

Trastuzumab-Induced HER Reprogramming in “Resistant” Breast Carcinoma Cells

Murli Narayan,¹ Jason A. Wilken,² Lyndsay N. Harris,^{1,3} Andre T. Baron,^{5,6} Kimberly D. Kimbler,⁵ and Nita J. Maihle^{2,4}

¹Division of Medical Oncology, Yale Cancer Center, Departments of ²Obstetrics, Gynecology, and Reproductive Sciences, ³Internal Medicine and School of Nursing, and ⁴Pathology and Pharmacology, Yale University, New Haven, Connecticut; and ⁵Department of Internal Medicine, Division of Hematology, Oncology, and Blood & Bone Marrow Transplantation, and ⁶Department of Epidemiology, University of Kentucky, Lexington, Kentucky

Abstract

Although trastuzumab (Herceptin) is an important advance in the treatment of breast cancer, a significant proportion of patients do not respond to trastuzumab either alone or in combination with chemotherapy. In this study, we observe that epidermal growth factor receptor (EGFR) and HER3 expression is substantially increased after long-term trastuzumab exposure of HER2-positive breast carcinoma-derived cell lines that show primary resistance to trastuzumab. Furthermore, long-term trastuzumab exposure of trastuzumab-resistant cell lines induces *de novo* sensitivity to the EGFR-targeted agents gefitinib or cetuximab in two of three cell lines accompanied by increased EGFR expression. Together, these results indicate that primary trastuzumab resistance is not synonymous with lack of responsiveness to trastuzumab and, importantly, suggest that trastuzumab priming may sensitize trastuzumab-resistant tumors to other HER family-directed therapeutics. [Cancer Res 2009;69(6):2191–4]

Introduction

Biologically targeted cancer therapeutics coupled with *in vitro* theragnostics have the potential to revolutionize the treatment of cancer patients. Personalized therapy for each cancer patient is now within our reach through genomic, transcriptomic, and proteomic profiling of the patient's tumor and normal tissues, yielding theragnostic information that can be used to target the specific phenotype of a cancer cell. The advent of “personalized” cancer therapies, through the introduction of drugs such as imatinib (Gleevec) for the treatment of chronic myelogenous leukemia (CML), and trastuzumab (Herceptin) for the treatment of both early and advanced stage HER2-positive breast cancer has revolutionized the treatment of these diseases. Unfortunately, acquired resistance to these two new classes of biologically targeted therapeutics is frequently seen in the advanced disease setting, and in a small proportion of early stage patients after adjuvant therapy (1, 2). Although the molecular basis of acquired resistance to imatinib has been determined, and new drugs have now been engineered to overcome acquired imatinib resistance (3), our understanding of trastuzumab resistance is still in its infancy.

This is somewhat surprising given that imatinib received Food and Drug Administration (FDA) approval more recently than trastuzumab, and that many more patients afflicted with breast cancer than CML are available to study acquired resistance to these drugs in clinical trials. One potential reason for the delay in the development of our understanding of trastuzumab resistance is the controversy that has surrounded the coupling of diagnostics to the selection of appropriately responsive breast cancer patients for trastuzumab treatment, in contrast to the identification of CML patients responsive to imatinib treatment based on the characteristic Philadelphia chromosome. Only one study has examined the molecular basis of secondary or acquired trastuzumab resistance (4). A second recent study suggests that primary Herceptin sensitivity can be overcome by activation of the hepatocyte growth factor receptor Met (5). In contrast, limited information is available regarding the basis for primary trastuzumab resistance in breast cancer patients.

