

Met Receptor Contributes to Trastuzumab Resistance of Her2-Overexpressing Breast Cancer Cells

David L. Shattuck, Jamie K. Miller, Kermit L. Carraway III, and Colleen Sweeney

University of California Davis Cancer Center, Sacramento, California

Abstract

Her2 is overexpressed in 20% to 30% of breast tumors and correlates with reduced disease-free and overall patient survival. Trastuzumab, a humanized monoclonal antibody directed against Her2, represents the first Her2-targeted therapy, which decreases the risk of relapse and prolongs patient survival. Resistance to trastuzumab, both inherent and treatment-acquired, represents a significant barrier to the effective treatment of Her2 (+) breast cancer. The Met receptor tyrosine kinase is aberrantly expressed in breast cancer and predicts poor patient prognosis. In this study, we find that Met is frequently expressed in Her2-overexpressing breast cancer cells, as well as Her2 (+) breast cancer. Importantly, Met contributes to trastuzumab resistance, as inhibition of Met sensitizes cells to trastuzumab-mediated growth inhibition, whereas Met activation protects cells against trastuzumab by abrogating p27 induction. Remarkably, Her2-overexpressing breast cancer cells rapidly up-regulate Met expression after trastuzumab treatment, promoting their own resistance. Our study suggests that a subset of Her2 (+) patients may benefit from combined inhibition of Her2 and Met. [Cancer Res 2008;68(5):1471–7]

Introduction

Overexpression and aberrant activation of members of the ErbB family of receptor tyrosine kinases (RTK), including epidermal growth factor receptor (EGFR)/ErbB1, Her2/Neu/ErbB2, ErbB3/Her3, and ErbB4, are frequently observed in human cancer and contribute to aggressive tumor behavior and poor patient prognosis. Members of this receptor family both homodimerize and heterodimerize in response to ligand binding, generating an elaborate signaling network that drives key cellular events, including proliferation, motility, and survival. Her2 enjoys a unique position in this hierarchy as the preferred dimerization partner. Notably, Her2 intensifies and prolongs the signaling of heterodimers into which it is incorporated through augmented ligand affinity and enhanced receptor recycling (1).

Her2 is amplified and/or overexpressed in 20% to 30% of invasive human breast cancers and is associated with reduced disease-free and overall survival. The development of trastuzumab (Herceptin), a humanized monoclonal antibody directed against the extracellular juxtamembrane domain of Her2, has transformed the management of breast cancer. It represents the first Her2-targeted therapy and was approved by the Food and Drug Administration in 1996 for the treatment of metastatic breast cancer (2). Subsequent

studies combining trastuzumab with taxanes showed a survival benefit compared with antibody monotherapy for patients with metastatic disease (2, 3). Trastuzumab has recently been evaluated in patients with Her2 (+) early breast cancer and has been found to decrease the risk of relapse by 50% and prolong survival when added to chemotherapy (4). Despite these successes, resistance to trastuzumab therapy, both primary and acquired, remains a significant clinical problem. Primary resistance to single-agent trastuzumab ranges from 66% to 88% and from 20% to 50% for combination therapy. Moreover, most patients that experience a response will show evidence of disease progression within 1 year (2–4).

Several mechanisms of action have been put forth for trastuzumab and, while no one mechanism is universally accepted, several consistent observations have been made. Initial studies pointed toward an antibody-induced down-regulation of Her2; subsequent studies have confirmed that trastuzumab impairs downstream signaling but the effect on Her2 stability is unclear (2). In any event, diminished downstream signaling through Her2 leads to the stabilization of p27^{kip1}, a Cdk2 inhibitor, leading to an antibody-induced G₁ cell cycle arrest. Trastuzumab has also been reported to block shedding of the Her2 extracellular domain, which leaves behind a constitutively active membrane fragment termed p95, as well as inhibit tumor angiogenesis through the suppression of angiogenic factor expression (2, 4). Antibody-dependent cellular cytotoxicity seems to play a significant role in the efficacy of trastuzumab in preclinical models but its role in immune compromised patients is unclear (2).

A number of studies have led to the identification of factors associated with inherent or treatment-acquired trastuzumab resistance. PTEN depletion *in vitro* and *in vivo* leads to trastuzumab resistance, and patients whose tumors display activation of the phosphoinositide 3-kinase (PI3K) pathway have a lower response rate and an increased risk of progression while on trastuzumab-containing combination therapy (5, 6). Importantly, the addition of PI3K inhibitors restores trastuzumab sensitivity to PTEN-depleted breast cancer cells (7). Activation of other ErbB family members, such as EGFR, through receptor or ligand overexpression counteracts the growth inhibitory properties of trastuzumab, and inclusion of an EGFR inhibitor restores growth suppression (8–10). Cross-talk between Her2 and the unrelated RTK, the insulin-like growth factor-I (IGF-I) receptor, rescues cells from trastuzumab-mediated growth inhibition. Inhibition of IGF-I receptor with an antireceptor antibody or an IGF-IR-specific tyrosine kinase inhibitor sensitizes resistant cells to trastuzumab (11–14). The common theme that emerges from these studies is the reversal of resistance by a multitargeting approach, suggesting that patients could benefit if such a strategy were used. These studies also suggest that it will be important to categorize tumors based not only on Her2 status but also on the status of “resistance factors.” This will require a highly individualized analysis but should ultimately improve patient response.

Requests for reprints: Colleen Sweeney, University of California Davis Cancer Center, Research Building III, Room 1400C, 4645 2nd Avenue, Sacramento, CA 95817. Phone: 916-734-0726; Fax: 916-734-0190; E-mail: casweeney@ucdavis.edu.

