; 68:10031–3. [PubMed: 19074866]
16. Larsen AB, Pedersen MW, Stockhausen MT, Grandal MV, van Deurs B, Poulsen HS. Activation of the EGFR gene target EphA2 inhibits epidermal growth factor-induced cancer cell motility. *Mol Cancer Res.* 2007; 5:283–93. [PubMed: 17374733]
17. Brantley-Sieders DM, Zhuang G, Hicks D, et al. The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling. *J Clin Invest.* 2008; 118:64–78. [PubMed: 18079969]
18. Rody A, Karn T, Ruckhaberle E, et al. Loss of Plexin B1 is highly prognostic in low proliferating ER positive breast cancers--results of a large scale microarray analysis. *Eur J Cancer.* 2009; 45:405–13. [PubMed: 19054665]
19. Rody A, Holtrich U, Pusztai L, et al. T-cell metagene predicts a favorable prognosis in estrogen receptor-negative and HER2-positive breast cancers. *Breast Cancer Res.* 2009; 11:R15. [PubMed: 19272155]
20. Alexe G, Dalgin GS, Scandfeld D, et al. High expression of lymphocyte-associated genes in node-negative HER2+ breast cancers correlates with lower recurrence rates. *Cancer Res.* 2007; 67:10669–76. [PubMed: 18006808]
21. Muraoka RS, Koh Y, Roebuck LR, et al. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Mol Cell Biol.* 2003; 23:8691–703. [PubMed: 14612410]
22. Ritter CA, Perez-Torres M, Rinehart C, et al. Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. *Clin Cancer Res.* 2007; 13:4909–19. [PubMed: 17699871]
23. Nahta R, Yuan LX, Zhang B, Kobayashi R, Esteva FJ. Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Res.* 2005; 65:11118–28. [PubMed: 16322262]
24. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods.* 2003; 30:256–68. [PubMed: 12798140]
25. Brantley-Sieders DM, Fang WB, Hicks DJ, Zhuang G, Shyr Y, Chen J. Impaired tumor microenvironment in EphA2-deficient mice inhibits tumor angiogenesis and metastatic progression. *FASEB J.* 2005; 19:1884–6. [PubMed: 16166198]
26. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med.* 2002; 347:1999–2009. [PubMed: 12490681]
27. Carter N, Nakamoto T, Hirai H, Hunter T. EphrinA1-induced cytoskeletal re-organization requires FAK and p130(cas). *Nat Cell Biol.* 2002; 4:565–73. [PubMed: 12134157]
28. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol.* 2001; 3:785–92. [PubMed: 11533657]
29. Belsches-Jablonski AP, Biscardi JS, Peavy DR, Tice DA, Romney DA, Parsons SJ. Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncogene.* 2001; 20:1465–75. [PubMed: 11313890]
30. Kim H, Chan R, Dankort DL, et al. The c-Src tyrosine kinase associates with the catalytic domain of ErbB-2: implications for ErbB-2 mediated signaling and transformation. *Oncogene.* 2005; 24:7599–607. [PubMed: 16170374]
31. Nagata Y, Lan KH, Zhou X, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell.* 2004; 6:117–27. [PubMed: 15324695]

32. Ouyang M, Sun J, Chien S, Wang Y. Determination of hierarchical relationship of Src and Rac at subcellular locations with FRET biosensors. *Proc Natl Acad Sci U S A*. 2008; 105:14353–8. [PubMed: 18799748]
33. Wang Y, Botvinick EL, Zhao Y, et al. Visualizing the mechanical activation of Src. *Nature*. 2005; 434:1040–5. [PubMed: 15846350]
34. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer*. 2005; 5:341–54. [PubMed: 15864276]
35. Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol*. 2006; 3:269–80. [PubMed: 16683005]
36. Berns K, Horlings HM, Hennessy BT, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell*. 2007; 12:395–402. [PubMed: 17936563]
37. Lu Y, Zi X, Zhao Y, Mascarenhas D, Pollak M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst*. 2001; 93:1852–7. [PubMed: 11752009]
38. Gori S, Sidoni A, Colozza M, et al. EGFR, pMAPK, pAkt and PTEN status by immunohistochemistry: correlation with clinical outcome in HER2-positive metastatic breast cancer patients treated with trastuzumab. *Ann Oncol*. 2009 Apr; 20(4):648–54. Epub 2009 Feb 2. [PubMed: 19188134]
39. Hosokawa S, Toyooka S, Fujiwara Y, et al. Comprehensive analysis of EGFR signaling pathways in Japanese patients with non-small cell lung cancer. *Lung Cancer*. 2009 Oct; 66(1):107–13. Epub 2009 Jan 31. [PubMed: 19185949]
40. Carracedo A, Ma L, Teruya-Feldstein J, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *J Clin Invest*. 2008; 118:3065–74. [PubMed: 18725988]
41. Grant S. Cotargeting survival signaling pathways in cancer. *J Clin Invest*. 2008; 118:3003–6. [PubMed: 18725993]
42. Kinkade CW, Castillo-Martin M, Puzio-Kuter A, et al. Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model. *J Clin Invest*. 2008; 118:3051–64. [PubMed: 18725989]
43. Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF, Arteaga CL. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo. *Cancer Res*. 2001; 61:8887–95. [PubMed: 11751413]

Figure 1. Overexpression of EphA2 in HER2-positive patients predicts poor prognosis
A previously published microarray data set from the fresh-frozen tissue bank of the Netherlands Cancer Institute for a panel of 295 breast cancer sample was analyzed. The resulting Kaplan-Meier kinetic analyses of survival data revealed that high levels of EphA2 mRNA expression correlated with a decrease in overall survival (A, $p=0.009$) and recurrence-free survival (B, $p=0.009$).

