HER Kinase Axis Receptor Dimer Partner Switching Occurs in Response to EGFR Tyrosine Kinase Inhibition despite Failure to Block Cellular Proliferation

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Abstract
The human epidermal receptor (HER) axis consists of a dynamic, interconnected family of receptors that make critical contributions to a number of malignancies. Therapeutics targeting epidermal growth factor receptor (EGFR) are unable to effectively inhibit tumor growth in a majority of cases. These tumors are assumed to possess primary resistance to anti-EGFR therapies, but the consequence of inhibiting EGFR in these tumors is unclear. We established isogenic cell lines by prolonged gefitinib treatment at concentrations that are in excess of that which is required for complete EGFR kinase inhibition but only minimally affected growth. Subsequently, we monitored the ligand-dependent HER profiles based on receptor expression, phosphorylation, and dimerization in conjunction with measurements of cellular susceptibility to gefitinib. Chronic EGFR kinase inhibition rapidly switched the HER network from dependence on EGFR to HER2. However, both receptors activated the critical signaling proteins AKT and mitogen-activated protein kinase, and in both cases, HER3 was the common association partner. Remarkably, the switch in receptor dimers caused diminished susceptibility to EGFR-targeted inhibitors gefitinib and cetuximab but acquired susceptibility to the HER2-targeted inhibitor pertuzumab. Overall, our study indicates that the EGFR pathway is responsive to EGFR inhibiting therapies that are not dependent on EGFR for their growth and survival, thus challenging the current definition of primary therapeutic resistance. Furthermore, EGFR kinase inhibition induces HER kinase receptors to engage in alternative dimerization that can ultimately influence therapeutic selection and responsiveness.

Introduction
The human epidermal receptor (HER) axis is a dynamic, interconnected family of receptors that can form receptor complexes that activate both common and distinct downstream signaling events critical for cell growth and proliferation. It includes four structurally related receptors: HER1 [epidermal growth factor (EGF) receptor (EGFR)], HER2, HER3, and HER4. These receptors are activated in response to ligand-dependent dimerization that initiates key signaling pathways to cellular proliferation, including the rat sarcoma/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/AKT pathways. However, receptor hyperactivation due to overexpression (1), amplification (2, 3), or activating mutations (4) can cause aberrant cellular proliferation and tumorigenesis.

Constitutively active receptors, such as EGFR or HER2, when overexpressed and/or deregulated, can trigger potent signaling events that confer a selective growth advantage and can lead to tumorigenesis. However, sustained activation also causes cell dependence on individual signaling pathways. This phenomenon, called "oncogene addiction" (5), predicts targets for directed therapeutics, as inhibition of the target oncogene effectively inhibits cellular proliferation. This is exemplified by tumors overexpressing HER2 that can be inhibited by the HER2-directed antibody trastuzumab (6), as well as EGFR-expressing tumors that are inhibited by EGFR–tyrosine kinase inhibitors (TKI; i.e., erlotinib and gefitinib) or EGFR-directed monoclonal antibodies (cetuximab and panitumumab). However, efficacy for both of these targeted inhibitors is limited to a subset of patients. Although there are some controversies concerning the measurement of HER2 amplification in breast cancers by both standard immunohistochemistry or fluorescence in situ hybridization (FISH) techniques (7), it is conventionally accepted that trastuzumab is only effective in a subset of breast cancers.
(8), those with elevated HER2 expression as assessed by these diagnostic assays. Similarly EGFR-TKIs are most effective in non–small cell lung carcinoma (NSCLC) tumors, in which EGFR is hyperactivated due to gene amplification, kinase domain–activating mutations (9–12), EGFR ligand overexpression (13), or activation by non-HER family members (14, 15).

