Receptors & Clinical Investigation 2014; 1: e340. doi: 10.14800/rci.340; © 2014 by Milos Dokmanovic, *et al.*
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REVIEW

Trastuzumab-induced HER2 phosphorylation: exploring the mechanisms and implications

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Received: September 13, 2014
Published online: November 15, 2014

Targeting of ErbB family of receptor tyrosine kinases (RTKs) is frequently used to inhibit the oncogenic signaling in different malignancies. Lapatinib, a dual selective tyrosine kinase inhibitor (TKI) of EGFR and HER2, inhibits their tyrosine kinase activities and receptor tyrosine phosphorylation. Cetuximab, a chimeric monoclonal antibody (mAb) directed against the extracellular domain of EGFR, prevents EGF-mediated receptor kinase activation and tyrosine phosphorylation. However, trastuzumab, a humanized mAb directed against HER2, induces EGFR and HER2 receptor tyrosine phosphorylation and this agonistic effect is correlated with its inhibition of cancer cell proliferation. This review will focus on the current understanding of molecular mechanisms and implications of trastuzumab-induced tyrosine phosphorylation of HER2.

Keywords: trastuzumab; monoclonal antibody; HER2/ErbB2; phosphorylation

To cite this article: Milos Dokmanovic, *et al*. Trastuzumab-induced HER2 phosphorylation: exploring the mechanisms and implications. Receptor Clin Invest 2014; 1: e340. doi: 10.14800/rci.340.

Introduction

ErbB family of receptor tyrosine kinases, which consists of four type I receptor tyrosine kinases (RTKs), known as ErbB1/EGFR/HER1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4, plays important roles in different malignancies and has been intensely pursued for therapeutic targeting. ErbB family members share common structural features, such as extracellular ligand binding domain, transmembrane domain and intracellular tyrosine kinase domain [1]. Upon binding to the extracellular domain of EGFR, HER3 and HER4, the ligand induces the formation of receptor homo-or heterodimers, leading to the increased tyrosine kinase activity followed by transphosphorylation and activation of downstream signaling pathways [2,3]. In contrast to the other ErbB family members, ligand specific to HER2 has not yet been identified. However, the extracellular domain of HER2 can adopt a fixed conformation that resembles a ligand-activated state that permits it to form homo- or heterodimers with other ErbB family receptors in the absence of a ligand [4-6].

Therapeutic targeting of ErbB family members for cancer therapy is either achieved by tyrosine kinase inhibitors (TKIs) which target intracellular tyrosine kinase domain or recombinant monoclonal antibodies (mAb) which target different regions of the extracellular domains.

Trastuzumab is a humanized monoclonal antibody directed against HER2 and approved for the treatment of HER2-positive breast and gastric cancers [6,7]. Since HER2 does not have a specific ligand, this may result in the complexity of the tumor-suppressive mechanisms of trastuzumab action. Overall, it is believed that binding of trastuzumab to HER2 extracellular domain IV inhibits HER2-mediated mitogenic and pro-survival signaling [2]. Binding of trastuzumab to HER2 results in induction of HER2 endocytosis, followed by HER2 degradation [8-12], inhibition of HER2 receptor homodimerization or heterodimerization [2,13], and prevention of HER2 extracellular domain from cleavage by metalloproteinase ADAM10 [14,15]. Furthermore, trastuzumab, an IgG1 subtype, is also capable of inducing antibody-dependent cell mediated cytotoxicity (ADCC) [2].

