Modulation of HER3 Is a Marker of Dynamic Cell Signaling in Ovarian Cancer: Implications for Pertuzumab Sensitivity

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Abstract
This study was designed to evaluate the expression of HER receptors as a marker of sensitivity to the humanized anti-HER2 monoclonal antibody pertuzumab in ovarian cancer cells. In a recent clinical trial, low levels of HER3 mRNA have been shown to associate with pertuzumab response when combined with gemcitabine. We sought to define how pertuzumab modulated HER expression levels in ovarian cancer using cell line models to better understand differential and dynamic receptor expression in therapeutic response. Changes in HER3 mRNA expression were also assessed in pertuzumab-treated xenografts. HER3 mRNA and, to a lesser extent, HER2, were down-regulated after stimulation both with heregulin-β1 and epidermal growth factor in a range of ovarian cancer cell lines either growth sensitive or growth resistant to pertuzumab. Pertuzumab reversed this down-regulation and the magnitude of the reversal correlated with pertuzumab sensitivity. The change in HER3 mRNA expression correlated inversely to how much the extracellular signal-regulated kinase and phosphoinositide 3-kinase pathways were dynamically activated with stimulation. Finally, up-regulation of HER3 mRNA was found in cancer xenografts treated with pertuzumab. We conclude that HER3 mRNA is down-regulated by both heregulin-β1 and epidermal growth factor activation. This suggests that in some tumors, low HER3 mRNA expression is driven by, or dependent on, growth factor. HER3 mRNA expression is effectively reversed in pertuzumab-sensitive tumors. These data are consistent with low HER3 mRNA identifying a pertuzumab-sensitive phenotype. (Mol Cancer Res 2009;7(9):1563–71)

Introduction
Pertuzumab (rhuMAb 2C4) is a humanized monoclonal antibody that binds to HER2, a member of the HER family of receptor tyrosine kinases (1). The HER family consists of four members: epidermal growth factor (EGF) receptor (also termed HER1/ErbB-1), HER2/ErbB-2/Neu, HER3/ErbB-3, and HER4/ErbB-4 (2). There are numerous HER-specific ligands that generate signaling diversity within the cell; EGF is a ligand specific to EGF receptor (3), whereas heregulin-β1 (HRG-β1) binds HER3 and HER4 (2, 4, 5). No naturally occurring direct ligand for HER2 has been discovered. Binding of ligands leads to the homodimer and heterodimer formation of the receptor tyrosine kinase (2, 4, 5). Dimerization stimulates the intrinsic tyrosine kinase activity of receptors, and activates downstream-signaling molecules such as AKT and extracellular signal-regulated kinase (ERK), which in turn determine cell proliferation and survival (6). Pertuzumab is the first of a new class of agents known as HER dimerization inhibitors, which act by binding to the dimerization arm of HER2 (domain II), thus preventing dimerization with other HER partners (7). This binding site differs from the binding domain of trastuzumab (Herceptin) and thereby pertuzumab differs in its actions from trastuzumab.

The growth and progression of many ovarian cancers has been linked to high levels of expression of either HER1 (8) or HER2 (9). Because high levels of HER2 expression have been identified in 6.6% to 40% of ovarian cancers and this has subsequently been associated with poor survival (9-12), ovarian cancer patients are prospective candidates for HER2-targeted therapies. Trastuzumab has been studied previously in HER2-overexpressing ovarian cancer and showed an overall response rate of 7.3% included one complete and two partial responses with 39% patients achieving stable disease (13). Because pertuzumab may also act in low as well as high HER2-expressing cancers, it has been evaluated in a phase II trial of advanced chemoresistant ovarian cancer against ovarian cancers with any level of HER2 expression (14). In that study, 117 patients were assessable for response and 5 patients (4.3%) had a partial response, 8 patients (6.8%) had stable disease for at least 6 months, and 10 patients showed a CA125 reduction of at least 50% (including two partial responses and four stable disease), indicating that 14.5% of ovarian cancer patients derived some clinical benefit from pertuzumab (14). This trial then led to a phase II study comparing gemcitabine with gemcitabine plus pertuzumab (15). Analysis of a number of biomarkers within that study suggested that expression levels of HER3 mRNA might provide prognostic and predictive...
information (15). In the group of pertuzumab/gemcitabine-treated patients, the hazard ratio for patients with lower HER3 mRNA-expressing tumors was 0.34 (95% confidence interval, 0.18-0.63), whereas that for high expressing tumors was 1.48 (95% confidence interval, 0.83-2.63; ref. 15). These results suggest that HER3 mRNA expression levels may be a potential prognostic and predictive diagnostic biomarker. Furthermore, the investigators postulated that a negative feedback loop might exist in tumor cells with HER2/HER3 dimerization leading to down-regulation of HER3 gene expression (15). The process of HER receptor down-regulation and degradation is well documented as a major negative feedback regulatory mechanism that controls the intensity and duration of receptor signaling (16). In another trial evaluating carboplatin-based chemotherapy with pertuzumab versus chemotherapy alone, the investigators suggested that low HER3 mRNA expression may be predictive for response to chemotherapy + pertuzumab, particularly in patients with a shorter treatment-free interval (17).

