Signal integration: a framework for understanding the efficacy of therapeutics targeting the human EGFR family

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The human EGFR (HER) family is essential for communication between many epithelial cancer cell types and the tumor microenvironment. Therapeutics targeting the HER family have demonstrated clinical success in the treatment of diverse epithelial cancers. Here we propose that the success of HER family–targeted monoclonal antibodies in cancer results from their ability to interfere with HER family consolidation of signals initiated by a multitude of other receptor systems. Ligand/receptor systems that initiate these signals include cytokine receptors, chemokine receptors, TLRs, GPCRs, and integrins. We further extrapolate that improvements in cancer therapeutics targeting the HER family are likely to incorporate mechanisms that block or reverse stromal support of malignant progression by isolating the HER family from autocrine and stromal influences.

Introduction
A solid tumor is composed of cancer cells embedded in abundant stroma consisting of nontransformed stromal cells and ECM (in this article we use “tumor” to refer to the mass of cancer cells together with nonmalignant stromal cells) (1). It is now widely accepted that within this specialized microenvironment, there is a complex interplay between the cancer cells and the stroma, which strongly influences the development, progression, and metastatic potential of the cancer cells (2, 3). One of the earliest recognized properties of cancer cells is their growth factor self-sufficiency, which is achieved by activation of cellular proto-oncogenes, by either mutation or overexpression (4–6). Further evidence of the importance of growth factors (a subclass of cytokines) and their receptors in tumor progression includes the association between cancer and aberrant signal transduction mediated by growth factor receptors, such as members of the human EGFR (HER) family (7, 8). Indeed, activation of HER family members leads not only to increased cell proliferation, but also to cancer cell resistance to growth-inhibitory cytokines and expression of selective immunosuppressive and proangiogenic cytokines and chemokines (9, 10), thereby creating an environment that favors tumor progression.

Therapeutic reagents targeting HER family members, in particular EGFR (also known as HER1) and the receptor tyrosine kinase (RTK) encoded by HER2, p185HER2 (also known as HER2, HER2/neu, and ErbB2), have proven successful for the treatment of breast, colon, lung, and pancreatic cancers (11, 12). However, not all tumors expected to respond to these therapeutics are in fact sensitive to them, and in many cases resistance eventually develops. We believe that broader success in developing therapies directed at HER family members depends upon an increased appreciation for how these receptors consolidate signals from diverse sources and how they cooperate toward functional homeostasis in cancer cells. This article discusses a diverse body of work that we hope provides a basis for the design of new therapeutic approaches to cancers associated with aberrant activation of HER family members.

The HER family and cancer
The HER family is composed of four receptors (Figure 1), plus a number of variants generated by alternative splicing, and 11 types of ligands, some of which are also generated by alternative splicing (e.g., the neuregulins [NRGs]) (13). Roles for these receptors and their ligands have been described in many types of human cancer, including breast, colon, pancreatic, ovarian, brain, and lung cancers (14). While appropriate focus has been on malignant cell expression of HER family members, expression of EGFR and TGF-α on tumor-associated stromal endothelial cells has also been reported and could be of therapeutic significance (15).

HER family members are activated through oligomerization, and the minimal active configuration is either a homodimer or a heterodimer (Figure 1) (16). Activation can be achieved by ligand binding. This is true even for p185HER2, which has no known soluble ligands and acts as a coreceptor for the other HER family members. In addition, constitutive activation can occur in the absence of endogenously added ligand if either EGFR or p185HER2 is overexpressed or if activating mutations occur in their tyrosine kinase domain (17–19). This constitutive activity can be increased if cells are treated with HER family ligands (20, 21). In addition, there are other proteins on the cell surface that may enhance the ability of p185HER2 to associate with other HER family receptors or to signal constitutively when it is overexpressed.