Upon chronic treatment "oncogene-addicted" tumors become resistant to HER-targeted therapeutics. However, tumors are heterogeneous tissues, and it is plausible that there is diversity for therapeutic susceptibility within a tumor. As such, it is expected that both oncogene-addicted and nonaddicted cells will be exposed to targeted therapies. The oncogene-addicted cells may undergo apoptosis via the recently explained mechanism of "oncogenic shock" (16), whereas the nonaddicted cells may remain viable. Eventually oncogene-addicted cells acquire resistance against the targeted therapeutic through readjustment of signaling networks (17–19), HER reprogramming (20, 21), and acquisition of secondary mutations (22, 23). In contrast, the effect of HER-targeted therapies in the nonaddicted cells that express HER family targets at functional, but not hyperactive, levels has not been thoroughly evaluated. These nonaddicted cells may harbor de novo or primary resistance; however, it is unclear if the HER network in these cells undergoes modulation in response to HER-targeted therapies. Recent studies have shown that, although trastuzumab is ineffective at altering the growth properties of cells that express normal levels of HER2, it causes a shift in HER family expression (24). We queried whether a similar phenomenon occurs in normal EGFR-expressing cells in response to EGFR-TKIs.

We have developed isogenic cell lines that are not dependent on EGFR for cell survival to evaluate HER family interactions that are important for EGFR-TKI sensitivity. We have shown that, whereas cellular proliferation is only moderately affected by prolonged exposure to supraphysiologic gefitinib concentrations, otherwise known to compromise viability of gefitinib-sensitive, EGFR-expressing cells (25), in the androgen-independent prostate cancer cells (22Rv1), the HER family receptor profile was profoundly altered. These gefitinib concentrations were shown previously to rapidly select for gefitinib-resistant cells (25). In response to gefitinib, there is a shift in the HER axis, and as EGFR expression is downregulated, HER2 expression is upregulated. In parallel, ligand-dependent receptor association patterns are altered and switch from primarily EGFR-containing dimers to HER2-HER3 heterodimers. Whereas MAPK activation is not altered by gefitinib exposure, AKT is less efficiently activated by EGFR-dependent EGFR homodimers but is activated more readily by heregulin β1 (HRGβ1)–dependent HER2-HER3 heterodimers. Ultimately, cells harboring these receptor profile alterations become susceptible to the HER2 heterodimer–inhibiting antibody pertuzumab (2C4). Our results show that EGFR-TKI treatment targets EGFR even in cells that are de novo resistant to EGFR-TKIs and causes a shift in HER axis signaling, potentially "priming" cells for susceptibility to alternative HER-targeting strategies.

Materials and Methods

All experiments were performed at least thrice. Representative data are shown.

Cell Lines and Tissue Culture

Cells were maintained in RPMI 1640 (American Type Culture Collection) supplemented with 10% fetal bovine serum (FBS; Omega Scientific), 1% penicillin-streptomycin (Invitrogen), and 1% glucose (Invitrogen).

**Gefitinib selection of 20GS.** 22Rv1 cells were serially passed in 20 μmol/L gefitinib (a gift from AstraZeneca) for ~4 mo until single-cell clones were obtained.

**Stable transfection of 20GS.** 20GS cells were transiently transfected with pEGFP-N1-EGFR (a gift from Alexander Sorokin; ref. 26) using Effectene (Qiagen). Single-cell clones were selected in presence of G418 and maintained in 200 μg/mL G418. Clonal populations were analyzed for EGFR expression, and fluorescence-activated cell sorting was done for green fluorescent protein (GFP) expression. GFP-positive population was labeled as 20GS-EGFR.

**Ligand and drug treatments.** Cells were serum-starved overnight in phenol red–free RPMI 1640 containing 0.1% charcoal-stripped FBS and supplements incubated with drugs [gefitinib, cetuximab (obtained from Cedars-Sinai Medical Center pharmacy), or 2C4 (pertuzumab, gift from Genentech)] at the indicated concentrations at 37°C for 2 h before ligand addition. Ligands were 30 nmol/L HRG (R&D Systems) at 37°C, 10 min or 4 nmol/L EGF (Invitrogen) at 37°C, 5 min.