Lapatinib is an EGFR and HER2 dual tyrosine kinase inhibitor which inhibits their kinase activities and tyrosine phosphorylation to mediate the reduction in oncogenic signaling and tumor growth [16]. Growing evidence suggests that targeting of extracellular domain of HER2 by trastuzumab increases HER2 tyrosine phosphorylation in trastuzumab-sensitive breast cancer cells [17,18]. However, trastuzumab-induced HER2 tyrosine phosphorylation is coordinated with its ability to inhibit cancer cell growth [17-19]. Furthermore, despite different effects on HER2 phosphorylation, treatment with either lapatinib or trastuzumab results in down-regulation of Akt activity and tumor growth inhibition in breast cancer cells. The molecular mechanisms underlining the direct correlation between trastuzumab-induced HER2 phosphorylation and tumor growth inhibition remain elusive and cannot be explained by established mechanistic features of ErbB family of RTKs and their receptor associated tyrosine kinase signaling pathways. In this review we highlight our proposed molecular mechanism by which trastuzumab induces HER2 tyrosine phosphorylation and a previously unappreciated relationship between trastuzumab action and the non-receptor kinase Csk-homologous kinase (CHK). We also discuss potential implications of our findings for the design of novel screens to identify new therapies that can be used for anti-HER2 targeting.

Molecular mechanism by which trastuzumab induces HER2 tyrosine phosphorylation

Much of our mechanistic knowledge on ErbB family of RTKs is based on EGFR. However, EGFR differs in many key aspects from HER2. Traditionally it is believed that increase in tyrosine phosphorylation and kinase activity of ErbB family receptors is associated with mitogenic and pro-survival signaling pathways. It has long been an open question in the field of HER2-targeted therapy as to how trastuzumab concomitantly induces HER2 tyrosine phosphorylation and down-regulation of HER2-coupled signaling. Recent studies by Dokmanovic *et al*. revealed some interesting findings that may provide clues to answer this question [17]. First of all, Dokmanovic *et al*. for the first time demonstrates that trastuzumab induces the activation of HER2 kinase activity in trastuzumab-sensitive breast cancer cells (BT474 and SKBR3 cells). This up-regulated HER2 tyrosine kinase activity partially contributes to the enhanced HER2 phosphorylation at tyrosine 1248 (Y1248), although the molecular mechanism(s) underlining trastuzumab-induced up-regulation of HER2 kinase activity is not clear. We propose that binding of trastuzumab to HER2 may induce structural changes in the HER2 cytoplasmic kinase domain, resulting in the allosteric activation of HER2 kinase followed by transphosphorylation of HER2. Secondly, using EGFR phosphorylation antibody array, which can simultaneously evaluate the relative levels of 17 potential phosphorylation sites among the ErbB family receptors, Dokmanovic *et al*. also found that while both EGF and trastuzumab induced phosphorylation of EGFR and HER2 in trastuzumab-sensitive breast cancer cells, different phosphorylation profiles of ErbB family receptors were observed when cells were treated with either EGF or trastuzumab [17]. This indicates that binding of trastuzumab to HER2 activates downstream signaling pathways that are different from EGF-coupled signaling, and such signaling pathways mediated by trastuzumab lead to growth inhibition. Thirdly, while lapatinib blocks trastuzumab-induced EGFR phosphorylation at tyrosine 845, trastuzumab is still able to induce HER2 phosphorylation at Y1248 in the presence of lapatinib in trastuzumab-sensitive cells, although the extent of phosphorylation of HER2 at Y1248 is lower than that in the absence of lapatinib. This suggests that trastuzumab-mediated HER2 phosphorylation at Y1248 is at least in part independent of HER2 kinase activity and that a tyrosine kinase, yet unidentified, plays a role in trastuzumab-mediated HER2-Y1248 phosphorylation. The report by Zrihan-Licht *et al*. suggested that some of the HER2 binding partners may play a role in growth inhibition and suppression of HER2-mediated oncogenic signaling [20]. In particular, binding of Csk-homologous kinase (CHK), a non-receptor tyrosine kinase also known as megakaryocyte-associated tyrosine kinase (MATK), to phosphorylated tyrosine residue at Y1248 of HER2 was reported to negatively regulate HER2 activity [20-22]. This raised a question of whether CHK is the downstream effector of HER2 to mediate trastuzumab-induced growth inhibition. Finally, Dokmanovic *et al*. provides several lines of evidence that support this idea: 1) trastuzumab induces phosphorylation of HER2 at Y1248, which is the docking site for CHK signaling, 2) trastuzumab increases the interaction between HER2 and CHK, 3) over expression of CHK enhances HER2