Understanding primary trastuzumab resistance is particularly important for breast cancer patients because only ~30% of patients selected for treatment (on the basis of positive HER2 expression) respond to trastuzumab monotherapy, and a significant proportion of patients also show primary resistance to trastuzumab plus chemotherapy regimens (6). Our inability to effectively stratify patients for trastuzumab therapy using currently available theragnostic biomarkers such as HER2 highlights the need for better predictors of therapeutic responsiveness. The clinical effect and limitations of HER2 testing is further underscored by a recent report demonstrating that even patients with low HER2 expression can benefit from trastuzumab treatment (7). Continued research to improve the selection of breast cancer patients for trastuzumab therapy is clearly warranted and urgently needed.

Conventional wisdom defines primary (or *de novo*) resistance to a given drug as a lack of some measurable (tumor) response such as cell proliferation, which connotes lack of drug activity. In the studies presented here, we show for the first time that breast cancer cells exhibiting primary resistance to trastuzumab are in fact, not only responsive to trastuzumab activity but are responsive in ways that may establish vulnerability to other classes of existing cancer therapeutics, such as some of the other biologically directed HER family inhibitors. This Kuhnian observation is in contrast to existing assumptions regarding the ability of HER2-positive breast cancer cells to respond to trastuzumab. Accordingly, our results suggest that the definition of responsiveness to trastuzumab based on cell proliferation may be too limited in breast (and perhaps other) cancer cell types. Moreover, primary trastuzumab resistance may actually prime the responsiveness of a tumor cell to other classes of therapeutic inhibitors. CML patients who acquire

Note: M. Narayan and J.A. Wilken contributed equally to this work.

Current address for M. Narayan: Agencourt Biosciences Corporation, 500 Cummings Center, Suite 2450, Beverly, MA, 01915, USA.

Requests for reprints: Nita J. Maihle, 300 George Street, Suite 770, New Haven, CT 06511. Phone: 203-737-1385; Fax: 203-737-2914; E-mail: nita.maihle@yale.edu.

©2009 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-08-1056

imatinib resistance develop tumor phenotypes that can be used to target vulnerable (and tumor specific) growth regulatory pathways. By analogy, here, we show that emergent phenotypic changes in trastuzumab-primed tumor cells also may be exploitable to more effectively target these cells. As such, this study is among the first to provide experimental evidence in support of the recent proposal by Yarden and colleagues (8) that the plasticity of signaling networks can be exploited to increase therapeutic efficacy, and also to expose novel therapeutic targets in cells that are resistant to growth inhibition by trastuzumab.

Materials and Methods

Cell culture. BT-474, SKBR-3, MDA-MB-361, MDA-MB-453, T47D, UACC812, and UACC893 breast carcinoma cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate (Invitrogen). MDA-MB-361, MDA-MB-453, T47D, UACC812, and UACC893 cells were cultured long-term for 12 wk in media without trastuzumab (parental) or supplemented with 100 µg/mL trastuzumab (T100).

Determination of trastuzumab primary resistance. BT-474, SKBR-3, MDA-MB-361, MDA-MB-453, T47D, UACC812, and UACC893 cells were rinsed with PBS, trypsinized, and seeded into 96-well plates in complete media (see above) supplemented with 0 to 75 µg/mL trastuzumab in quintuplicate. Cell proliferation of each trastuzumab-treated cell line was measured after 120 h by a WST-1-based colorimetric assay (Roche Diagnostics) and compared with untreated cells.

Immunoblot analysis of HER expression. Near-confluent (80–90%) dishes of cells cultured with (T100) or without (parental) 100 µg/mL trastuzumab were harvested and lysed with 2.5% SDS, 0.5% NP40, and 0.5% sodium deoxycholate with boiling for 10 min, or per manufacturer's instructions using the Human Phospho-MAPK Array kit (R&D Systems, Inc.). Cell lysate total protein concentration was determined by Bio-Rad DC assay using bovine serum albumin (BSA) as the quantitative standard. Equal total protein quantities of cell lysates were resolved by SDS-PAGE. Gel proteins were transferred to polyvinyl difluoride membrane by semidry immunoblot (Millipore) and blocked with TBS [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 7.4)] with 5% nonfat dry milk for 1 h. Membranes were rinsed 6 times for 5 min each with TBS with 0.1% Tween 20 (TBS-TW20), and incubated with TBS with 1% BSA and primary anti-epidermal growth