**Cell viability assays.** Cells were treated with drugs and/or ligands at the indicated concentrations for 5 d at 37°C. Cell viability was assessed using the ATPLite 1step Luminescence Assay (Perkin-Elmer).

Western Blotting

Immunoblots were performed, as described previously (27). Antibodies used were phosphorylated MAPK p44/42 (#9101), phosphorylated HER3 (Y1289; #4791), phosphorylated EGFR (Y1068; #2236), phosphorylated AKT (#9271), EGFR (#2232), AKT (#9272; Cell Signaling), HER3 (Neomarkers MS-201), ERK1/2 (Santa Cruz Biotechnology), and β-actin (Sigma).

Real-time Quantitative Reverse Transcription–PCR

RNA extraction and real-time quantitative reverse transcription–PCR methods were performed, as described previously (27), using gene-specific primers (900 nmol/L each) and probe (250 nmol/L) from Applied Biosystems. Samples were analyzed using the ΔΔCt method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Animal Studies

All experiments were conducted under CSMC Institutional Animal Care and Use Committee approval. Single-cell tumor suspensions were filtered, incubated for 5 min in RBC lysis buffer (Sigma), and washed in 1× PBS. Cell pellets resuspended
in 50% RPMI 1640/50% Matrigel (BD Biosciences) were injected s.c. into female nude mice (Taconic Labs) right flank at ∼2 × 10⁶ cells per animal.

Gefitinib tablets were ground, resuspended in 0.5% methylcellulose/0.5% Tween 80, and given p.o. 5× a week (100 mg/kg animal body weight). 2C4 was given i.p. 2× a week at 20 mg/kg body weight. The control group received vehicle alone. Treatments typically started 3 to 6 d after tumor implantation. Tumor volumes were measured twice a week with a digital vernier caliper and calculated as π/6 × larger diameter × (smaller diameter)². The data represent three to four experiments of 8 to 10 mice per group, with mice distributed randomly between control and treated groups.

**Statistical Analysis**

We used a Box-Cox transformation (28) of the tumor volume to account for the nonnormality of the distributions, and statistical significance of the treatment effect was tested by a two-sided t test. P values of <0.05 were considered significant. The analyses were performed using the R software package.5

**VeraTag Lysate HER Assays**

VeraTag lysate assays were performed using the VeraTag technology (29). These proximity-based assays were used to measure HER protein complexes using tagged receptor-specific antibodies combined with photo-activated cleavage. The detailed protocol is described in the supplementary data. Monoclonal antibodies were purchased from various sources: EGFR-Ab11, EGFR-Ab5, HER2-Ab5, HER2-Ab4, and HER3-Ab7 (Lab Vision); HER3-IB4C3 (Santa Cruz Biotechnology); and PT100 (Cell Signaling Technology). Optimized antibody concentrations were as follows: EGFR multiplex assay: all antibodies at 0.3 μg/mL; HER2 multiplex assay: HER2 Ab5-biotin at 1 μg/mL, EGFR Ab11-Pro10 and HER3 Ab7-Pro99 at 0.2 μg/mL, HER2 Ab4-Pro14 and PT100-Pro2 at 0.1 μg/mL; HER3 multiplex assay: HER3 IB4-C3-biotin at 2 μg/mL, EGFR Ab11-Pro10 and HER2 Ab4-Pro14 at 0.2 μg/mL, HER3 Ab7-Pro99 and PT100-Pro2 at 0.1 μg/mL. Results are reported as slopes ± SEM expressed as relative peak area (RPA) per milligram of input protein. The statistical significance of different slopes was generated by linear regression analysis using GraphPad prism software.