The contribution of each HER family member to cancer cell behavior is generally regulated by a combination of its level of expression and the availability of its cognate ligand(s) in the tumor microenvironment. Overexpression of single HER family members and their ligands is now known to predict more aggressive behavior of many epithelial malignancies (including breast cancer, non–small cell lung cancer, and bladder cancer) (13, 14, 22). Furthermore, the idea that cooperativity among HER family members creates a malignant phenotype is strongly supported by mechanistic and other nonclinical data. One fundamental...
HER2 became the focus of work in patients whose cancers are characterized by the coexpression of HER family members in vitro (24, 25) and that coexpression of HER family members can be transphosphorylated and achieve signaling competence (23). Other work has proven that coexpression of HER family members and their ligands synergizes for cellular transformation in vitro (24, 25) and that coexpression of HER family members can activate cell signaling and tumor cell invasion pathways not activated by single receptors (26). Other data further emphasize the complex cooperative nature of the HER family, not only with each other, but also with other inflammatory proteins, in driving tumor progression. For instance, overexpression of p185HER2 in breast cancer cells is associated with enhanced expression of the angiogenic proteins IL-8 and VEGF (9, 10). Similarly, both overexpression of EGFR and activating mutations in the tyrosine kinase domain of EGFR induce cytokine production (in particular, VEGF, IL-6, and IL-8) in animal models of human cancer and in primary human lung adenocarcinomas (27–29). Other recent results have demonstrated a tyrosine kinase–independent role for EGFR in preventing autophagic cell death by maintaining intracellular glucose levels through interaction with the sodium/glucose transporter SGLT-1 (30). Taken together, these data demonstrate cooperativity among HER family members, not just in the malignant behavior of cancer cells, but also in modifying the tumor microenvironment to favor tumor progression.

A significant amount of literature relating to prognostic outcome and HER family coexpression supports the concept that more successful therapeutics will require simultaneous suppression of the aberrant activation of multiple HER family members. Most importantly, analysis of patient samples has shown that patients whose cancers are characterized by the coexpression of two or more HER family members demonstrate a more aggressive malignancy than is observed when only one member is expressed in the cancer cells (31–33). In one study (35), patients with breast cancer expressing a single HER family member had a median ten-year survival rate of 75%, as compared with 40% for patients whose cancer expressed two or more HER family members.

Link between TNF-α, incipient tumor progression, and the HER family: the conception of trastuzumab

Incipecnt cancer cells progress toward malignancy as a result of epigenetic and genetic changes coupled with continuous in vivo selection for the most malignant cells (5, 36). One mechanism of host protection against incipient tumor progression involves the production of TNF-α by immune cells in the stroma as part of the antitumor innate immune response (36, 37). Overcoming this component of host immunosurveillance, for example by selecting for resistance to TNF-α in vitro, can convert a regressing syngeneic mouse tumor into a progressively growing lesion (38). This phenomenon is also relevant to human cancer cells: an extensive screen of human cancer cell lines surprisingly demonstrated that most are inherently resistant to TNF-α–induced cytotoxicity (39). Investigations into the specific mechanisms that give rise to the TNF-α–resistant phenotype were initiated with the goal of exploiting defined resistance mechanisms as targets for the development of novel therapeutics. These experiments showed that cancer cell resistance to the cytotoxic effects of TNF-α could be induced by the EGFR ligands EGF and TGF-α, as well as other growth factors (40). The ability of growth factors to inhibit TNF-α–mediated cytotoxicity suggested that one mechanism by which cancer cells could escape immunosurveillance is through autocrine or stromal cell production of growth factors.