factor receptor (EGFR; sc-03; Santa Cruz Biotechnologies; 1:500 dilution) or anti-HER3 (sc-285; Santa Cruz Biotechnologies; 1:250 dilution) overnight at 4°C. Membranes were rinsed 6 times for 10 min each with TBS-TW20 and incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce; 1:4,000 dilution) for 1 h at room temperature. Membranes were rinsed 6 times for 10 min each with TBS-TW20, and chemiluminescence was visualized with a NucleoVISION camera station after incubation with ECL reagent (Pierce).

HER inhibitor response assay. Parental or T100 MDA-MB-361, MDA-MB-453, T47D, UACC812, and UACC893 cells were rinsed with PBS, trypsinized, and seeded at a density of 2.5×10^3 cells per well into 96-well plates in a total volume of 50 µL per well in assay medium without trastuzumab consisting of RPMI 1640 supplemented with penicillin, streptomycin, glutamine, sodium pyruvate, and 10 µg/mL transferrin, and 200 µg/mL BSA. After overnight incubation, 50 µL of culture media with FBS (described above) with 2 µmol/L gefitinib, 400 µg/mL cetuximab, or 20 µg/mL ErbB3 inactivating antibody (H3.105; NeoMarkers) was added to wells in quintuplicate. After 120 h, cell proliferation was measured by a WST-1-based colorimetric assay.

Results and Discussion

The purpose of this study was to examine the long-term effect of trastuzumab exposure on so-called trastuzumab-resistant breast cancer cells to gain insight into the 20–50% of patients who show primary resistance to trastuzumab therapy in the adjuvant setting, and 70% of patients who show primary resistance to trastuzumab monotherapy (6, 9). In agreement with previous reports (10, 11), trastuzumab inhibited proliferation of HER2-positive BT-474 and SKBR-3 breast carcinoma cells (Fig. 1). In contrast, several other HER2-positive breast carcinoma cell lines (MDA-MB-361, MDA-MB-453, T47D, UACC812, and UACC893) were far less sensitive to optimal patient serum trastuzumab concentrations (i.e., ~10 µg/mL). We, therefore, used these trastuzumab-resistant breast cancer cell lines as experimental model systems of primary trastuzumab resistance, to further study the mechanism(s) underlying resistance *in vitro*.

To approximate breast cancer trastuzumab resistance *in vitro*, HER2-positive breast cancer cell lines were cultured for 12 weeks in media containing 100 µg/mL trastuzumab, hereafter designated as T100 cells. Parental cell lines also were cultured without