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The Met RTK and its ligand, hepatocyte growth factor (HGF), are frequently overexpressed in breast cancer and correlate with decreased relapse-free and overall survival (15–20). Indeed, Met receptor overexpression is an independent predictor of poor prognosis in breast cancer (21–23). A recent immunohistochemical study of Her2 (+) breast tumors found that a subset (5 of 20) were strongly positive for Met receptor expression (24). This is of particular importance as Met and Her2 have been shown to synergize in promoting cellular invasion, suggesting that tumors that express both receptors may be more aggressive (25). From a therapeutic standpoint, the expression of other tyrosine kinases in Her2 (+) tumors may provide tumor cells with an “escape hatch.” In the face of Her2 inhibition, such tumors may shift their growth dependence from Her2 to other available tyrosine kinases or may be less dependent on Her2 to begin with, vastly influencing the response to Her2-targeted therapy. For example, colorectal cancer cells develop resistance to the anti-EGFR therapeutic antibody Cetuximab by using Src-mediated signaling to bypass their dependency on EGFR for growth (26).

In this study, we consider whether the Met receptor contributes to trastuzumab resistance of Her2 (+) breast tumor cells. We show that attenuation of Met activity leads to sensitization to trastuzumab treatment, whereas Met activation protects cells from the growth inhibitory effects of trastuzumab by preventing trastuzumab-induced p27 induction. In addition, we show that Met is coexpressed along with Her2 in Her2-overexpressing breast cancer cells, as well as Her2 (+) clinical breast cancer samples, emphasizing the potential translational relevance of our findings.

Materials and Methods

Reagents and cell culture. Human cell lines MDA-MB-361, BT474, and SKBR3 were purchased from American Type Culture Collection and cultured in recommended media in the presence of 10% fetal bovine serum (FBS; Invitrogen) and penicillin-streptomycin antibiotics (Mediatech). Serum-starved media contained just 0.1% FBS. HGF was purchased from RDI/Fitzgerald, and neuregulin-1 β was produced as described (27). Antibodies used here include anti-Met C12 and anti-p27 (Santa Cruz Biotechnology); anti-phosphorylated p44/42 MapK [phosphorylated extracellular signal-regulated kinase (ERK)], anti-phosphorylated AKT S473, and anti-phosphorylated Met Y1349 (Cell Signaling Technology, Danvers, MA); anti-actin AC15 (Sigma); and anti-Her2 Ab3 (EMD Biosciences). Trastuzumab was purchased from Genentech.

Human breast tissue analysis and Western blot. Frozen human breast tissues with pathology reports were obtained from the National Cancer Institute Cooperative Human Tissue Network.¹ All samples were deidentified, and the study was approved by the institutional review board of the School of Medicine, University of California at Davis. Her2, ER, and PR status was obtained from the accompanying pathology reports. Samples were homogenized on ice in 10 μ L of T-PER (Pierce)/mg of tissue in the presence of 4 μ g/mL leupeptin, 4 μ g/mL pepstatin, 4 μ g/mL aprotinin, and 100 nmol/L ABESF and then centrifuged to remove insoluble products. Protein expression was analyzed by SDS-PAGE and Western blot analysis. Blots were incubated first with primary antibodies and next with horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratories), followed by developing with SuperSignal West chemicals (Pierce). An Alpha Innotech imaging station with FluorChem software was used to capture images.

Analysis of combined Her2 and Met activation. SKBR3 cells were plated at 2.5×10^4 cells into 24-well plates and allowed to settle overnight

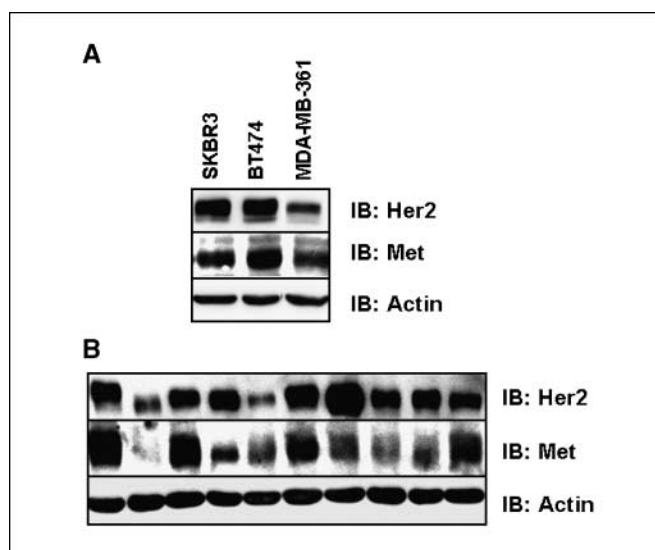


Figure 1. Met is expressed in Her2-overexpressing cell lines and Her2 (+) breast tumors. *A*, whole-cell lysates from the Her2-overexpressing cell lines SKBR3, BT474, and MDA-MB-361 were analyzed for Met, Her2, and actin content by SDS-PAGE followed by Western blot. *B*, tissue lysates from 10 Her2 (+) human tumors were analyzed for Met, Her2, and actin expression by SDS-PAGE followed by Western blot.

and then serum-starved for 24 h. They were then treated with 1.5 nmol/L HGF, 5 nmol/L neuregulin or 1.5 nmol/L HGF, and 5 nmol/L neuregulin for 48 h. An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted as described (28) to measure cellular proliferation. Additionally, cells were plated at 2.5×10^5 cells per well in 12-well plates to measure downstream signaling. After settling, cells were treated with serum-starved media overnight. Wells were then treated in the presence or absence of 5 nmol/L neuregulin or 1.5 nmol/L HGF in serum-starved media for the indicated times. Whole-cell lysates were collected and analyzed via Western blot.

Data analysis for microarray expression. Publicly available microarray data² from U133 Plus 2.0 Affymetrix Gene Array analysis of Her2 (+) tumors was queried, and normalization of data was conducted using dCHIP software (29, 30). Within this data set, 3 of 22 tumors were identified as nonresponsive (lack of response or relapse within 6 months).