phosphorylation at Y1248, 4) over expression of CHK mimics trastuzumab leading to the down regulation of Akt activity and inhibition of breast cancer cell growth. Taken together, data published by Dokmanovic *et al*. suggest at least two distinct mechanisms of trastuzumab-mediated HER2 phosphorylation in trastuzumab-sensitive breast cancer cells [17]. While trastuzumab-induced increase in HER2 tyrosine kinase activity contributes to the phosphorylation of HER2, trastuzumab-mediated recruitment of non-receptor tyrosine kinase, CHK, to HER2 further enhances HER2 phosphorylation at Y1248 to mediate growth inhibition. Consistent with trastuzumab-mediated changes in HER2 phosphorylation, sensitivity to trastuzumab was also reported to be associated with expression of phosphorylated HER2 at Y1248 in different breast cancer-derived cells [23].

Different mechanism of trastuzumab-induced HER2 phosphorylation was proposed by Gijsen *et al* [18]. Based on the study conducted by Gijsen *et al*. trastuzumab-induced HER2 phosphorylation is maintained by trastuzumab-mediated release of ErbB family ligands which can then result in ligand-dependent activation of other ErbB family members followed by dimerization with HER2 and HER2 phosphorylation [18]. Gijsen *et al*. and others also noted paradoxical activation of ERK1/2 signaling concomitant with suppression of Akt signaling following trastuzumab treatment [17, 18]. It is still unclear how increased HER2 phosphorylation and activation of HER2 downstream oncogenic signaling pathway, such as ERK1/2, is associated with anti-tumor effects mediated by trastuzumab. The model proposed by Gijsen *et al*. is consistent with other reports in literature which suggest that the activation of other receptors from ErbB family following HER2 targeting may be associated with compensatory feedback mechanisms to limit the efficacy of anti-HER2 targeting and possibly induce resistance in tumor cells [24].

Implications for the development of HER2 phosphorylation at Y1248 as a predictive biomarker for trastuzumab resistance

Testing for HER2 expression is performed on patient tumor samples before administration of trastuzumab, and the American Society of Clinical Oncology/College of American Pathologists has published guideline recommendations regarding determination of HER2 expression and HER2 positivity for trastuzumab treatment in clinics [25]. In the study reported by Vogel *et al*., the rate of primary trastuzumab resistance was 74%. These data indicated that the vast majority of HER2-positive metastatic breast cancer was intrinsically resistant to single agent-trastuzumab [26]. Moreover, the majority of patients with HER2-positive breast cancer develop acquired resistance within one year [27, 28]. Better understanding of the mechanisms of trastuzumab resistance is critical for future development of predictive biomarkers and novel therapies for trastuzumab-resistant cancers. Currently, no predictive biomarker is clinically useful for differentiating between trastuzumab sensitive and trastuzumab resistant tumors [6,29]. Among many candidates, phosphorylation of ErbB family receptors was also tested in different clinical samples obtained from trastuzumab-resistant and trastuzumab-sensitive patients. In the study by Hudelist *et al*. it was shown that the presence of phosphorylation at EGFR-Y845 and HER2-Y1248, the two sites also reported to be selectively up-regulated following trastuzumab treatment, was an independent predictor of better progression-free survival following trastuzumab treatment [30]. Ramic *et al*. found that reduction in HER2 phosphorylation at Y1248 correlated with acquisition of resistance to trastuzumab therapy [31]. Guiliani *et al*. reported that positive HER2 phosphorylation is associated with higher response rates following treatment with trastuzumab and chemotherapy [32]. Our data also indicate that trastuzumab-resistant tumor biopsies are characterized by reduction in HER2-Y1248 phosphorylation while trastuzumab- sensitive tumor samples have high levels of HER2-Y1248 phosphorylation [17]. We also found that little or no HER2 phosphorylation at Y1248 was detected in trastuzumab-resistant breast cancer cells (JIMT1 cells) and that trastuzumab was unable to induce HER2-Y1248 phosphorylation in JIMT1 cells [17]. Based on our findings and the results from other groups, we suggest that the HER2-Y1248 phosphorylation status be considered together with HER2 expression for further evaluation as a potential biomarker of trastuzumab response in well controlled clinical studies.