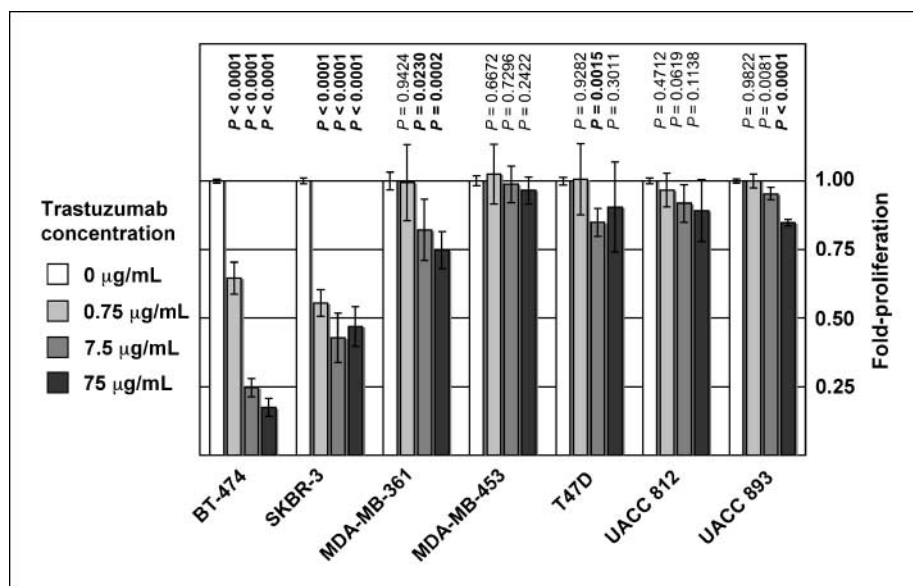


Figure 1. Trastuzumab-mediated growth inhibition in breast carcinoma-derived cell lines. BT-474, SKBR-3, MDA-MB-361, MDA-MB-453, T47D, UACC812, and UACC893 cells were exposed to trastuzumab (0–75 µg/mL) for 120 h, and cell proliferation was measured by a WST-1-based colorimetric assay in quintuplicate ($n = 2$). BT-474 and SKBR-3 cells were significantly growth inhibited by trastuzumab (i.e., primary trastuzumab sensitive), whereas MDA-MB-361, MDA-MB-453, T47D, UACC812, and UACC893 cells were not significantly growth inhibited by trastuzumab (primary trastuzumab resistant).

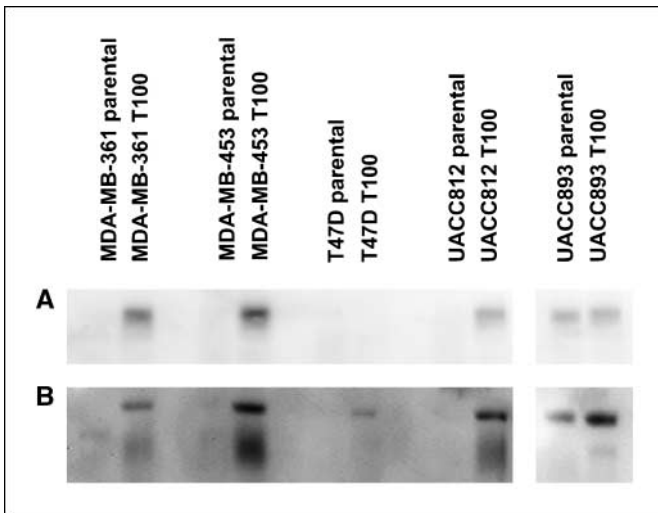


Figure 2. HER expression reprogramming in primary trastuzumab-resistant breast carcinoma-derived cell lines after long-term trastuzumab treatment. Immunoblot of EGFR and HER3 expression in parental versus trastuzumab-treated breast carcinoma cell lines MDA-MB-361, MDA-MB-453, T47D, UACC812, and UACC893 ($n = 1$). Cell lysates, normalized for protein content, were probed after SDS-PAGE with (A) anti-EGFR (sc-03; Santa Cruz Biotechnologies) or (B) anti-HER3 (sc-285; Santa Cruz Biotechnologies) antibody.

trastuzumab. In agreement with others, we observed low but detectable levels of EGFR expression in T47D cells by immunoblot analysis (see Fig. 2); notably, at least two other laboratories have shown functional EGFR signaling in T47D cells (12, 13). Strikingly, EGFR expression was up-regulated in three of five primary trastuzumab-resistant T100 cell lines compared with parental cells, and HER3 expression was up-regulated in five of five primary trastuzumab-resistant T100 cell lines after long-term trastuzumab exposure (see Fig. 2). These results show that primary trastuzumab resistance as defined by no change in cell population number is not equivalent to a lack of trastuzumab activity; rather, continuous trastuzumab exposure induces a measurable reprogramming of HER receptor tyrosine kinase expression in 5 of 5 resistant cell lines.