**Results**

**Gefitinib blocks EGFR activation and signaling, but cell proliferation is only moderately inhibited.** The 22Rv1 cell line expresses a functional EGFR pathway. EGFR is the most highly expressed receptor at the mRNA level, whereas HER3 is expressed ∼5-fold less and HER2 and HER4 are barely detectable (Fig. 1A). These cells express modest levels of the EGFR-specific ligands, transforming growth factor-α (TGF-α), amphiregulin, and β-cellulin. In contrast, EGF and HRGβ1 were not detected (Fig. 1B). The EGFR pathway in 22Rv1 cells is efficiently activated by exogenous EGF, shown by increased EGFR phosphorylation and activation of both MAPK and AKT, the downstream molecular correlates of cell proliferation and survival (Fig. 1C). Moreover, EGF-stimulated EGFR phosphorylation was inhibited with low doses (0.1 μmol/L) of the reversible EGFR-TKI, gefitinib. Phosphorylated AKT and MAPK were also inhibited with 1 and 10 μmol/L of gefitinib, respectively. Also, supporting the presence of a functional EGFR signaling pathway, 22Rv1 xenograft tumors s.c. grown in immunodeficient nude mice show ∼50% tumor growth inhibition in the presence of 100 mg/kg gefitinib (Fig. 1D). However, it should be noted that the gefitinib-mediated inhibition in tumor growth shown in this system is significantly lower compared with systems that either have gene-amplified EGFR or EGFR mutations, such as in NSCLC. These analyses show that the 22Rv1 cell line harbors a functional EGFR pathway that is efficiently inhibited with gefitinib, but the effect on growth and survival is minimal. Therefore, the 22Rv1 cell line can serve as a model to study the apparent disconnect between sensitivity to EGFR-TKIs and the presence of its target, EGFR.

**Chronic exposure to gefitinib downregulates EGFR and upregulates HER2.** To evaluate the role of EGFR and whether the other HER kinase axis receptors contribute to the failure of gefitinib to inhibit growth of 22Rv1 cells, cells were treated with gefitinib concentrations reported to kill cells expressing either wild-type or mutant EGFR, with the exception of those that grow as gefitinib-resistant colonies (25). Chronic exposure to 20 μmol/L gefitinib rapidly selected for 22Rv1 cell clones (20GS) that no longer exhibited the limited responsiveness to gefitinib, as previously measured in vivo as xenograft tumors (Fig. 2A). The expression pattern of the HER kinases was altered in the 20GS cell line both at the level of mRNA (Fig. 2B) and protein (Fig. 2C). EGFR mRNA was reduced ∼100-fold, HER2 mRNA was increased ∼2-fold, and HER3 mRNA remained constant. EGFR-specific ligands, EGF, and TGF-α mRNA levels were increased 10-fold, possibly compensating for reduced EGFR expression (data not shown).

As chronic exposure of cells to gefitinib resulted in reduced EGFR expression (as seen in the 20GS line), recombinant EGFR was stably reintroduced into the 20GS cells generating the 20GS-EGFR cells. The 20GS-EGFR cell line expressed EGFR at ∼25% the level (based both on mRNA and protein expression) of the parental 22Rv1 cells (Fig. 2B and C). Reintroduction of EGFR in the 20GS cell line did not alter HER2 and HER3 receptor levels. Protein expression of the HERs in 22Rv1, 20GS, and 20GS-EGFR cells was verified using the VeraTag assay (Supplementary Fig. S1). The relative receptor protein expression in the three 22Rv1 cell lines was in concordance with the mRNA measurements (Fig. 2B).

However, modified HER kinase expression patterns in the cell lines caused only minor modifications in the cellular response to gefitinib (Fig. 2D). As expected, downregulation of the primary molecular target for gefitinib, EGFR, led to decreased sensitivity to high doses of gefitinib in the 20GS cell line and interestingly conferred a growth advantage at gefitinib concentrations sufficient for inhibition of EGFR signaling. Reintroduction of EGFR in the 20GS cells (20GS-EGFR) was sufficient to restore gefitinib sensitivity that paralleled the
parental 22Rv1 cell line (Fig. 2D). However, growth inhibition by gefitinib was minimal for all of the cell lines with only some decrease in growth seen at very high gefitinib doses.