Figure 2. EphA2 overexpression confers cellular intrinsic resistance to trastuzumab

(A) Constitutively activated (CA-EphA2) or kinase dead (KD-EphA2) EphA2 receptor were introduced into MCF10A or MCF10A.HER2 cells by retroviral transduction. Pooled G418-resistant cell populations were cultured in 3-dimensional Matrigel and stained for Ki67 (green) to assess proliferation and counter-stained for To-Pro-3 (red) to visualize nuclei. Overexpression of CA-EphA2, but not KD-EphA2, desensitizes MCF10A.HER2 cells to trastuzumab. Cell proliferation was quantified in (B) * $P < 0.01$; Student's t test. (C) MCF10A or MCF10A.HER2 cells were cultured in 3-dimensional Matrigel and treated with antibodies as indicated. Anti-EphA2 antibody inhibited cell growth in MCF10A.HER2 cells. Cell proliferation in panel (C) is quantified in (D). * $P < 0.01$, ** $P < 0.05$; Student's t test.

Figure 3. EphA2 elevation contributes to acquired trastuzumab resistance

(A) Trastuzumab-sensitive (WT) or trastuzumab-resistant (HR) SK-BR-3 or BT-474 cells were subjected to western blot analysis to assess EphA2 expression levels. (B) Sensitive or resistant SK-BR-3 or BT-474 cells were treated with IgG control, anti EphA2, trastuzumab, or a combination of anti-EphA2 antibody and trastuzumab. Anti-EphA2 antibody restores cellular sensitivity to trastuzumab. (C) Sensitive or resistant SK-BR-3 or BT-474 cells were cultured in 3-dimensional Matrigel. Colonies were photographed at day 7 and colony size quantified. * $P < 0.01$; Student's t test.

Figure 4. Targeting EphA2 inhibits trastuzumab-resistant tumor growth

(A) Trastuzumab-resistant BT474 cells were orthotopically transplanted into mammary gland of nude female mice. Two weeks after transplantation, tumors were treated with control IgG, anti-EphA2 antibody (10mg/kg), trastuzumab (20mg/kg) or a combination of anti-EphA2 antibody and trastuzumab twice weekly via intraperitoneal injection. Data are presented as mean $\pm$ SEM of 10 mice per treatment group from 2 independent experiments. (B) Tumors were harvested and photographed. (C) Cell proliferation and apoptosis in tumor sections were evaluated by PCNA immunohistochemistry and TUNEL assay, respectively. * $P < 0.01$. Arrowheads indicate PCNA+ or TUNEL+ nuclei.

Figure 5. EphA2 regulates breast cancer sensitivity to trastuzumab by modulation of Akt and MAPK activity

(A) EphA2 was knocked down by siRNA in either parental or trastuzumab-resistant (HR1) SK-BR-3 cells. EphA2, phosphor-Akt and phospho-Erk levels were assessed by western blot analysis. Ratio of phospho-protein/total protein was determined by densitometry and expressed in arbitrary units. (B) Trastuzumab-sensitive or -resistant cells were treated with control, trastuzumab, anti-EphA2 antibody, or a combination of trastuzumab and anti-EphA2 antibody in the presence of 10% serum. Quantification of phospho-protein/total protein was determined as above. (C, D) Trastuzumab-sensitive (red) or -resistant (HR1, blue or black) SK-BR-3 cells were treated with increasing dose of either PI3K inhibitor LY294002 or MEK inhibitor U0126 for 3 days and cell viability was determined.

Figure 6. Trastuzumab treatment activates EphA2 through Src kinase

(A) MCF-7.HER2 cells expressing Src reporter were treated with trastuzumab over a time course. Normalized CFP/YFP emission ratio of the Src biosensor over time in response to trastuzumab is shown. (B) MCF-7.HER2 cells expressing Src reporter were treated with trastuzumab for 0, 1 and 24 hours. EphA2 levels were not changed during treatment (Supplemental Figure 4C). Changes in CFP/YFP emission ratio were quantified in 20 cells per experimental group at given time point. * $P < 0.01$. (C) Sensitive (WT) or resistant (HR) SK-BR-3 cells were treated with Src inhibitor PP2 or vehicle control. Phospho-Src and phospho-EphA2 levels were assessed by immunoprecipitation and western blot analysis. (D) Trastuzumab-sensitive or -resistant (HR) SK-BR-3 cells were treated with control, dasatinib, trastuzumab, or a combination of dasatinib and trastuzumab for 3 days. Cell growth was determined by luminescent cell viability assay. * $P < 0.05$; Student's t test.