To evaluate what, if any, potential clinical benefit could be derived by exploiting trastuzumab-induced HER expression reprogramming, trastuzumab-resistant parental and T100 cells were treated with two different classes of FDA-approved EGFR-targeted therapeutics using methods described previously (14). As illustrated in Fig. 3, cell growth patterns were altered in T100 cells compared with parental cell lines after EGFR inhibitor treatment, demonstrating that HER function as well as expression is reprogrammed in trastuzumab-resistant breast cancer cells after long-term trastuzumab exposure. Specifically, *de novo* sensitivity to either gefitinib or cetuximab was observed in two of the three T100 cell populations that showed an increase in EGFR expression; furthermore, one T100 cell population acquired sensitivity to a HER3-targeted antibody (15). These results are consistent with the previous observation that HER expression levels do not always predict (a) a dose-dependent increase in responsiveness to inhibitor (reviewed in ref. 16), (b) HER mutations/overexpression resulting in constitutive receptor activation, and (c) alterations in the expression of HER ligand levels or downstream effectors. All of these conditions may contribute to HER signal transduction in primary resistant cells.

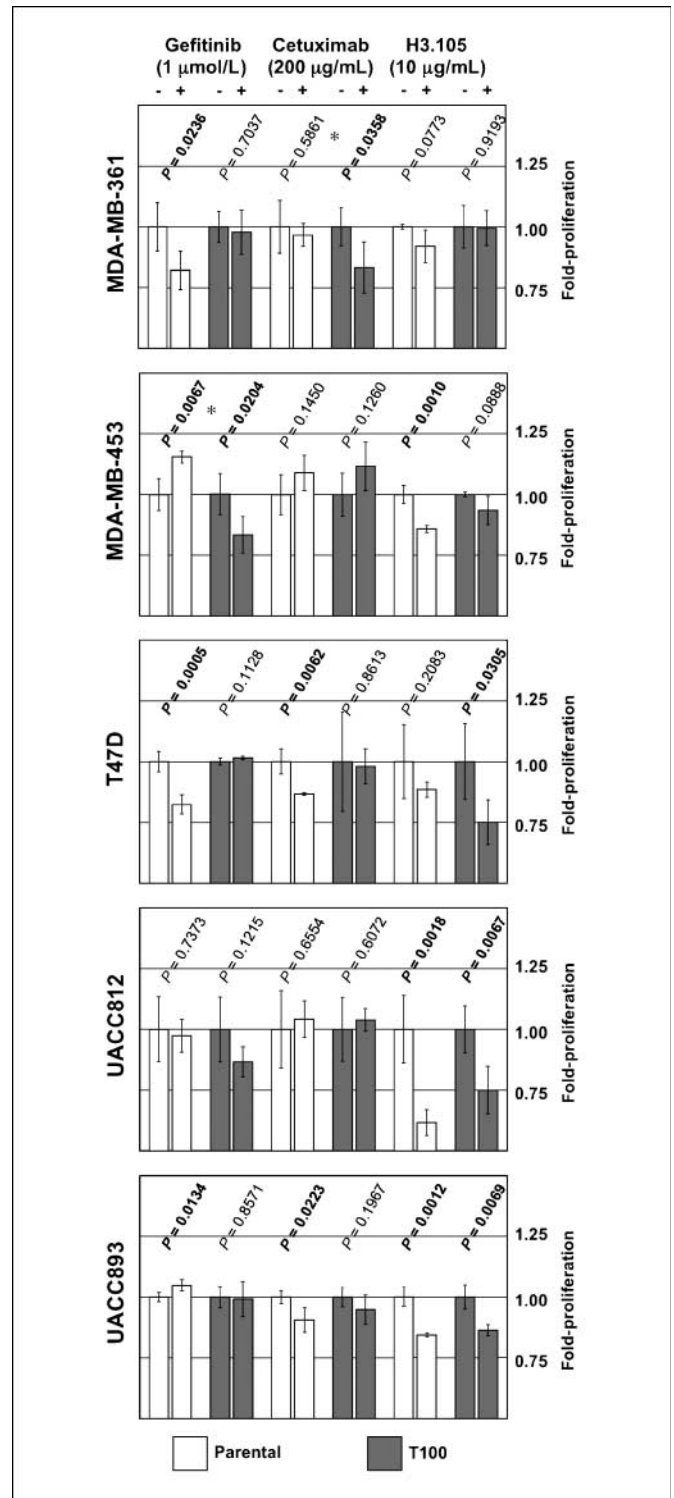


Figure 3. HER inhibitors and cell proliferation in long-term trastuzumab-treated breast carcinoma cell lines. Parental versus long-term trastuzumab-treated (T100) MDA-MB-361, MDA-MB-453, T47D, UACC812, and UACC893 cells were treated with gefitinib, cetuximab, or H3.105 for 120 h, and cell proliferation was measured by a WST-1–based colorimetric assay in quintuplicate ($n = 1$; except *, $n = 2$). The inter-quartile rule was used to eliminate data outliers before calculating the mean absorbance for untreated and treated cell populations. Fold change in cell population number is normalized against untreated cells. Student's *t* test was used to determine whether significant differences in cell proliferation exist between untreated and treated parental and T100 cell populations. **Bold text**, a statistically significant difference in cell proliferation between untreated and treated cell lines ($P < 0.05$).

Together, these results show that HER reprogramming after long-term trastuzumab exposure of primary resistant breast cancer cells is not only correlated with functional changes in the HER receptor tyrosine kinase axis but also unmasks *de novo* targets for therapeutic intervention. These observations are consistent with Friedman and Perrimon's (17) suggestion that robust signaling via a given receptor tyrosine kinase axis