Growth inhibition analysis. SKBR3 and BT474 cells were plated at 2.5×10^4 cells per well in 24-well plates and allowed to settle for 24 h. For Met knockdown experiments, cells were then treated with 100 nmol/L of On-Target Plus siControl nontargeting pool small interfering RNA (siRNA) or On-Target Plus Met targeting pool siRNA per manufacturer's directions (Dharmacon). After 48 h, cells were serum-starved in the presence and absence of 21 μ g/mL trastuzumab and allowed to incubate with the drug for 24 h. An MTT assay was then conducted to measure cell proliferation. For Met inhibition experiments, 24 h after plating, cells were treated with 1 μ mol/L (3Z)-N-(3-chlorophenyl)-3-((3,5-dimethyl-4-[(4-methylpiperazin-1-yl)carbonyl]-1H-pyrrol-2-yl)methylene)-N-methyl-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide (SU11274; EMD Biosciences) in the presence or absence of 21 μ g/mL trastuzumab for 48 h before cell proliferation was measured via MTT. Results are the average of four replicates performed in three independent experiments with representative experiments shown. Met knockdown and inhibition was confirmed by Western blot analysis of whole-cell lysates.

Isobologram analysis. Isobologram analysis was conducted as described by Menendez et al. (31). To examine the interaction between

¹ <http://chtn.nci.nih.gov/>

² <http://caarraydb.nci.nih.gov/caarray/>

trastuzumab and SU11274, cells were exposed to decreasing concentrations of SU11274 and a range of trastuzumab concentrations to empirically determine the complimentary concentration of trastuzumab necessary to achieve 20% inhibition. Each point on the graph represents the average of duplicates. Percentage inhibition was determined using the MTT analysis 48 h after treatment with drugs.

Real-time PCR. Total RNA was harvested from SKBR3 cells using Trizol followed by the Micro-to-Midi Total RNA Purification System (Invitrogen). High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to convert 5 μ g RNA into cDNA. Real-time analysis was conducted using Applied Biosystems TaqMan Gene expression primers and probes which were labeled with FAM and EuroGentec Two-Step qRT-PCR Master Mix on a Bio-Rad iCycler iQ Real-Time PCR instrument.

Results

Met and Her2 are coexpressed in Her2 (+) cell lines and tumors. A recent immunohistochemical study reported the robust coexpression of Met in a subset of Her2 (+) breast tumors [5 of 20 Her2 (+) tumors; ref. 24]. To investigate this further, we examined the expression of Met in a panel of Her2 (+) breast cancer cell lines and primary breast tumors by Western blot analysis. As shown in Fig. 1A, analysis of three Her2-overexpressing breast cancer cell lines (SKBR3, BT474, and MDA-MB-361) shows that Met is expressed to varying degrees in all three lines. We next examined the expression of Met in Her2 (+) primary breast tumors. As shown in Fig. 1B, Met is expressed to varying degrees in the majority of this collection of Her2 (+) breast tumors. Three tumors showed low to moderate Met expression, and four tumors showed high to very high levels of expression. These results are in agreement with the immunohistochemical study and indicate that Met and Her2 are frequently coexpressed in breast cancer, providing an opportunity for cross-talk between the two receptors.

Combined activation of Her2 and Met provides breast cancer cells with a growth advantage. Her2-overexpressing breast cancer cells generally display constitutive Her2 tyrosine phosphorylation, signaling pathway activation, and a high rate of basal cellular growth. Despite this, growth factor stimulation can further promote tumor cell proliferation (9) and RTKs are frequently found overexpressed along with their cognate ligands. Indeed, growth factor availability regulates several key aspects of tumor cell behavior, including therapeutic response (8). To determine whether the activation of Met enhances the growth of Her2-overexpressing breast cancer cells, SKBR3 cells were treated with HGF, a ligand for the Met receptor, or neuregulin-1 β , a ligand for Her3, the dimeric partner of Her2. As shown in Fig. 2A, treatment of SKBR3 cells with either HGF or neuregulin-1 β resulted in a modest but reproducible growth response, whereas treatment with both growth factors led to an additive increase in cellular proliferation. This was paralleled in the activation of downstream signaling pathways as shown in Fig. 2B. HGF stimulation led to a rapid but transient phosphorylation of AKT and p42 ERK, whereas neuregulin-1 β resulted in a delayed but sustained phosphorylation of these signaling molecules. The combination of the two growth factors yielded the most robust phosphorylation, which was both rapid and sustained. These results indicate that Met activation enhances the growth of Her2-overexpressing cells and that the combined stimulation of Her2 and Met further promotes growth.

A recent study from Harris et al. (32) examined the response of patients with Her2 (+) early breast cancer (stage II/stage III) to preoperative trastuzumab and vinorelbine therapy. Gene expres-

sion analysis of resistant tumors, defined as those that did not respond or relapsed within 6 months of therapy, identified several genes that differed in expression from the remaining tumors. Of 22 Her2 (+) tumors, three were found to be resistant. Analysis of the publicly available dataset² shows that HGF and Met expression is significantly increased in the resistant tumors. The average increase in expression in the resistant tumors compared with responsive tumors is ~1.5-fold for HGF and 2.0 fold for Met receptor (Fig. 2C). These data suggest that Met receptor signaling may play a role in Trastuzumab resistance. However, because these patients were treated with combination therapy, more defined experiments are necessary to implicate Met in trastuzumab resistance specifically, as described below.

Met depletion or inhibition sensitizes Her2-overexpressing breast cancer cells to Trastuzumab. Her2-overexpressing breast cancer cells are growth inhibited *in vitro* by trastuzumab