**Chronic EGFR kinase inhibition modulates HER ligand-dependent signaling.** Chronic exposure to gefitinib altered EGFR and HER2 gene expression. As low doses of gefitinib effectively inhibited EGF-dependent signaling in the parental 22Rv1 cells (Fig. 1C), we investigated whether changes in the HER kinase axis also altered EGFR- and HER2-driven signaling pathways. All cell lines (22Rv1, 20GS, and 20GS-EGFR) were growth factor starved and stimulated with either EGF or HRGβ1 in the presence or absence of gefitinib to assess downstream signaling.

The level of EGF-stimulated EGFR phosphorylation, measured by either Western blot using phosphorylated site-specific antibodies or proximity-based pan-phosphorylated antibodies using VeraTag assays (Fig. 3A), EGFR-dependent AKT activation correlated with both EGFR expression and phosphorylation (Fig. 3A). AKT was activated by EGF in 22Rv1 cells and was undetectable in 20GS cells, but AKT activation was restored by the reintroduction of recombinant EGFR in 20GS-EGFR cells. The VeraTag assay corroborated these observations (Fig. 3C). EGFR-dependent phosphorylated AKT signals in both 22Rv1 and 20GS-EGFR cells were inhibited with comparable doses of gefitinib (Fig. 3A). These observations indicate a direct role for EGFR expression in modulating EGF-dependent phosphorylated AKT signaling that correlates with response to gefitinib.

In contrast, HRGβ1-stimulated phosphorylated AKT was detectable in all cell lines consistent with similar HER3 expression (Fig. 3C and D). However, HRGβ1-stimulated
phosphorylated AKT was four to five times greater in 20GS and 20GS-EGFR relative to 22Rv1 (Fig. 3C and D). This directly paralleled HRGβ1-mediated HER3 phosphorylation (Fig. 3D). Furthermore, a log-fold greater gefitinib concentration of 100 nmol/L was necessary to partially inhibit HRGβ1-stimulated HER3 phosphorylation and phosphorylated AKT in the 20GS and 20GS-EGFR cells compared with the 22Rv1 cell line. These differences could be due to higher HER2 expression in the 20GS and 20GS-EGFR cells, gefitinib cross-reactivity with HER2 (30, 31), or possibly increased HER2 sensitivity to gefitinib in HER2-overexpressing cells (32). An analysis of the EGFR, HER2, and HER3 phosphorylation patterns in the presence of HRGβ1 stimulation combined with gefitinib inhibition revealed that HER2 phosphorylation was indeed inhibited with 100 nmol/L gefitinib (Supplementary Fig. S2). Furthermore, gene-specific knockdown of HER2, but not EGFR, in 20GS cells downregulated HRGβ1-mediated HER3, AKT, and MAPK phosphorylation signals showing a role for HER2 in gefitinib-selected cells (Supplementary Fig. S3).

EGFR and HER2 expression levels influence both HER kinase receptor phosphorylation and dimerization. As distinctive receptor profiles were identified in response to chronic gefitinib exposure, we evaluated which receptors were responsible for the altered downstream signaling observed in the 22Rv1, 20GS, and 20GS-EGFR cell lines. We compared the relative phosphorylation of EGFR, HER2, and HER3 in response to specific ligand stimulations in all cells using VeraTag assays. EGF stimulation of 22Rv1, 20GS, and 20GS-EGFR cells did not alter total protein levels of EGFR, HER2, or HER3. However, in response to HRG, EGFR and HER2 levels were unaltered, but there was a small but reproducible decrease in HER3 protein (Fig. 4A).