The overexpression of p185HER2 became the focus of work related to the mechanisms of cancer cell resistance to TNF-α when experiments showed that ligand (EGF or TGF-α) activation of EGFR resulted in diminished TNF-α–mediated cytotoxicity (40). Simultaneously, it was also shown that overexpression of p185HER2 in NIH 3T3 fibroblasts resulted in oncogenic transformation (41), without the accompanying mutations reported for transformation of NIH 3T3 cells by the NEU oncogene (42). In addition, soon after its initial characterization (43–45), amplification/overexpression of HER2 was linked to aggressive breast cancer and other malignancies (46–49). Subsequent studies tested whether overexpression of p185HER2 was associated with cancer cell resistance to TNF-α. In vitro analysis of NIH 3T3 fibroblasts transformed by overexpression of p185HER2 demonstrated that these...
cells were resistant both to TNF-α and to cytotoxicity mediated by LPS/IFN-γ-activated macrophages (50), which use TNF-α as a major mechanism of immunosurveillance against incipient cancer cells (38). Similarly, increased resistance to the cytotoxic effects of TNF-α is demonstrated by breast tumor cells that express elevated p185HER2-associated tyrosine kinase activity (50). Further experiments demonstrated that TNF-α resistance of transformed cells in vitro was associated with an increased rate of dissociation of TNF-α from its receptor (50). More recently, cancer cell resistance to TNF-α has been associated with upregulation of cell survival pathways involving Akt and NF-κB (51, 52). Overexpression of p185HER2 has also been shown to interfere with the antiproliferative activity of IFN-γ and TGF-β, both of which may be important for inhibition of incipient tumor progression (2, 37, 53, 54).

These initial findings stimulated a search for an antagonistic mAb that could recognize the extracellular domain of p185HER2 and inhibit cancer cell growth while enhancing the sensitivity of HER2-overexpressing cancer cells to TNF-α. Of the more than 100 mAbs derived as specific for the extracellular domain of p185HER2 (55) and studied in detail (56), the antibody designated muMAb4D5 demonstrated the highest degree of correlation between p185HER2 expression and growth inhibition of both breast cancer cell lines in vitro and human breast cancer xenografts in nude mice (56–58). Treatment with muMAb4D5 was also shown to convert TNF-α-induced growth inhibition to a cytotoxic response in HER2-overexpressing breast cancer cells in vitro, but it did not have this effect on cancer cells that did not overexpress HER2 (57). The link between TNF-α resistance and proto-oncogene overexpression was further substantiated by subsequent work showing that selection of NIH 3T3 fibroblasts for resistance to the cytotoxic effects of TNF-α leads to enrichment in the remaining cells for those with a transformed morphology and is often associated with amplified copy number and overexpression of the c-MET proto-oncogene, which encodes hepatocyte growth factor receptor (59). It is interesting to speculate that some incipient cancer cells might escape immune cells secreting TNF-α in vivo, as part of the antitumor innate immune response, leading to the formation of tumors characterized by aberrant constitutive RTK activation or RTK overexpression.

The demonstration that muMAb4D5 inhibited tumor cell growth in vitro in a manner that correlated with overexpression of HER2, that it induced a cytoxic response to TNF-α in vitro (also specific for HER2-overexpressing tumor cells), and that it inhibited the growth of HER2-overexpressing human breast cancer in xenograft models (58) led to the development of humanized muMAb4D5 (trastuzumab) and its subsequent application in breast cancer. Trastuzumab was designed as an IgG1 subtype to provide the potential for antibody-dependent immune cell killing (ADCC) of breast cancer cells that overexpress p185HER2 (56, 60). This aspect of trastuzumab might be especially relevant for its clinical effects, as, when overexpressed, p185HER2 tends to cluster with about 104 receptors per cluster in SK-BR-3 tumor cells in culture, an effect that is potentiated by trastuzumab (61–63). Following binding to cell surface p185HER2, trastuzumab can subsequently couple to and induce clustering of Fc receptors (FcyRIIa) on stromal immune cells, especially NK cells, resulting in tumor cell killing mediated by release of perforin, granzyme, and cytokines (64). The activation of ADCC enables a mechanism for antitumor activity of trastuzumab that is independent of its other mechanisms of action and that results in preferential targeting of immune cells to HER2-overexpressing tumor cells in vitro (56). The activation of ADCC may enable a mechanism for the antitumor activity of trastuzumab that is independent of its other mechanisms of action and that allows preferential targeting of immune cells to p185HER2-overexpressing cancers (56, 65). Because HER2 expression persists in trastuzumab-resistant tumor cells (66), ADCC tumor cell cytotoxicity may provide an independent mechanism for efficacy mediated by trastuzumab (67).