consists of interconnected graded components, such that disruption of one pathway is compensated for by other pathways. Alternatively, one might envision HER2 functioning as an addictive oncogene in some (i.e., trastuzumab-sensitive) but not all HER2-positive breast cancer cell lines. In this latter case, the phenotype of primary trastuzumab resistance might cosegregate with other gene mutations, and identification of such patterns of mutation may one day allow physicians to predict which breast tumors will be sensitive to HER-targeted therapy after trastuzumab treatment (18), as is already being done in imatinib-resistant CML patients (3). Admittedly, in some instances long-term trastuzumab treatment induced *de novo* resistance to EGFR and/or HER3-targeted therapeutics in this study. However, because no EGFR or HER3-targeted therapeutics have yet been FDA approved for breast cancer treatment, and also given the availability of EGFR inhibitors and the ongoing development of HER3-directed inhibitors, we propose that the unexplored strategy of priming primary trastuzumab-resistant breast cancer patients with trastuzumab therapy, followed by treatment with one (or more) newly unmasked EGFR/HER3 targets warrants further investigation.

In conclusion, we recognize that as intriguing as these results are, it will be important to confirm and extend our observations in other studies before any benefit may be realized in the clinic. Our results suggest, however, that trastuzumab-induced reprogramming of the HER axis may be a predictable outcome in breast cancer patients after trastuzumab treatment. We propose that trastuzumab-induced alterations in cell signaling occur in *de novo* resistant tumor cells, and we further show that these changes are therapeutically exploitable. Specifically, trastuzumab may unmask

other targets in primary resistant breast tumors by reprogramming members of the HER axis, such as EGFR, the therapeutic target of gefitinib and cetuximab. Considering the significant number of HER2-positive breast cancer patients who exhibit primary trastuzumab resistance, we conclude that further study of trastuzumab-induced gene expression within trastuzumab-resistant tumors is warranted. Our findings suggest that, contrary to widely held assumptions, primary trastuzumab resistance is *not* synonymous with lack of trastuzumab activity in tumor cells, and as such, is a far more complex phenomenon than previously considered. As such, this work opens up important new avenues of investigation for testing the use of this novel therapeutic agent as a priming agent in the development of new treatment interventions for breast, and perhaps other cancer patients.