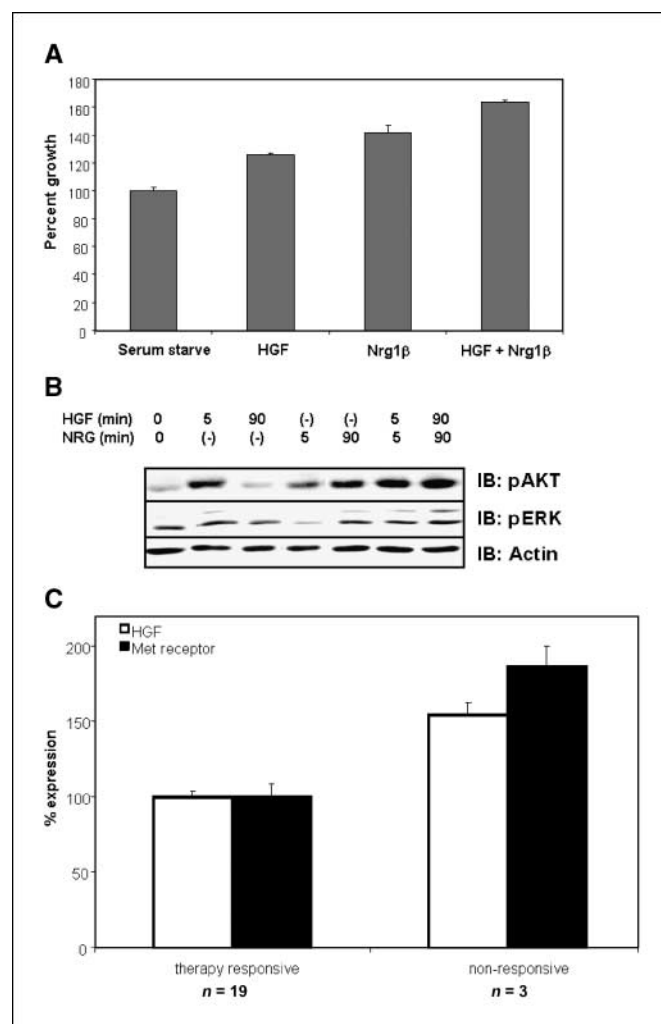


Figure 2. Combined activation of Met and Her2 enhances SKBR3 cell proliferation. A, SKBR3 cells were cultured for 48 h in serum-starved media or treated with HGF, neuregulin-1 β , or both HGF and neuregulin. An MTT assay was performed to measure proliferation. Columns, average of replicates of six; bars, SE. B, SKBR3 cells were treated with HGF, neuregulin, or both. Time points were collected as indicated, and whole-cell lysates were analyzed for phosphorylated Akt, phosphorylated Erk, and actin content by SDS-PAGE followed by Western blot. C, relative HGF (white columns) and Met expression (black columns) in 22 Her2 (+) tumors that were deemed "responsive" and "nonresponsive" to a combination of trastuzumab and vinorelbine therapy. Columns, average transcript levels; bars, SD.