However, the molecular basis of the HER3 mRNA down-regulation and how it relates to pertuzumab sensitivity remains undefined. Here, we investigated the HER receptor family mRNA and protein modulation in ovarian cancer cell lines and found that HER3 undergoes significant change following growth factor stimulation and pertuzumab treatment, in vitro and in vivo. Moreover, we found HER3 mRNA down-regulation correlates with the dynamic activation of ERK and phosphoinositide 3-kinase (PI3K) signaling, measured by pERK and pAKT, respectively. Taken together with the clinical data, these results identify HER3 mRNA as a potential candidate biomarker that may reflect sensitivity and response to pertuzumab treatment.

Results

HRG-β1 and EGF-Dependent Cell Growth in Ovarian Cancer Cells and Inhibition by Pertuzumab

Ligand-dependent stimulation of ovarian cancer cell growth was first examined to define the responsiveness of a panel of cell lines to growth factors. Five ovarian cancer cell lines (PE01, 41M, OAW42, OVCAR4, and SKOV-3) were cultured in medium with charcoal-stripped serum for 48 hours and then treated with either HRG-β1 (1 nmol/L) or EGF (1 nmol/L) for 72 hours. Cell viability was assessed by the sulforhodamine B assay (Fig. 1A and B). The PE01 and 41M cell lines were growth stimulated by HRG-β1, whereas OVCAR4 and SKOV3...
cell lines showed no growth change and OAW42 showed minimal change (Fig. 1A). EGF addition increased the growth of the PE04 and 41M cell lines significantly, and produced smaller effects in the other 3 cell lines (Fig. 1B). These results confirm previous findings with this panel of cell lines (18).

Simultaneous addition of pertuzumab (100 nmol/L) produced varying degrees of growth inhibition in the five cell lines tested. In PE04 cells, HRG-β1 stimulation was almost completely reversed by pertuzumab, and in 41M cells, HRG-β1 stimulation was partially reversed (Fig. 1A). No significant effects were seen in the cell lines where HRG-β1 was not growth stimulatory. Pertuzumab had only minor impact on EGF-stimulated growth (Fig. 1B). These results show that PE04 and 41M both are growth factor and pertuzumab sensitive, whereas others are less sensitive or insensitive cell lines. In the absence of growth factor, pertuzumab inhibited PE04, 41M, and SKOV3 cells (Fig. 1C).

The expression levels of the HER receptors in the pertuzumab-sensitive (PE04 and 41M) and pertuzumab-insensitive (OAW42 and OVCAR4) cell lines were also determined using qRT-PCR and Western blotting under stripped serum conditions (Fig. 1D and E). Messenger RNA expression (Fig. 1D) and protein expression (Fig. 1E) levels associated well. Comparison of the HER1-3 receptor expression levels under such nonstimulated conditions suggested that low HER1/HER3 ratios were associated with pertuzumab sensitivity (Fig. 1D).

Growth Factor-Induced Down-Regulation of HER3 mRNA and Its Reversal by Pertuzumab in PE04 and 41M Ovarian Cancer Cell Lines

In primary ovarian cancers, both HRG-β1 and EGF (or its related ligand transforming growth factor α) are expressed and can act in an autocrine/paracrine manner on HER receptor-positive cells (19, 20). To test whether these growth factors modulate HER3 expression, we first analyzed HER3 mRNA levels in the two pertuzumab-sensitive cell lines (PE04 and 41M) treated with HRG-β1 and EGF over a 72-hour time course (Fig. 2). Values are shown relative to no treatment control. HRG-β1 markedly decreased HER3 mRNA in both the PE04 and 41M cell lines. The reduction was most significant at 8 hours for PE04 cells where the HER3 mRNA value decreased to 10% of the control value (Fig. 2A). A similar but lesser reduction was observed in the 41M cell line (Fig. 2B). These results show that HRG-β1 can induce down-regulation of HER3 mRNA. In parallel to the growth response, we found that pertuzumab reversed the HRG-β1-induced HER3 mRNA decrease. This pertuzumab-reversal was smaller in 41M cells relative to PE04 cells.