Trastuzumab therefore serves as a model for the development of therapeutics that can simultaneously target malignant cell behavior and interactions between stromal cells and cancer cells. Trastuzumab directly inhibits breast cancer cell proliferation by disrupting the function of p185HER2 (58). Other consequences of trastuzumab treatment of HER2-overexpressing breast tumor cells include: increased sensitivity to growth inhibition by the cytokine TNF-α (57); downregulation of tumor cell production of IL-8, an inflammatory chemokine with proangiogenic activity (10); downregulation of tumor cell VEGF production (10); and downregulation of CXCR4, the receptor for CXCL12 (also known as stromal-derived growth factor 1). Downregulation of CXCR4 may inhibit metastasis of breast cancers that overexpress p185HER2 (68). These results suggest that overexpression of p185HER2 plays a central role in breast cancer tumor progression by inducing the expression of multiple protumorigenic signals and that trastuzumab exerts its efficacy by multiple mechanisms that depend on downregulating expression of p185HER2 (21, 58).

Several conclusions can be drawn from the results discussed to this point. The constitutive activation of a single HER family member creates more aggressive malignancy, and cooperation among HER family members enhances this effect. The mechanisms that contribute to more aggressive malignancy include increased tumor cell proliferation, as well as HER-associated tumor cell resistance to the innate immune system (including the growth inhibitory cytokines TNF-α, TGF-β, and IFN-γ). Last, as described earlier, aberrant activation of HER family members induces cancer cell production of cytokines and chemokines that impact stroma and encourage tumor progression. mAb antagonists that target HER family members, in particular trastuzumab (which targets p185HER2) and cetuximab (which targets EGFR), are successful therapeutics. A possible framework to explain the success of these therapeutics is that the HER family integrates signals from a multitude of other receptor systems, many of which are involved in cytokine and chemokine signaling, and that trastuzumab and cetuximab interfere with both the response of the tumor cell to these signals and the ability of the tumor cell to produce protumorigenic cytokines that activate the stroma.

**Signal integration by the HER family**

Transactivation of the HER family occurs when the cytoplasmic domain of one or more family members becomes tyrosine phosphorylated and signaling competent following an event initiated by a distinct “upstream” receptor family (Figure 2). As discussed here, we believe that this places the HER family at the center of converging signals for cell proliferation, motility, and other cancer cell behaviors.

The accumulated evidence supports a model in which HER family transactivation and cooperation represents a primary driver of HER function and subsequent modification of cancer cell behavior (Figure 2), as suggested earlier by Hynes and Britten (69, 70). The most common mechanism of HER transactivation is through...
upstream activation of GPCRs by their requisite ligands (71–73), including lysophospholipids, oligopeptide hormones (such as angiotensin II), and chemokines (74–77), followed by metalloproteinase-catalyzed release of HER ligands. In the best-characterized examples, GPCR activation results in G-protein–mediated activation of ADAM (a disintegrin and metalloprotease) family members, which then cleave HER pro-ligands. The mature HER ligands that are subsequently released activate their corresponding receptors on cancer or stromal cells, resulting in HER activation (71, 73, 78). In addition to GPCRs, many other ligand/receptor systems have been documented by several groups to elicit transactivation of the HER family, with most studies focusing on EGFR (Figure 2) (70, 73). Examples include cytokine receptors (79–81) and TLRs (Figure 2). TLRs are classically activated by pathogen-derived ligands and are primarily associated with the innate immune response (chemokine and cytokine release). Importantly, TLRs can also be activated as a result of release of malignant cell contents (such as HSP70; Figure 2) (82, 83), which occurs frequently in progressing cancer cells (37). Although the main objective of this article is to discuss the central role of the HER family in malignancy, it should be appreciated that the HER family acts as a conduit for diverse signaling systems. Most of the receptor systems described here are present in both nonmalignant stromal cells and cancer cells.
Important support for HER family cooperation in resistance to monotherapy with a mAb targeted to a single HER family member is provided by the demonstration that resistance to the HER2-targeted mAb trastuzumab in HER2-overexpressing BT474 breast tumors, selected in vivo, is associated with increased expression of EGFR, increased numbers of EGFR-HER2 heterodimers, and increased levels of mRNAs encoding TGF-α, heparin-binding EGF (HB-EGF), and NRG (66). In addition, simultaneous mAb targeting of multiple epitopes and members of the HER family has demonstrated synergistic antiproliferative effects on cancer cells in vitro (61). Thus, cooperation among HER family members and their associated growth factors appears to be a major culprit in acquired resistance to mAb therapeutics targeting the HER family (66, 92, 94, 95).