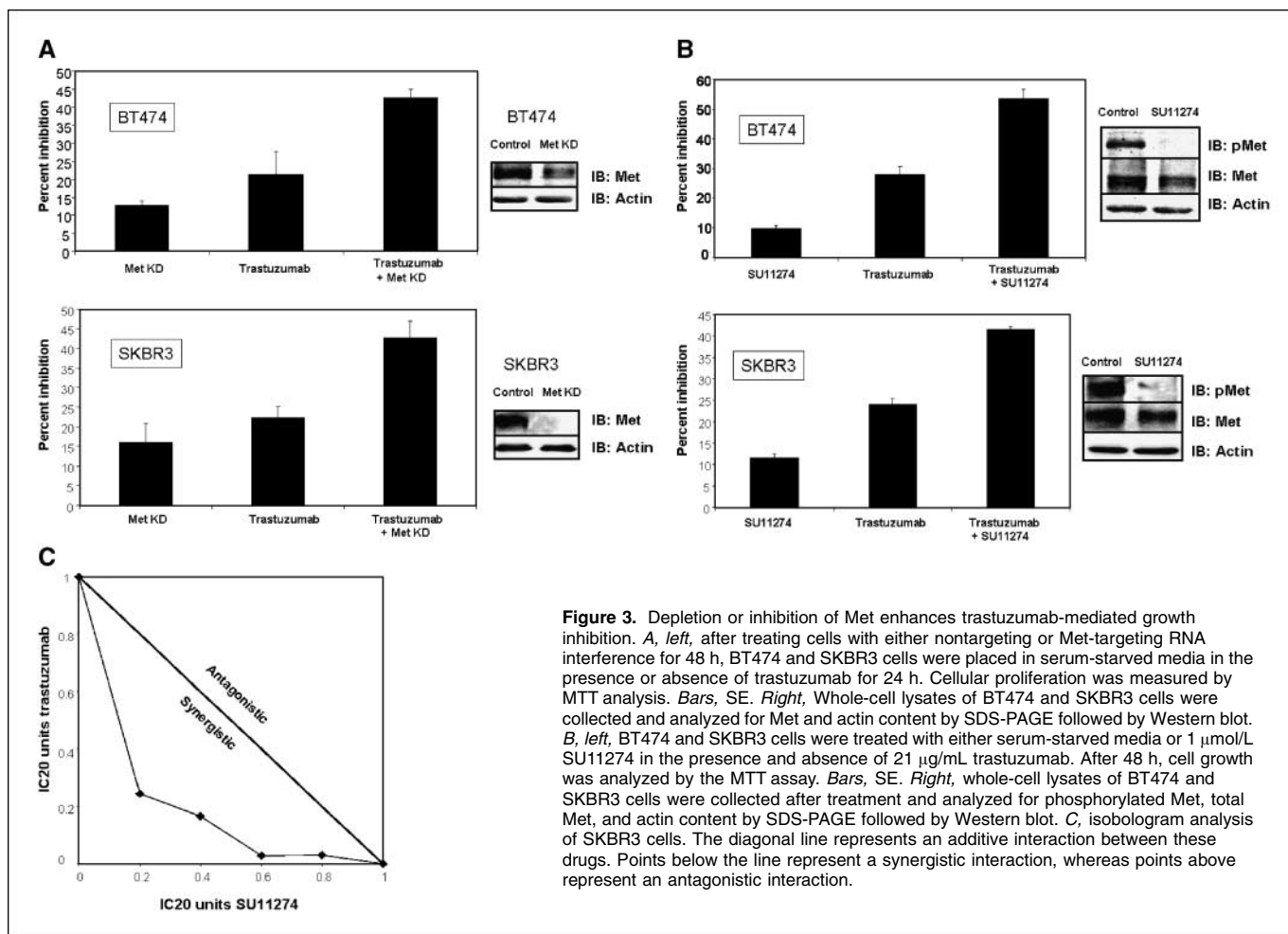


Figure 3. Depletion or inhibition of Met enhances trastuzumab-mediated growth inhibition. *A, left*, after treating cells with either nontargeting or Met-targeting RNA interference for 48 h, BT474 and SKBR3 cells were placed in serum-starved media in the presence or absence of trastuzumab for 24 h. Cellular proliferation was measured by MTT analysis. *Bars*, SE. *Right*, Whole-cell lysates of BT474 and SKBR3 cells were collected and analyzed for Met and actin content by SDS-PAGE followed by Western blot. *B, left*, BT474 and SKBR3 cells were treated with either serum-starved media or 1 μ M SU11274 in the presence and absence of 21 μ g/mL trastuzumab. After 48 h, cell growth was analyzed by the MTT assay. *Bars*, SE. *Right*, whole-cell lysates of BT474 and SKBR3 cells were collected after treatment and analyzed for phosphorylated Met, total Met, and actin content by SDS-PAGE followed by Western blot. *C*, isobologram analysis of SKBR3 cells. The diagonal line represents an additive interaction between these drugs. Points below the line represent a synergistic interaction, whereas points above represent an antagonistic interaction.

treatment; however, the extent of inhibition is modest and typically ranges from 20% to 40% (33). This suggests that these cells are either not completely dependent on Her2 for their growth or that they are capable of rapid adaptation, allowing them to bypass some of the effects of trastuzumab treatment. Met and Her2 activate many of the same downstream signaling pathways that may allow for growth and survival signaling to persist in the face of Her2 inhibition, decreasing the efficacy of targeted agents, such as trastuzumab.

To determine whether the expression of Met might lessen the response of Her2 (+) breast cancer cells to trastuzumab, Met was depleted by siRNA, and the proliferative response of BT474 (*top*) and SKBR3 (*bottom*) cells to trastuzumab was examined. As shown in Fig. 3A, treatment of cells with trastuzumab and control RNA interference led to 25% to 30% inhibition of growth, in agreement with previous reports. Treatment of cells with trastuzumab and Met-specific RNA interference significantly increased the response of both cell lines, leading to 40% to 45% inhibition of growth. To further implicate Met in trastuzumab resistance, cells were treated with a small molecule inhibitor of Met, SU11274 (Fig. 3B). SU11274 is an ATP-competitive inhibitor with \sim 10-fold selectivity for Met compared with other tyrosine kinases; however, it should be noted that only a subset of tyrosine kinases have been tested (34, 35). Met was constitutively phosphorylated in both SKBR3 and BT474 cells, and treatment with SU11274 significantly decreased

this phosphorylation (Fig. 3B, *right*). The combination of trastuzumab and SU11274 was more efficient at inhibiting the growth of BT474 and SKBR3 cells than trastuzumab alone (Fig. 3B), and isobologram analysis showed a synergistic interaction between the two agents (Fig. 3C). Collectively, these data show that Met expression and activity can modulate the response of Her2 (+) breast cancer cells to trastuzumab and that inhibition of Met sensitizes cells to trastuzumab therapy.

To examine the mechanism by which Met contributes to trastuzumab resistance, the activation status of downstream signaling pathways was evaluated. BT474 (Fig. 4A) and SKBR3 (Fig. 4B) cells were treated with trastuzumab alone or a combination of trastuzumab and SU11274 and the activation of the PI3K/AKT and Ras/mitogen-activated protein kinase pathways were examined by Western blotting with phosphorylated specific antibodies. As shown in Fig. 4, treatment of cells with trastuzumab diminished downstream signaling, as expected; however, residual phosphorylation of AKT and ERK was readily detected. By comparison, treatment of cells with trastuzumab and SU11274 significantly improved the impairment of downstream signaling. These results indicate that growth and survival signaling persist in the presence of trastuzumab in large part due to Met activity. Indeed, Met inhibition alone significantly inhibited AKT and ERK phosphorylation, demonstrating that Met contributes to constitutive activation of signaling pathways in Her2-overexpressing breast

cancer cells. However, it should be noted that the activity of SU11274 toward other kinases may contribute to the decline in AKT and ERK phosphorylation. A significant decrease in phosphorylated Akt and phosphorylated Erk was also observed with Met knockdown (data not shown), supporting the interpretation that Met plays an important role in AKT and ERK phosphorylation in these cells. Met inhibition may improve trastuzumab efficacy in Her2 (+) breast tumors through enhanced inhibition of key downstream signaling pathways. This finding has important clinical implications, as a significant percentage of Her2 (+) breast cancers express Met (Fig. 1B).

Ligand-mediated activation of Met protects cells from trastuzumab. Because Met depletion or inhibition improves the response to trastuzumab, we next examined whether Met activation by HGF protects cells from trastuzumab. HGF is normally expressed in the mesenchyme and acts in a paracrine manner to stimulate epithelial cells expressing Met. In breast cancer, HGF is frequently overexpressed and is an independent predictor of recurrence and poor survival (19). To examine the effect of HGF on the response to trastuzumab, SKBR3 cells were treated with trastuzumab without and with HGF. Growth inhibition relative to untreated cells was quantified as shown in Fig. 5A. HGF abrogated the growth inhibitory response to trastuzumab. To examine the mechanism by which HGF protects cells from trastuzumab, expression of the cyclin-dependent kinase inhibitor p27 was examined by Western blotting. Trastuzumab-mediated inhibition of AKT signaling leads to p27 stabilization, and this accumulation is crucial for antibody-induced G₁ arrest and growth inhibition (36). As shown in Fig. 5B, treatment of BT474 cells with trastuzumab increased p27 expression, as expected. Treatment with trastuzumab in the presence of