EGF treatment also produced a reduction of HER3 mRNA in both PE04 and 41M cells (Fig. 2B). Pertuzumab produced a minimal reversal in PE04 cells and a negligible reversal in 41M cells consistent with the effects on growth.

These results indicate that growth factors acting through the HER receptors cause HER3 mRNA down-regulation, and that the effect can be reversed or prevented by pertuzumab.

Effects of HRG-β1, EGF, and Pertuzumab on HER Receptor Expression in Ovarian Cancer Cell Lines

We next examined whether HER3 mRNA modulation was observed in cell lines that were less sensitive to growth factors and pertuzumab, and also modulation of other key growth-promoting members of the HER family, HER1 and HER2 (Fig. 3).

Data are shown as percentage values relative to nontreatment control and were measured at 8 hours. Although HER3 mRNA was markedly down-regulated in the PE04 and 41M cell lines by HRG-β1, the effect was reduced in the less sensitive OAW42 cell line and further reduced in the insensitive OVCAR4 cell line (Fig. 3A). EGF caused a reduction of HER3 mRNA in all four cell lines. The pattern of change for HER2 mRNA was similar to that of HER3 mRNA (Fig. 3B); the relative change in HER2 mRNA again broadly reflecting the growth changes. By contrast, HER1 mRNA expression (Fig. 3C) was relatively unchanged in the growth factor and
pertuzumab-sensitive cell lines (PE04 and 41M). In the OAW42 and OVCAR4 cell lines, expression was decreased.

Therefore, among the three major sets of HER receptors, HER3 mRNA is the most dynamically regulated and relates best to sensitivity to growth factors and pertuzumab, although HER2 also shows a reasonable association. The effect of pertuzumab on HER3 mRNA in HRG-β1–stimulated cell lines correlates with the pattern of growth sensitivity (e.g., PE04>41M>OAW42>OVCAR4; Fig. 3D). However, a similar analysis with EGF-stimulated cells did not show any marked difference (Fig. 3E).

We next sought to assess whether HER protein changes reflected mRNA expression changes (Fig. 4). HER receptor expression was assessed after HRGβ1 treatment over a 48-hour time course. Consistent with mRNA expression changes, HER3 protein decreased in PE04 and 41M cells but not in OVCAR4, OAW42, or SKOV3 cells. HER2 protein expression decreased in PE04, OVCAR4, and OAW42 cells. By contrast, HER1 expression increased in PE04 cells and decreased a little in 41M cells and more so in OAW42 cells in parallel with mRNA findings. These data strongly support the idea that growth factors can dynamically modulate HER gene and protein expression.

**Negative Feedback on HER3 mRNA Is Associated with Activation of ERK and PI3K Pathways**

We next investigated which signaling pathways are associated with HER3 down-regulation. Specific PI3K (LY294002) and MAP/ERK kinase inhibitors (PD98059 and U0126) were used to assess the involvement of the PI3K and ERK pathways on the feedback process. Cells were incubated with HRGβ1 (1 nmol/L) in the absence or presence of pertuzumab (100 nmol/L), LY 294002 (20 mmol/L), PD 98059 (20 mmol/L), or U0126 (20 mmol/L for PE04 because PD 98059 was ineffective even at

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**FIGURE 3.** Effects of HRG-β1 and EGF with or without pertuzumab on HER3 (A), HER2 (B), and HER1 (C) receptor expression. Quantification of mRNA expression was carried out by QRT-PCR normalized to actin mRNA levels. Results are shown as percentage change compared with unstimulated control cells. Cells were incubated with each stimuli for 8 h. HER receptor expression changes induced by pertuzumab addition were calculated relative to HRG-β1 (D) or EGF (E) alone, from Fig. 2A to C. Statistically significant differences are shown as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student's t-test).
40 mmol/L) for 8 hours. The effects of these inhibitors were first checked on phosphoHER2, phosphoHER3, phosphoAKT, and phosphoERK (Fig. 5A). OAW42 and SKOV3 cell lines were mutant for the PIK3CA gene (H1047L and H1047R), respectively, whereas the remaining cell lines were confirmed as wild-type (21). Similarly, PTEN expression is lowest in these two cell lines relative to the other three (21).