Our study also revealed several other interesting findings regarding relationship between trastuzumab response/resistance and Akt activity [17]. Our data show that while trastuzumab was unable to induce HER2-Y1248 phosphorylation in JIMT1 cells, it was still capable of downregulating Akt activity in trastuzumab-resistant breast cancer cells, JIMT1 cells. This result indicates that although trastuzumab inhibition of PI3K-Akt signaling is one of major mechanisms to mediate growth inhibition, resistance to trastuzumab may not be due to the inability of trastuzumab to inhibit Akt signaling. Clinical studies to evaluate Akt activity in both trastuzumab- sensitive and resistant breast cancers are warranted.

Implications for the design of novel strategies for the discovery of new therapeutic agents

Targeting of ErbB family of RTKs is frequently used in different malignancies to inhibit the oncogenic signaling [33]. While lapatinib and cetuximab both inhibit tyrosine

kinase activities of their targeted kinases, trastuzumab induces activation of HER2 tyrosine kinase and HER2 phosphorylation to mediate signaling to inhibit breast cancer cell growth. Our findings suggest a novel screening strategy for the identification of potential modulators of HER2 activity, including therapeutic monoclonal antibodies, such that activation of HER2 kinase activity is included in the screening assays together with modulation of downstream signaling pathways and suppression of cell growth. Lessons learnt from studying the mechanisms of trastuzumab-mediated induction of HER2 tyrosine kinase activity suggest that activation of HER2 tyrosine kinase activity may not be necessarily associated with the lack of inhibition of breast cancer cell growth and signaling. Activation of HER2 phosphorylation and tyrosine kinase activity needs to be considered in the context of other signaling pathways, such as CHK-associated non-receptor tyrosine kinase signaling.

Future directions in research

Identification of novel mechanisms of trastuzumab action, such as trastuzumab-mediated activation of HER2 kinase activity, recruitment of CHK non-receptor tyrosine kinase followed by induction in HER2 phosphorylation and suppression of oncogenic signaling opens up several questions that await further understanding. First, the role of non-receptor tyrosine kinase CHK in the modulation of HER2-induced oncogenesis and response to trastuzumab by regulation of HER2 receptor phosphorylation and HER2 downstream signaling pathways needs to be better understood using available cell culture and animal model systems [21, 34, 35]. Second, the mechanism(s) contributing to the paradoxical activation of ERK1/2, concomitant with suppression in Akt signaling following trastuzumab treatment, needs to be better characterized both *in vitro* and *in vivo*, including clinical studies, and possibly exploited in future therapeutic directions, such as using trastuzumab in combination with more potent ERK1/2 inhibitors [36] if it is truly associated with compensatory feedback mechanisms to limit the efficacy of anti-HER2 targeted therapy. Third, identification of predictive biomarkers for trastuzumab efficacy in appropriately designed and well controlled clinical studies may also include HER2-Y1248 phosphorylation. Taken together, availability of better therapeutic options, in conjunction with better monitoring and earlier detection of trastuzumab-resistant tumors may have the potential to improve treatment outcomes in HER2-positive breast cancer patients.

Acknowledgments

We thank Drs. Zhaohua Zhou and Weiming Ouyang for the critical review of the manuscript.

Grant support

This study was supported by the U.S. Food and Drug Administration Critical Path Funding for FY2011.

Conflict of interests

The information presented in this article reflects the views of the authors and does not represent the policy of the U.S. Food and Drug Administration.

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