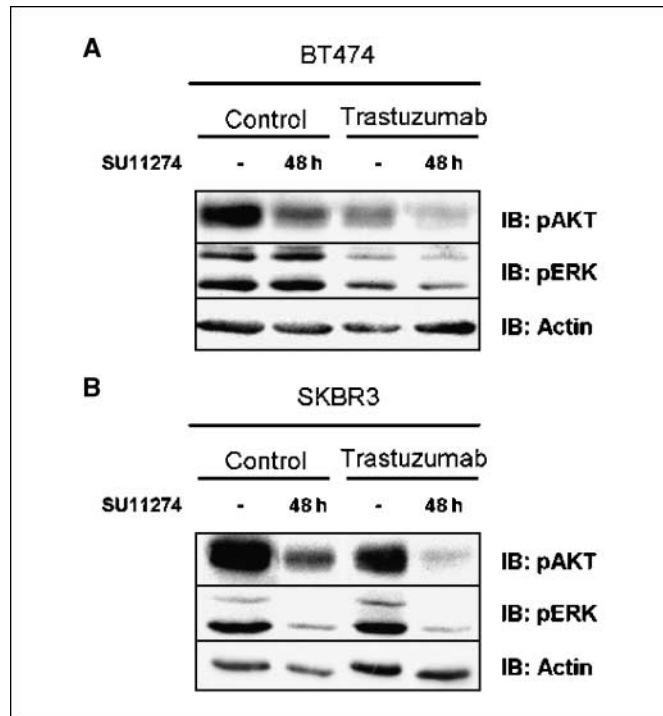


Figure 4. The combination of trastuzumab and SU11274 abrogate Akt phosphorylation. Serum-starved BT474 cells (A) and SKBR3 cells (B) were treated with 1 μ M SU11274 in the presence or absence of 21 μ g/mL trastuzumab. Whole-cell lysates of BT474 and SKBR3 cells were collected after treatment and analyzed for phosphorylated Akt, phosphorylated Erk, and actin content by SDS-PAGE followed by Western blot.

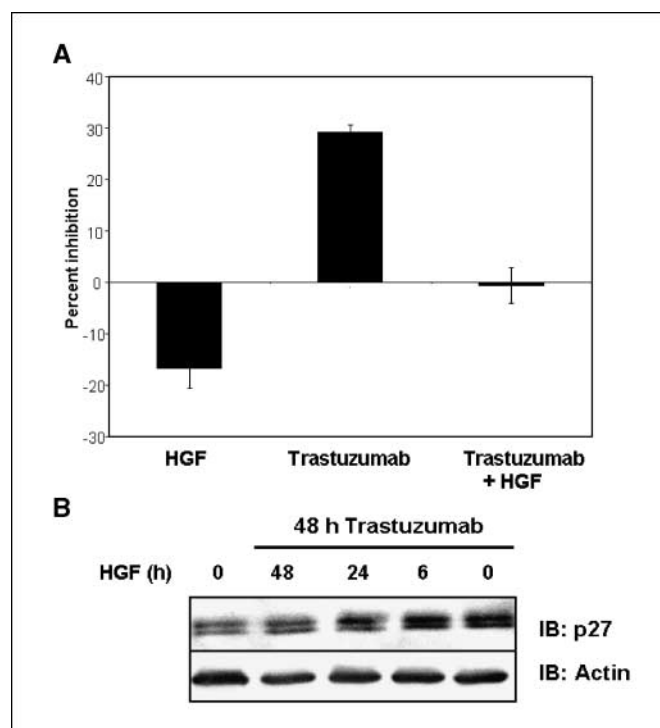


Figure 5. HGF protects cells from trastuzumab-mediated growth inhibition by preventing p27 induction. A, serum-starved SKBR3 cells were treated with trastuzumab, HGF, or the combination of both. Cellular growth was measured with the MTT assay. Bars, SE of four replicates. B, serum-starved BT474 cells were treated with trastuzumab for 48 h, during which HGF was added for the indicated times. Whole-cell lysates were collected after treatment and analyzed for p27 and actin content by SDS-PAGE followed by Western blot.

HGF abrogated p27 induction. Similar results were observed in SKBR3 cells (data not shown). These results indicate that ligand-mediated activation of Met receptor protects cells from trastuzumab-mediated growth inhibition by preventing the induction of the key cell cycle inhibitor p27.

Her2 (+) breast cancer cells up-regulate Met receptor during trastuzumab treatment. Analysis of publicly available microarray data (Fig. 2C) indicates that Met receptor transcript is elevated in tumors resistant to trastuzumab and vinorelbine therapy. A separate study examining mechanisms of acquired resistance to trastuzumab found that resistant cells express elevated levels of EGFR message (10), suggesting that tumor cells recognize their “plight” during trastuzumab therapy and may respond by up-regulating growth and survival pathways. *In vitro* models of acquired resistance are treated continually with trastuzumab for weeks to months at which time resistant clones are isolated and analyzed for changes that contribute to resistance. We reasoned that Her2 (+) breast cancer cells may have inherent protective mechanisms that are deployed rapidly (hours to days) upon initiation of trastuzumab therapy. We therefore examined Met expression in BT474 (Fig. 6A) and SKBR3 (Fig. 6B) cells after treatment with trastuzumab. SKBR3 cells were isolated in 1970 from a pleural effusion,³ whereas BT474 cells were isolated in 1978 from an invasive ductal carcinoma (37). Both cell lines predate the development of trastuzumab and are naive to trastuzumab

³ <http://www.atcc.org>

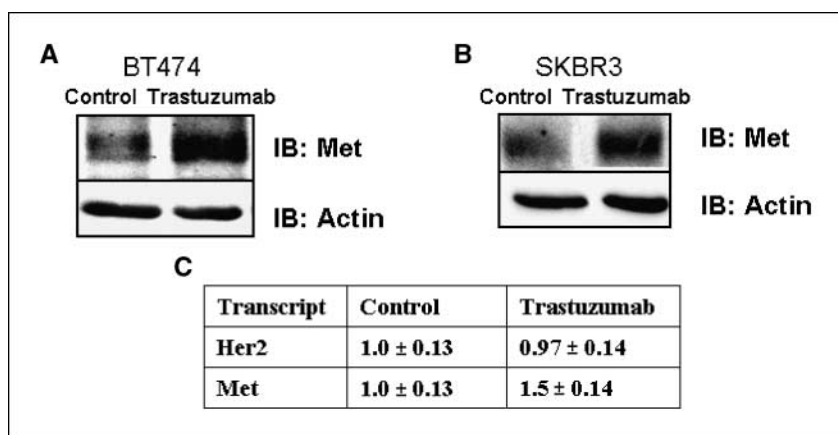


Figure 6. Trastuzumab treatment increases Met expression. BT474 (A) and SKBR3 (B) cells were serum-starved overnight and then treated with trastuzumab for 48 h. Whole-cell lysates were collected and analyzed for Met and actin content by SDS-PAGE followed by Western blot. C, total RNA of SKBR3 cells treated as in B were analyzed by real-time PCR. Results shown are the result of three independent experiments. The reported error represents the SD standardized to actin.