EGFR was phosphorylated in all cell lines at levels that paralleled receptor expression (Fig. 4B). Phosphorylation of

Figure 2. Characterization of 22Rv1, 20GS, and 20GS-EGFR cell lines. A, gefitinib-treated (100 mg/kg, 5× a week) 20GS xenograft tumors. Arrow, initiation of dosing. B, EGFR, HER2, and HER3 mRNA expression normalized to GAPDH and presented as a percentage of receptor expression in 22Rv1 cells. C, immunoblot analysis of receptor protein expression in 22Rv1, 20GS, and 20GS-EGFR cells with β-actin as the normalization control. D, cell viability assays in response to increasing doses of gefitinib. Results plotted as percentage of control.
EGFR occurred exclusively in response to EGF. In contrast, whereas HER2 phosphorylation tracked with receptor expression, both EGF and HRGβ1 stimulated HER2 phosphorylation. HER3 phosphorylation seemed to follow unique regulation as HRGβ1-dependent HER3 phosphorylation paralleled HER2 expression whereas EGF-dependent HER3 phosphorylation paralleled EGFR expression. VeraTag assays were used to assess which ligand-stimulated receptor dimers correlated with the observed receptor phosphorylation. In parental 22Rv1 cells, which primarily express EGFR, EGFR-EGFR homodimers were observed exclusively in response to EGF stimulation (Fig. 4C) in agreement with the observation that EGFR phosphorylation was detected only in response to EGF. Although low levels of EGF-stimulated phosphorylated HER2 and phosphorylated HER3 were observed, we did not detect ligand-stimulated EGFR-HER2 or EGFR-HER3 heterodimers, but rather there seemed to be baseline ligand-independent heterodimerization in the high EGFR-expressing lines. HRG stimulation did not induce any detectable receptor dimer partners in the 22Rv1 cells.

Similar analysis on the 20GS cells showed ~2-fold increase in EGFR homodimers in response to EGF stimulation. The fold-change value was comparable with homodimer measurements in 22Rv1 cells. However, overall there were fewer homodimers in the 20GS cells relative to the 22Rv1 cells, corresponding to EGFR expression and phosphorylation. Unlike the direct correlation of the EGFR homodimers with EGFR expression, the ~3-fold increase in EGF-dependent HER2-EGFR heterodimerization does not correlate with EGFR expression but rather seems to correlate with both EGFR and HER2 phosphorylation. There was very little EGF-stimulated HER3 phosphorylation in 20GS cells, and consequently only
minor levels of EGF-stimulated phosphorylated AKT were detected. However, HRGβ1 stimulated high levels of HER2-HER3 heterodimers (Fig. 4C) correlating with the observed HRGβ1-dependent HER2 and HER3 phosphorylation. Lastly, the 20GS-EGFR cells expressed both EGF-stimulated and HRGβ1-stimulated HER dimers in concordance with the observed phosphorylation patterns. These cells displayed EGF-mediated EGFR homodimerization, increased basal ligand-independent EGFR dimerization, and HRGβ1-mediated HER2-HER3 heterodimerization comparable with 20GS cells. Overall, EGFR homodimers are the major HER dimer species of the HER kinase signaling network in the parental 22Rv1 cells, whereas HER2 heterodimers predominate upon EGFR downregulation in 20GS cells. 20GS-EGFR cells, which express both EGFR and HER2, display both EGFR homodimers and HER2 heterodimers upon ligand stimulation.

Figure 4. VeraTag assay measurements of total receptor expression (A), receptor phosphorylation (B), and receptor dimerization (C) in response to EGF or HRGβ1 stimulation. Statistically significant differences are indicated (*, P < 0.05; **, P < 0.0001).
**Receptor dimers determine the dominant HER signaling pathway and subsequent response to HER-targeted therapeutics.** In response to chronic gefitinib treatment, EGFR expression is reduced while HER2 expression is increased. This altered receptor profile is less responsive to EGFR-targeted therapies. Therefore, we evaluated whether these cells would be susceptible to HER2-targeting therapies. 22Rv1, 20GS, and 20GS-EGFR cells were treated with either EGFR-directed therapeutics, including gefitinib or cetuximab, or a HER2-targeted therapeutic, pertuzumab (2C4).