As expected, HRGβ1 strongly activated HER2, HER3, and downstream AKT and ERK in PE04 and 41M cells. Here, we observed phosphorylated p95HER2 together with full-length p185HER2. Pertuzumab significantly inhibited HRGβ1–stimulated phosphorylation of HER2 and HER3 in PE04 cells, but only partly HER3 phosphorylation in 41M cells; it reversed AKT and ERK phosphorylation in both of these cell lines but less so in 41M cells, corresponding to their relative sensitivity to pertuzumab. In the other three cell lines, HRG-β1 activation was marginal and pertuzumab did not influence AKT and ERK phosphorylation. LY 294002 or PD 98059/U0126 inhibited AKT or ERK activation, respectively, although inhibition of each pathway activates the other to a certain degree.

Under the same conditions, we determined HER3 mRNA expression (Fig. 5B), and the comparison revealed that HER3 mRNA modulation correlated with AKT/ERK activation. In PE04, 41M, and OAW42 cells (despite each inhibitors' effects being less marked due to cross talk signaling between AKT and ERK), the sum of AKT and ERK activation, but not HER2 and/or HER3 activation, correlated with HER3 down-regulation. In OVCAR4 and SKOV3 cells, the association between AKT/ERK activation and HER3 down-regulation is more direct and it is noteworthy that if AKT/ERK is inhibited to lower than untreated control levels, HER3 mRNA expression increased to greater than untreated control values. We also checked HER2 and HER1 mRNA changes again and found that the pattern of HER2 changes is similar to HER3, whereas HER1 is less significant (data not shown).

Taken together, these results suggest that HER3 down-regulation involves both AKT and ERK pathways, in at least some of the pertuzumab-sensitive and pertuzumab-insensitive cell lines. From these observations, we hypothesized how HER3 mRNA down-regulation and pertuzumab sensitivity might be connected (Fig. 6A). If there is a dynamic activation of AKT and/or ERK induced by HER2 dimerization that is driven by growth factors such as HRG-β1 or EGF, it also induces HER3 mRNA down-regulation. These cell lines are pertuzumab sensitive because the dimerization is blocked by pertuzumab. On the other hand, if the cell has a constitutive signaling activation from other factors (e.g., HER2 overexpression/amplification, PIK3CA mutation, or low PTEN expression, for example in OAW42 and SKOV3 lines) and becomes less dependent on growth factor–induced HER2 dimer signaling, dynamic activation of AKT/ERK, i.e., HER3 down-regulation, is partial or not observed. Even if pertuzumab partially reduces AKT/ERK activation, cell survival and proliferation persist so that the cell remains pertuzumab-insensitive.
Confirmation of a Dynamic HER3 mRNA Modulation on Xenograft Tumors

To assess whether pertuzumab could modulate HER3 mRNA expression in vivo, we evaluated dynamic regulation of HER3 mRNA by pertuzumab in a xenograft model. For this, we used the BT474 breast cancer xenograft model as this has been reported to be pertuzumab responsive (22, 23).

Treatment of the BT474 xenograft model with pertuzumab produced a clear growth inhibition relative to control treatment with PBS only (Fig. 6B). After 3 and 7 days of pertuzumab treatment, HER3 mRNA expression (as measured by qRT-PCR) was increased in pertuzumab-treated tumors relative to controls (Fig. 6C). We also investigated HER1 and HER2 mRNA expression changes but they were not as significant as HER3 changes (data not shown). HER3 therefore seems to be most discriminative in vivo. These results support the idea that pertuzumab dynamically reverses the growth factor down-regulation of HER3 mRNA in vivo.

Discussion

Pertuzumab has shown activity as a single agent in ovarian cancer but, in common with other active agents thus far identified, it acts in only a subset of ovarian cancers (14). If a biomarker of sensitivity to pertuzumab could be identified, patients could be selected for treatment with a higher probability of response. Analysis of biomarkers within a clinical study of pertuzumab combined with gemcitabine identified low HER3 mRNA expression as being associated with improved time to progression and survival (15). This would be consistent with HRG-β? down-regulating HER3 mRNA expression and it is feasible that this down-regulation is a means of attenuating the response to ligand. Here, we provide experimental support for these previous findings, and importantly, we show that EGF also induced HER3 mRNA down-regulation and pertuzumab could reverse this.