treatment. Remarkably, within 48 h of trastuzumab treatment, there was a significant increase in Met protein expression in both cell lines. This increase in protein expression was accompanied by a 1.5-fold increase in Met transcript levels as revealed by real-time PCR analysis of SKBR3 cells (Fig. 6C). These results indicate that Her2 (+) breast cancer cells respond to trastuzumab treatment with a rapid up-regulation of Met receptor expression, which in turn promotes trastuzumab resistance through the sustained activation of downstream signaling pathways.

Discussion

The development of targeted therapies, such as tamoxifen and trastuzumab, has had a major effect on the clinical management of breast cancer, improving disease-free and overall patient survival, whereas lessening deleterious side effects. The premise of targeted therapy, the absolute dependence of a tumor on a single oncogene or "oncogene addiction" is supported by both preclinical and clinical evidence (38). The best example of this is given by the success of Gleevec, which targets the Bcr-Abl oncogene in chronic myelogenous leukemia (39). However, the vulnerability of targeted therapy lies in its very basis: the exquisite specificity of targeted agents leaves open multiple avenues by which tumors may evade therapy enabling therapeutic resistance. Indeed, tumors clearly escape oncogene addiction as the success with Gleevec is the exception rather than the rule.

Resistance to trastuzumab, both inherent and acquired, presents a significant barrier to the effective treatment of Her2 (+) breast cancer and has been the focus of numerous studies (3–14). The theme that has emerged underscores the importance of key downstream signaling pathways, particularly the PI3K/AKT pathway, in promoting resistance (6, 7). Coexpression of RTKs, such as EGFR (8–10) or IGF-IR (11–14), or loss of the negative regulator PTEN (5–7) allows PI3K/AKT signaling to be sustained despite Her2 inhibition. In other words, redundancy in mechanisms of AKT activation is one way by which trastuzumab resistance may be achieved. Presumably, coexpression of any number of RTKs could support this resistance; however, such studies must be grounded in the analysis of clinical samples to determine which kinases are coexpressed with Her2 *in vivo*. Interestingly, Met may also be a player in IGF-IR-mediated trastuzumab resistance, as Met is required for some IGF-IR-driven cellular responses (40, 41).

In this manuscript, we identify Met as an RTK that is frequently coexpressed with Her2 in Her2 (+) breast cancer and contributes to

trastuzumab resistance of Her2-overexpressing breast cancer cells through sustained AKT activation. Loss of Met function, either through RNA interference-mediated depletion or small molecule-mediated inhibition, significantly improves the response to trastuzumab; conversely, Met activation protects cells against trastuzumab. Significantly, Her2-overexpressing breast cancer cells respond to trastuzumab treatment with a rapid up-regulation of Met receptor expression, in essence promoting their own resistance. Met has also been found to be involved in acquired resistance to the EGFR-targeted agent gefitinib and is amplified in gefitinib-resistant lung cancer cell lines and tumors (42). The mechanism of resistance lies in the ability of Met to drive EGFR-independent/ErbB3-dependent PI3K/AKT activation. Met amplification does not seem to be necessary for its role in trastuzumab resistance, however, as Met is *not* amplified in any of the cell models used in this paper.

A hallmark of the evolving nature of targeted therapy lies in the number of agents that are in phase I or phase II clinical trials, which inhibit multiple tyrosine kinases.⁴ With respect to the Met receptor, there are a number of therapeutics in clinical development, including tyrosine kinase inhibitors, monoclonal antibodies, and molecular decoys. For example, XL880 (Exelixis), an orally available small molecule compound currently in phase II development, targets Met along with the vascular endothelial growth factor receptor, platelet-derived growth factor receptor, c-Kit, Flt3, and Tie2 RTKs. Our studies suggest that some Her2 (+) patients may benefit from the addition of a Met inhibitor to trastuzumab therapy. However, this must be approached with caution, as the combination of the EGFR-targeted agent gefitinib with trastuzumab was found to be unexpectedly antagonistic in the clinic (43). Future studies will seek to evaluate whether Met receptor coexpression predicts a worsened response to trastuzumab.

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⁴ <http://www.multi-targetedtherapy.com/targetedTherapies.asp>