Note Added in Proof

Recent studies on the mechanism of HER2 degradation may provide a rationale for the observations presented here regarding EGFR and ErbB3 receptor reprogramming in trastuzumab-resistant cells (19-22).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 3/20/2008; revised 1/6/2009; accepted 1/7/2009; published OnlineFirst 3/10/09.

Grant support: M. Narayan and L.N. Harris are supported by a grant from the Department of Defense (USMARC). J.A. Wilken is supported by a grant from Susan G. Komen for the Cure, the Elsa U. Pardee Foundation, and a Seed Grant from the Nancy R. Gelman Foundation. N.J. Maible is supported by NIH CA 79808 and a "Senior Women in Medicine Professorship" from Yale University School of Medicine.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. Elliott Bedows and Yingqun Huang for the critical review of this manuscript.

References

- Apperley JF. Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia. *Lancet Oncol* 2007;8:1018-29.
- Nahta R, Esteva FJ. Trastuzumab: triumphs and tribulations. *Oncogene* 2007;26:3637-43.
- Apperley JF. Part II: management of resistance to imatinib in chronic myeloid leukaemia. *Lancet Oncol* 2007;8:1116-28.
- 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.
- Shattuck DL, Miller JK, Carraway KL, III, Sweeney C. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. *Cancer Res* 2008;68:1471-7.
- Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007;25:1118-45.
- Paik S, Kim C, Wolmark N. HER2 status and benefit from adjuvant trastuzumab in breast cancer. *N Engl J Med* 2008;358:1409-11.
- Amit I, Wides R, Yarden Y. Evolvable signaling networks of receptor tyrosine kinases: relevance of robustness to malignancy and to cancer therapy. *Mol Syst Biol* 2007;3:151.
- 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.
- 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.
- 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.
- Lichtner RB, Menrad A, Sommer A, Klar U, Schneider MR. Signaling-inactive epidermal growth factor receptor/ligand complexes in intact carcinoma cells by quinoxaline tyrosine kinase inhibitors. *Cancer Res* 2001;61:5790-5.
- Pattarozzi A, Gatti M, Barbieri F, et al. 17 β -estradiol promotes breast cancer cell proliferation-inducing stromal cell-derived factor-1-mediated epidermal growth factor receptor transactivation: reversal by gefitinib pretreatment. *Mol Pharmacol* 2008;73:191-202.
- Lee H, Akita RW, Sliwkowski MX, Maible NJ. A naturally occurring secreted human ErbB3 receptor isoform inhibits heregulin-stimulated activation of ErbB2, ErbB3, and ErbB4. *Cancer Res* 2001;61:4467-73.
- van der Horst EH, Murgia M, Treder M, Ullrich A. Anti-HER-3 MAb inhibits HER-3-mediated signaling in breast cancer cell lines resistant to anti-HER-2 antibodies. *Int J Cancer* 2005;115:519-27.
- Arteaga CL. Epidermal growth factor receptor dependence in human tumors: more than just expression? *Oncologist* 2002;7 Suppl 4:31-9.
- Friedman A, Dar AA, Peng DF, et al. Expression of t-DARPP mediates trastuzumab resistance in breast cancer cells. *Clin Cancer Res* 2008;14:4564-71.
- Kang SH, Kang KW, Kim KH, et al. Upregulated HSP27 in human breast cancer cells reduces Herceptin susceptibility by increasing Her2 protein stability. *BMC cancer* 2008;8:286.
- Diermeier S, Horvath G, Kneuechel-Clarke R, Hofstaedter F, Szollosi J, Brockhoff G. Epidermal growth factor receptor coexpression modulates susceptibility to Herceptin in HER2/neu overexpressing breast cancer cells via specific erbB-receptor interaction and activation. *Experimental cell research* 2005;304:604-19.