Our analysis of five ovarian cancer cell lines revealed contributory components of the underlying mechanism of HER3 mRNA down-regulation and pertuzumab sensitivity (Fig. 6A). In PE04 and 41M cells, where this negative-feedback loop is fully in place, growth factors produce a marked stimulation, and on addition, strong activation of pAKT and pERK that results in 90% reduction of HER3 mRNA expression. Pertuzumab is growth inhibitory in this situation and inhibits the growth factor activation process with consequent blockade of all these downstream effects. We observed differences between PE04 and 41M that probably reflect different dimerization patterns due to different receptor expression levels (e.g., HER2/HER3 ratio versus HER1/HER2 ratio).

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**FIGURE 5.** Inhibitor effects on the HER3 mRNA negative feedback. Cells were pretreated with 100 nmol/L pertuzumab (per), 20 mmol/L LY 294002 (L), and 20 mmol/L PD 98059 except for PE04, 20 mmol/L U0126 used (P/U) for 15 min following incubation with HRG-β1 (1 nmol/L) for 8 h. A. Western blot analysis of the phosphorylation status of HER2(Tyr 877), HER3 (Tyr 1289), AKT, and ERK. Lysates are prepared and blotted as described in Materials and methods. AKT and ERK were used as loading control. B. QRT-PCR of HER3 mRNA. Under the same conditions, total RNA was isolated from the cells. Statistically significant differences are shown as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Student’s t test).
In the other three cell lines, there is a constitutive activation of pAKT and/or pERK in the absence of growth factor. For example, in SKOV3 cells, overexpression of HER2 would be non-ligand driven and lead to constitutive activation, and in addition, it harbors an activating mutation in PIK3CA. Likewise, OAW42 harbors an activating mutation of the PIK3CA gene, which would be expected to decouple phosphorylation of AKT from receptor-mediated activation (24). Other upstream activation via non-HER receptor pathways that might be present includes oncogenic mutations, loss of tumor suppressors (e.g., PTEN), and insulin-like growth factor-IR-mediated regulation. In this situation, the stimulation through HER ligand activation is negligible relative to the other activation processes leading to minimal down-regulation of HER3 mRNA. Pertuzumab is hardly effective on HER3 mRNA or growth response in these cell lines.

In this study, we observed an intrinsic difference between HRG-β1 and EGF stimuli. The observation that pertuzumab is less effective at reversing EGF-induced HER3 mRNA down-regulation than the HRG-β1-induced one is consistent with two explanations: First, pertuzumab blocks HER2:HER3 dimer formation more effectively than HER2:HER1 dimerization (7), and the former provides the most intensive stimulation of the PI3K/AKT pathway among all possible HER family dimers (25), because HER3 contains at least six phosphorylation sites recruiting the p85 regulatory subunit of PI3K (5). Second, EGF may induce HER1:HER1 and HER1:HER2 dimerization, whereas HRG-β1 predominantly promoted HER2:HER3 dimer formation, and this could be part of the reason why the pertuzumab reversal effect on EGF-induced HER3 down-regulation is small relative to the effect of pertuzumab on HRG-β1 (Fig. 3E).

Because pertuzumab reverses the down-regulation, this suggests that HER2 is at least partially implicated in this process. It is possible for example that in PE04 cells, the HRG-β1 effect is superior to that of EGF as the relatively higher HER3 expression may allow more HER3:HER2 dimerization than HER1:HER2 dimerization. In 41M cells, a higher level of HER1 expression allows a more potent EGF effect. Analysis of HER expression in unstimulated conditions showed that a high HER3 versus HER1 ratio before stimulation is correlated with pertuzumab sensitivity. This could suggest a larger HER3 pool to be activated by growth factor stimuli that could be inhibited by pertuzumab.

A recent study of the relative roles of HER3 and HER1 in amplified HER2 breast cancers suggested that HER3 plays a central role in HER2-amplified breast cancer. Inhibition of HER1 had little effect on growth, whereas inhibition of HER3 was as potent as inhibition of HER2 (26). These data argue that pertuzumab may have a valuable role in inhibiting growth in these tumors (27). We are currently investigating which kind of homodimer and heterodimer pattern is predominant with each

FIGURE 6. A. Schematic presentation of proposed association between HER3 mRNA feedback and pertuzumab sensitivity. B. Measurement of BT474 xenograft tumor volume treated with PBS control or pertuzumab. C. HER3 mRNA expression normalized to actin was assessed by QRT-PCR and found to be increased in pertuzumab treated BT474 xenograft (black columns) relative to control (white columns). P values for Student’s t test are shown.
growth factor stimulus and inhibited or not in both pertuzumab sensitive and insensitive cell lines.