References

1. Hynes NE. Tyrosine kinase signaling in breast cancer. *Breast Cancer Res* 2000;2:154-7.
2. Nahta R, Esteva FJ. Trastuzumab: triumphs and tribulations. *Oncogene* 2007;26:3637-43.
3. Hudis CA. Trastuzumab-mechanism of action and use in clinical practice. *N Engl J Med* 2007;357:39-51.
4. Valabrega G, Montemurro F, Aglietta M. Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Ann Oncol* 2007;18:977-84.
5. 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.
6. 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.
7. Lu CH, Wyzomierski SL, Tseng LM, et al. Preclinical testing of clinically applicable strategies for overcoming trastuzumab resistance caused by PTEN deficiency. *Clin Cancer Res* 2007;13:5883-8.
8. Motoyama AB, Hynes NE, Lane HA. The efficacy of ErbB receptor-targeted anticancer therapeutics is influenced by the availability of epidermal growth factor-related peptides. *Cancer Res* 2002;62:3151-8.
9. Diermeier S, Horváth G, Knuechel-Clarke R, et al. Epidermal growth factor receptor coexpression modulates susceptibility to Herceptin in HER2/neu overexpressing breast cancer cells via specific erbB-receptor interaction and activation. *Exp Cell Res* 2005;304:604-19.
10. 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.
11. Lu Y, Zi X, Zhao Y, et al. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst* 2001;93:1852-7.
12. Camirand A, Lu Y, Pollak M. Co-targeting HER2/ErbB2 and insulin-like growth factor-I receptors causes synergistic inhibition of growth in HER2-overexpressing breast cancer cells. *Med Sci Monit* 2002;8:BR521-6.
13. Nahta R, Yuan LX, Zhang B, et al. 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.
14. Lu Y, Zi X, Pollak M. Molecular mechanisms underlying IGF-I-induced attenuation of the growth-inhibitory activity of trastuzumab (Herceptin) on SKBR3 breast cancer cells. *Int J Cancer* 2004;108:334-41.
15. Edakuni G, Sasatomi E, Satoh T, et al. Expression of the hepatocyte growth factor/c-Met pathway is increased at the cancer front in breast carcinoma. *Pathol Int* 2001;51:172-8.
16. Jin L, Fuchs A, Schnitt SJ, et al. Expression of scatter factor and c-met receptor in benign and malignant breast tissue. *Cancer* 1997;79:749-60.
17. Kang JY, Dolled-Filhart M, Ocal IT, et al. Tissue microarray analysis of hepatocyte growth factor/Met pathway components reveals a role for Met, matriptase, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer. *Cancer Res* 2003;63:1101-5.
18. Nagy J, Curry GW, Hillan KJ, et al. Hepatocyte growth factor/scatter factor expression and c-met in primary breast cancer. *Surg Oncol* 1996;5:15-21.
19. Yamashita J, Ogawa M, Yamashita S, et al. Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. *Cancer Res* 1994;54:1630-3.
20. Yao Y, Jin L, Fuchs A, et al. Scatter factor protein levels in human breast cancers: clinicopathological and biological correlations. *Am J Pathol* 1996;149:1707-17.
21. Camp RL, Rimm EB, Rimm DL. Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. *Cancer* 1999;86:2259-65.
22. Ghoussoub RA, Dillon DA, D'Aquila T, et al. Expression of c-met is a strong independent prognostic factor in breast carcinoma. *Cancer* 1998;82:1513-20.
23. Lee WY, Chen HH, Chow NH, et al. Prognostic significance of co-expression of RON and MET receptors in node-negative breast cancer patients. *Clin Cancer Res* 2005;11:2222-8.
24. Lindemann K, Resau J, Nährig J, et al. Differential expression of c-Met, its ligand HGF/SF and HER2/neu in DCIS and adjacent normal breast tissue. *Histopathology* 2007;51:54-62.
25. Khoury H, Naujokas MA, Zuo D, et al. HGF converts ErbB2/Neu epithelial morphogenesis to cell invasion. *Mol Biol Cell* 2005;16:550-61.
26. Lu Y, Li X, Liang K, et al. Epidermal growth factor receptor (EGFR) ubiquitination as a mechanism of acquired resistance escaping treatment by the anti-EGFR monoclonal antibody Cetuximab. *Cancer Res* 2007;67:8240-7.
27. Funes M, Miller JK, Lai C, et al. The mucin Muc4 potentiates neuregulin signaling by increasing the cell-surface populations of ErbB2 and ErbB3. *J Biol Chem* 2006;281:19310-9.
28. Shattuck DL, Miller JK, Laederich M, et al. LRIG1 is a novel negative regulator of the Met receptor and opposes Met and Her2 synergy. *Mol Cell Biol* 2007;27:1934-46.
29. Harris L, Luftner D, Jager W, et al. c-erbB-2 in serum of patients with breast cancer. *Int J Biol Markers* 1999;14:8-15.
30. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A* 2001;98:31-6.
31. Menendez JA, Vazquez-Martin A, Colomer R, et al. Olive oil's bitter principle reverses acquired autoresistance to trastuzumab (Herceptin) in HER2-overexpressing breast cancer cells. *BMC Cancer* 2007;7:80.
32. Harris LN, You F, Schnitt SJ, et al. Predictors of resistance to preoperative trastuzumab and vinorelbine for HER2-positive early breast cancer. *Clin Cancer Res* 2007;13:1198-207.
33. Jerome L, Alami N, Belanger S, et al. Recombinant human insulin-like growth factor binding protein 3 inhibits growth of human epidermal growth factor receptor-2-overexpressing breast tumors and potentiates herceptin activity *in vivo*. *Cancer Res* 2006;66:7245-52.
34. Wang X, Le P, Liang C, et al. Potent and selective inhibitors of the Met [hepatocyte growth factor/scatter factor (HGF/SF) receptor] tyrosine kinase block HGF/SF-induced tumor cell growth and invasion. *Mol Cancer Ther* 2003;2:1085-92.
35. Sattler M, Pride YB, Ma P, et al. A novel small molecule met inhibitor induces apoptosis in cells transformed by the oncogenic TPR-MET tyrosine kinase. *Cancer Res* 2003;63:5462-9.
36. Nahta R, Takahashi T, Ueno NT, et al. p27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. *Cancer Res* 2004;64:3981-6.
37. Lasfargues EY, Coutinho WG, Redfield ES. Isolation of two human tumor epithelial cell lines from solid breast carcinomas. *J Natl Cancer Inst* 1978;61:967-78.
38. Weinstein IB, Joe AK. Mechanisms of disease: oncogene addiction-a rationale for molecular targeting in cancer therapy. *Nat Clin Pract Oncol* 2006;3:448-57.
39. Schiffer CA. BCR-ABL tyrosine kinase inhibitors for chronic myelogenous leukemia. *N Engl J Med* 2007;357:258-65.
40. Bauer TW, Fan F, Liu W, et al. Insulin like growth factor-I-mediated migration and invasion of human colon carcinoma cells requires activation of c-Met and urokinase plasminogen activator receptor. *Ann Surg* 2005;241:748-56.
41. Bauer TW, Somcio RJ, Fan F, et al. Regulatory role of c-Met in insulin-like growth factor-I receptor-mediated migration and invasion of human pancreatic carcinoma cells. *Mol Cancer Ther* 2006;5:1676-82.
42. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039-43.
43. Normanno N, Campiglio M, Perrone F, et al. Is the gefitinib plus trastuzumab combination feasible in breast cancer patients? *Ann Oncol* 2005;16:1709.