22Rv1 cells were serum-starved and stimulated with either EGF or HRGβ1. EGF stimulated EGFR phosphorylation that, in turn, activated AKT and MAPK. Cetuximab and gefitinib, but not pertuzumab, inhibited EGF-stimulated EGFR and AKT phosphorylation (cetuximab was more effective than gefitinib). EGF-stimulated MAPK was not inhibited by any of the HER-targeted therapeutics (Fig. 5A). Alternatively, HRGβ1 stimulated HER3 phosphorylation but caused only a slight increase in the basal EGFR phosphorylation. The HRGβ1-dependent activation of EGFR, HER3, AKT, and MAPK was inhibited by the EGFR inhibitors but not by the HER2 dimerization inhibitor pertuzumab, indicating dependence on EGFR.

In contrast, in 20GS cells in which there is a preponderance of HRGβ1-stimulated HER2 heterodimers, only pertuzumab effectively blocked dimerization as evaluated by inhibition of HRGβ1-induced phosphorylated HER3, phosphorylated AKT, and phosphorylated MAPK measurements.
HER Kinase Dimer Switching

Discussion

Although there are a number of HER-targeted therapies in clinical development or Food and Drug Administration approved for cancer treatment, some tumors, despite the presence of the HER target, either are not inhibited or show a short-term response (23, 33, 34). The current definition of drug responsiveness is based on inhibition of cellular proliferation, although HER-targeting inhibitors can cause alterations in the HER axis (24) even in the absence of this critical phenotype. Our data show that cells, defined as “resistant” to EGFR-targeted therapeutics, undergo similar shifts in receptor profiles as the “responsive” cells. Potentially, these altered receptor profiles can be used to guide therapy and may be inhibited by alternative HER-targeting therapies.

A functional receptor pathway, such as EGFR, is often not sufficient to predict sensitivity (35) to tumor growth inhibition by EGFR-targeted therapeutics. However, despite the absence of measurable growth inhibition, which typifies “primary therapeutic resistance,” the dynamic network of the HER family receptors and ligands (reviewed in ref. 36) still undergoes transient and long-term adaptation in response to targeted inhibition. This reprogramming of the HER axis may not only activate alternate receptor/signaling pathways that contribute to resistance but may also reveal escape routes from targeted inhibition. Our results show that in 22Rv1 cells, which contain functional EGFR activity, cell proliferation was not dramatically affected by prolonged gefitinib exposure, rather the HER axis was reorganized and EGFR was downregulated while HER2 was upregulated. This possible gefitinib escape route through HER2 could allow for the formation of HER2-HER3 heterodimers, which could be targeted by pertuzumab. Interestingly, a subtle but reproducible growth advantage was observed in the 20GS cells relative to both the parental 22Rv1 or the add-back 20GS-EGFR cells in the presence of gefitinib. The parallel growth pattern of both the EGFR-expressing cell lines compared with the EGFR-negative 20GS cell line supports the dominant role of EGFR for growth. Although growth was only minimally affected in 22Rv1 cells, gefitinib effectively blocked the EGFR pathway based on decreased EGFR phosphorylation and inhibition of downstream signals, including EGF-dependent AKT phosphorylation. However, inhibition of both EGFR and AKT phosphorylation by gefitinib did not accurately reflect gefitinib sensitivity based on cellular proliferation but may perhaps have been an indicator for potential HER axis switching.