There is another interesting difference between HER receptor behavior in that HRG-β1 and EGF down-regulated both HER3 and HER2 mRNAs but not HER1 mRNA. These effects on mRNA expression were then reflected in decreased HER2 and HER3 protein expression for the growth-sensitive PEO4 cell line. The decrease in HER3 (and HER2) mRNA correlated with the response to the growth factor in a panel of cell lines and its reversal in the presence of pertuzumab again correlated with response to drug. These results support the view that if HER3 mRNA expression is low within an ovarian cancer, that tumor may be growth factor driven. We have previously observed in a panel of ovarian cancer cell lines that response to transforming growth factor α was not linked to EGF receptor expression and cell lines with very low levels could be very responsive to transforming growth factor α (20). Although the paradigm for trastuzumab sensitivity is a requirement for a high level of HER2 receptor, this does not seem to necessarily be the case for pertuzumab.

Finally, we could show HER3 mRNA modulation in vivo, too. Consistent with a growth response, HER3 expression was increased with pertuzumab. If serial samples of ovarian cancer ascites could be obtained from patients, a raised level of this biomarker after treatment with pertuzumab might provide information on response.

In conclusion, HRG-β1–induced HER3 down-regulation is a surrogate of active HER signaling–dependent tumor, which would be accompanied with pertuzumab sensitivity. These data lend support for the idea of measuring HER3 expression in ovarian cancers as it may indicate a growth factor driven cancer.

Materials and Methods

Cell Lines

The ovarian cancer cell line PEO4 was established within the Edinburgh Cancer Research Centre (28). SKOV3 cells were obtained from the American Type Culture Collection. OV-CAR4 was obtained from Dr. T.C. Hamilton (Fox Chase Institute). 41M and OAW42 cells were obtained from the European Collection of Cell Cultures. All cells were grown routinely as monolayer cultures in RPMI 1640 supplemented with 10% heat-inactivated FCS and 100 IU/mL penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37°C.

Western Analysis

Cells were preincubated in RPMI 1640 without phenol red but containing 5% double charcoal-stripped serum for 24 h before treatment with EGF (1 nmol/L; E9644 Sigma) or HRG-β1 (1 nmol/L; R&D Systems). Inhibitors pertuzumab (100 nmol/L; Roche Diagnostics GMBH Penzberg), LY 294002 (Merck), PD 98059 (Merck), and U0126 (Merck) were added 15 min before growth factors. To harvest, cells were washed with PBS and lysed in 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EGTA, 10 μg/mL aprotinin (Sigma), Complete Protease Inhibitor Cocktail (Roche), and Phosphatase Inhibitor Cocktail 1 and 2 (Sigma), and spun for 10 min at 16,000 × g at 4°C. The protein content of the resulting supernatant was determined by the Bicinchoninic acid protein assay (Sigma). Protein lysates were electrophoretically resolved on 7.5% to 10% SDS-PAGE and transferred to Immobilon-P membranes. After transfer, membranes were blocked with 1% blocking agent (Roche) in TBS before probing overnight at 4°C with the appropriate primary antibody. Antibodies used for Western blotting were as follows: anti-total HER3 (Cell Signaling Technology) at 1:1000; anti-phosphorylated HER3 (pHER3; Tyr1289; Cell Signaling Technology) at 1:1000; anti-total HER2 (Cell Signaling Technology) at 1:1000; anti-phosphorylated HER2 (pHER2; Tyr473; Cell Signaling Technology) at 1:1000; anti-total EGF receptor (Cell Signaling Technology) at 1:1000; anti-total ERK 1/2 (Cell Signaling Technology) at 1:1000; anti-phosphorylated ERK (pERK) 1/2 (Cell Signaling Technology) at 1:1000; anti-total Akt (Cell Signaling Technology) at 1:1000; anti-phosphorylated Akt (pAkt; Ser473; Cell Signaling Technology) at 1:1000; and anti-actin (Merck). Immunoreactive bands were detected using enhanced chemiluminescent reagents (Roche) and Hyperfilm-enhanced chemiluminescence film (Amersham). Integrated absorbance values were obtained by densitometric analysis using a gel scanner and analyzed by Labworks gel analysis software (UVP Life Sciences).