The current challenge
Trastuzumab and cetuximab are groundbreaking anticancer therapeutics, but they are active in only a fraction of their targeted patient populations (11, 12). Resistance to trastuzumab monotherapy and trastuzumab in combination with chemotherapy is multifactorial, but we believe that the best-documented association with acquired resistance is the upregulation and/or increased association of the therapeutically targeted receptor with other nontargeted members of the HER family (23, 66, 92, 95). This conclusion is supported by the discussion above and by Arpino et al. (96), who used two xenograft models of HER2-overexpressing breast cancer to show that a combination of gefitinib (a nonproteinaceous tyrosine kinase inhibitor [TKI] specific for the EGFR), trastuzumab, and pertuzumab (which targets a distinct HER2 epitope to trastuzumab) (58, 97) provided significantly improved antitumor efficacy compared with any single agent and any dual combination of the agents. These results provide a valuable rationale for developing new therapeutics that can more broadly interfere with HER family function.

Clinical efforts for the purpose of developing mAbs targeting multiple members of the HER family (“pan-HER” approaches) have been focused on p185HER2, because of its central role as a driver for HER family signaling in HER2-overexpressing breast cancer and its proposed role as a signaling amplifier for other HER family members (98). So far, these efforts have yielded limited success (99, 100). A possible explanation is that HER2 may not play as dominant a role in most cancers as it does in HER2-overexpressing breast cancers. Instead, in many malignancies, HER2 may function as a coreceptor, responding primarily to activation by other HER family members. Different approaches are therefore needed and should take into account the multiplicity of ligands that activate the HER family (Figure 1) as well as the cooperative and self-compensating behavior of the receptors themselves (95). Treatment with a mixture of mAbs and TKIs (including SFK inhibitors) that simultaneously target multiple receptor epitopes and tyrosine kinases has shown promise in a number of preclinical models (96, 101) and is one approach currently undergoing clinical evaluation (102).

A second pan-HER approach targets the ADAM17 metalloproteinase, which is important in HER ligand maturation (Figure 2) (103). Because soluble growth factors driving HER family transactivation originate in a metalloproteinase-dependent manner from both cancer cells and nonmalignant stromal cells, an ADAM17 antagonist might provide a means of therapeutically targeting both cancer cells and hyperactivated stromal cells to downregulate HER family function. Some disadvantages of this approach are that previous attempts to inhibit metalloproteinases resulted in off-target activity (104) and that the ADAM family exhibits tremendous redundancy (105).

An approach with the inherent specificity of a biologic therapeutic is “ligand trap” technology, first proven successful with etanercept, a fusion protein comprising the extracellular domain of human p75-TNFR linked to the Fc component of human IgG1 (IgG1Fc) (106). To create a pan-HER ligand trap, the ligand domains of EGFR and HER3 have been combined into a human IgG1Fc-mediated heterodimeric configuration designated as RB200 (21). EGFR was chosen as one arm of the ligand trap in order to sequester most growth factors that activate the HER family (7 of the 11 ligands of HER family members bind to EGFR) (Figure 1). HER3 was chosen as the second subunit because its activation appears to have an important role in resistance to HER-targeted therapeutics (23, 92, 95). Blocking ligand-modulated activation in this way might isolate cancer cells from stromal or autocrine growth factors, the protumorigenic effects of which are consolidated through the HER family. Because of the importance of transactivation by GPCRs in activating the HER family, initial in vitro studies of RB200 (the first-generation pan-HER ligand trap) have focused on inhibition of cancer cell proliferation induced by lysophosphatidic acid (LPA) (21). LPA was chosen for these experiments because the GPCRs to which it binds (LPA receptor-1 [LPAR1], LPAR2, and LPAR3) couple with multiple ADAMs, resulting in release of ligands that activate both EGFR and HER3 (107). In addition, overexpression of LPAR occurs in multiple malignancies that are also associated with aberrant HER activation (75). RB200 demonstrated a broad range of activity in this assay (Table 1), indicating its potential for interrupting autocrine growth stimulation mediated by the GPCR/ADAM/HER family pathway (21).