Despite the altered HER axis profiles, the ability to activate AKT was sustained in all cell lines consistent with its central role in both tumorigenesis (reviewed in ref. 37) and resistance (18, 19, 38). However, the mechanism of activation was influenced by altered HER family expression, as well as susceptibility to alternative targeted inhibitors. HRGβ1-dependent AKT activation was poorly inhibited by gefitinib in cells with higher levels of HER2 (20GS and 20GS-EGFR), whereas AKT was potently inhibited in cells with low HER2 expression (22Rv1). At high HER2 expression, the predominant mechanism of HRGβ1-dependent AKT activation is likely to occur in response to HER2-HER3 heterodimerization, which is not targeted by gefitinib, whereas at low HER2 expression, the HRGβ1-dependent AKT activation could be a consequence of HRG-activated HER3 that comes into contact with overexpressed EGFR, the target of gefitinib. It seems that AKT activation and inhibition may be a downstream marker of receptor switching and consequential susceptibility to alternate HER-targeted therapeutics.

The roles of both EGFR and HER3 in EGFR-TKI susceptibility have been well studied. Coexpression of EGFR and HER3 has been shown previously to correlate with erlotinib sensitivity in both pancreatic and colorectal cell lines (39). Furthermore, inhibition of EGF-stimulated AKT phosphorylation in EGFR-TKI sensitive systems suggests that signaling occurs between EGFR and HER3 (40). However, the mechanism by which EGF stimulates HER3 phosphorylation and consequently AKT activation is unclear. In our study, EGF-stimulated AKT activation and subsequent inhibition by EGFR-TKI were only observed in the gefitinib-sensitive cell lines (22Rv1 and 20GS-EGFR). EGFR and HER3 were measured in close proximity by VeraTag assays in these cells. However, there was no evidence for ligand-dependent EGFR-HER3 heterodimerization. It is possible that a fraction of the detected EGFR-HER3 heterodimers is ligand-activated, as suggested by the complementary receptor phosphorylation measurements, but that these heterodimers are transient. Alternatively, it is possible that the receptors flux between open and closed conformations and in the absence of ligand, particularly at higher receptor expression, both ligand-dependent as well as ligand-independent heterodimers, are detected but are indistinguishable in the assay. In cells...
with high EGFR expression basal EGFR and HER3 phosphorylation was detected, which correlated with EGFR-HER3 close proximity dimers. However, in these cell lines, there was no detectable growth advantage in the presence of gefitinib.

In summary, our study provides insight into EGFR-TKI-mediated HER kinase axis modulations in systems that are supposedly “resistant” to EGFR kinase inhibition. Our data indicate that receptor switching mechanisms are likely to occur in tumors both as a resistance mechanism for EGFR-TKI “responsive” cells and as a priming mechanism in receptor-expressing but not growth-inhibited cells (EGFR-TKI “resistant” cells). The heterogeneous nature of tumors, coupled with the plasticity of the HER axis, makes receptor-use switching and altered response to targeted therapies very probable. Multinode strategies targeting multiple HER family members or the signaling pathways common for the HER axis may lead to more effective treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank James Mirocha, Jeff Sperinde, and Agnes Paquet for statistical analysis; John Curran, Jack Altura, and Rob Fannon for technical assistance; and AstraZeneca and Genentech for the gifts of gefitinib and pertuzumab, respectively.

Grant Support

Jerry and Joyce Monkarsh Young Investigator Award (Prostate Cancer Foundation; A. Jain), NIH 1-R21 NS059381-01 (A. Jain), and Sumner Redstone Prostate Cancer Research Program (D.B. Agus and A. Jain).

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Received 09/08/2009; revised 12/17/2009; accepted 12/21/2009; published OnlineFirst 02/16/2010.

References


39. Buck E, Eyzaguirre A, Haley JD, Gibson NW, Cagnoni P, Iwata KK. Inactivation of Akt by the epidermal growth factor receptor inhibitor erlotinib is mediated by HER-3 in pancreatic and colorectal tumor cell lines and contributes to erlotinib sensitivity. Mol Cancer Ther 2006;5:2051–9.

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Cancer Res  Published OnlineFirst February 16, 2010.

Updated version Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-3326

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