Sulforhodamine B Growth Assays

Log-phase cells were seeded into 96-well flat-bottomed tissue culture plates (optimized between 1,000 and 4,000 cells per well). The following day, cells were washed in PBS and transferred to RPMI 1640 containing 5% double charcoal-stripped serum for 48 h before treatment. Cells were treated with either EGF (1 nmol/L) or HRG-β1 (1 nmol/L) ± pertuzumab (100 nmol/L). Cells were removed from the incubator after 72 h, and ice-cold 25% TCA solution (50 μL) was added to each well. All plates were placed at 4°C for 60 min after which the TCA solution was removed. Plates were washed under running tap water (10 times) and dried before staining with sulforhodamine B dye solution for 30 min at room temperature. Trays were again washed with 1% glacial acetic acid (four times) at room temperature, air dried, and resuspended in 10 mmol/L Tris buffer (pH 10.5; 150 μL) before reading at 540 nm.

Statistics

Relationships between variables were analyzed by Student’s t test (Microsoft Excel).

RNA Extraction and qRT-PCR

Total RNA was extracted from cells using Absolutely RNA Miniprep kit (Stratagene) as per instruction manual. RNA concentration was determined using a Nanodrop (Thermo Scientific). qRT-PCR reactions were done on a Rotorgene 3000 thermal cycler (Qiagen) according to the manufacturers instructions for one-step QRT-PCR. Reaction volumes of 15 μL included 40 ng RNA and 0.5 μmol/L primers. The Rotorgene PCR cycle conditions were as follows: reverse transcription step 50°C for 30 min, TAQ activation step 95°C for 15 min, PCR 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 45 s. After a final extension of 72°C for 5 min, a PCR product melt curve was done from 60°C to 99°C. The following primers were used as follows: β-actin: 5'-CTACGTGCGACTGACTGACGACG-3' (forward) and 5'-GATGGAGGCCGCAGATCCACACGG-3' (reverse); HER1: 5'-CAAGCCTACTTGTGATCCA-3' and
5'-TGACTCAGAGGCTCAGA-3'; HER2: 5'-CTCGTT-GGAAAGGAGACG-3' and 5'-CTGAGGCTC- TTTGTT-3'; HER3: 5'-ACACACTTGAGGACGAA-3' and 5'-TAGCCACAGCAATTTCC-3'.

In vivo Tumor Models

For xenograft studies, adult female nu/nu mice were implanted s.c. with BT474 tumor fragments (previously established from the cell line) in the flank and groups of tumors were grown. Animals were at least 8-wk-old at the time of experimentation and were maintained in negative pressure isolators. Tumor fragments were implanted s.c. into both flanks of nude mice and allowed to grow to 4 to 6 mm in diameter (over a period of ~1 mo). Animals were then allocated to treatment (5 mice/group) or control (10 mice/group) groups and treatment was commenced (defined as day 0). Pertuzumab (20 mg/kg) was given via the i.p. route in saline on days 0, 3, 7, and 10. Tumor size was measured twice weekly using calipers and the volume was calculated according to the formula \( \pi/6 \times \text{length} \times \text{width}^2 \). Relative tumor volumes (%) were then calculated for each individual tumor by dividing the tumor volume on day \( t \) (V\(_t\)) by the tumor volume on day 0 (V\(_0\)) and multiplying by 100.

Total RNA was then prepared from 10 to 50 mg of frozen tissue preincubated with RNAlater-ICE (Ambion) using the miRNAasy Mini kit (Qiagen) and TissueRuptor (Qiagen) following the manufacturers' instructions. The RNA quality was checked by the RNA 6000 Nano assay on the Agilent Bioanalyzer (Agilent Technologies). RNA Integrity Numbers were >8.5. qRT-PCR was done as described above.

PIK3CA Mutation Analysis

PIK3CA mutation analysis was done using the ARMS/Scorpions multiplexed PCR assay as previously described (29) to detect the four most common mutations in PIK3CA [H1047L, H1047R (Exon 20), E545K and E542K (Exon 9)].

Disclosure of Potential Conflicts of Interest

Dr. Max Hasmann is an employee of Roche and some support for this study was provided by Roche.

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