Combinations of traditional therapeutics together with novel immunologic approaches will enable further general strategies that can simultaneously target the stromal and cancer cell components of a tumor. One possible direction is the engineering of

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Table 1
Inhibition of LPA-stimulated proliferation of tumor cells in vitro by RB200

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Tumor type</th>
<th>Inhibition of LPA-induced proliferation by RB200</th>
</tr>
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<tbody>
<tr>
<td>H1975</td>
<td>Lung</td>
<td>++++</td>
</tr>
<tr>
<td>H441</td>
<td>Lung</td>
<td>+++</td>
</tr>
<tr>
<td>H1993</td>
<td>Lung</td>
<td>+++</td>
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<tr>
<td>A549</td>
<td>Lung</td>
<td>+++</td>
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<tr>
<td>Calu-1</td>
<td>Lung</td>
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<tr>
<td>H358</td>
<td>Lung</td>
<td>++</td>
</tr>
<tr>
<td>HCC4006</td>
<td>Lung</td>
<td>+</td>
</tr>
<tr>
<td>H2122</td>
<td>Lung</td>
<td>–</td>
</tr>
<tr>
<td>A431</td>
<td>Epidermoid</td>
<td>++</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
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</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>–</td>
</tr>
<tr>
<td>N-87</td>
<td>Gastric</td>
<td>++</td>
</tr>
<tr>
<td>HT-29</td>
<td>Colon</td>
<td>–</td>
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Assays were performed as described in ref. 21 in serum-free medium with or without 5.0 μM LPA and with or without RB200 (EGFR:HER3/Fc bispecific ligand trap). Inhibition of GPCR ligand–stimulated (LPA) proliferation by RB200: –, <10%; +, 10%–20%; ++, 21%–50%; ++++, 51%–70%; ++++, >70% (21).
immunological effectors cells (108) so that they target specific peptide fragments derived from HER family members overexpressed in cancer cells. Such peptides have been shown to be presented in the context of MHC class I at the cell surface of cancer cells (direct presentation) and at the cell surface of neighboring stromal cells (indirect or cross-presentation), creating a system in which both cell types can become targets for CTLs (109, 110). The process of antigen presentation by cancer and stromal cells is quite different from that of direct recognition of an epitope by a therapeutic mAb and is therefore likely to successfully target tumors that over-express multiple HER family members, even if they have become resistant to the mAbs as a result of compensatory mechanisms. This is because expression of the targeted receptor (either EGFR or p185HER2) is usually maintained in resistant cells in vitro and in patients (11, 12, 66, 92). Multiple independent studies have identified amino acids 369–377 of HER2 as a prominent HLA-A2.1–restricted CTL epitope (originally identified by T cells of a patient with ovarian cancer) (111–113). Since identical epitopes are processed in humans and mice (114), possible side effects on somatic cells expressing the autochthonous mouse HER2 should be revealed in preclinical models, making them more relevant. The success of this approach depends on engineering a high-affinity TCR for the targeted epitope (115).

Summary

The HER family integrates stimuli originating from the serum, nonmalignant stromal cells, and the cancer cells themselves. We believe that this property of the HER family is likely to be an important reason for the remarkable success of cetuximab and trastuzumab and probably the TKIs that target EGFR and p185HER2. Other properties of these therapeutics, such as their ability to mediate ADCC, may also contribute to their efficacy. Building on this success to achieve efficacy in a wider range of malignancies associated with aberrant activity of HER family members is the current challenge. Technologies described in this Review, which target both stromal and cancer cells, will be critical keys to success in meeting this challenge.

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