EPHA2 Blockade Overcomes Acquired Resistance to EGFR Kinase Inhibitors in Lung Cancer

Katherine R. Amato, Shan Wang, Li Tan, Andrew K. Hastings, Wenqiang Song, Christine M. Lovly, Catherine B. Meador, Fei Ye, Pengcheng Lu, Justin M. Balko, Daniel C. Colvin, Justin M. Cates, William Pao, Nathanael S. Gray, and Jin Chen

Abstract

Despite the success of treating EGFR-mutant lung cancer patients with EGFR tyrosine kinase inhibitors (TKI), all patients eventually acquire resistance to these therapies. Although various resistance mechanisms have been described, there are currently no FDA-approved therapies that target alternative mechanisms to treat lung tumors with acquired resistance to first-line EGFR TKI agents. Here we found that EPHA2 is overexpressed in EGFR TKI-resistant tumor cells. Loss of EPHA2 reduced the viability of erlotinib-resistant tumor cells harboring EGFR T790M mutations in vitro and inhibited tumor growth and progression in an inducible EGFR L858R-T790M-mutant lung cancer model in vivo. Targeting EPHA2 in erlotinib-resistant EGFR kinase activity as well as exquisite sensitivity to first-generation EGFR-specific tyrosine kinase inhibitors (TKI), such as erlotinib (4–6). Unfortunately, approximately a year after commencing treatment all patients treated with EGFR TKIs acquire resistance to these therapies (7). Sequencing efforts have revealed that tumors with acquired resistance to EGFR TKIs commonly gain an additional mutation, T790M, in the gatekeeper position of the kinase domain of EGFR (8). Currently, there are limited options for the treatment of first-generation EGFR TKI (erlotinib)-resistant tumors, although some success has been observed with administration of second-generation (9) and third-generation (10) EGFR TKIs or combining antibody therapy targeting EGFR with second-generation inhibitors (11). Risks of persistent and/or mutation-specific targeting of EGFR include likely development of alternative mechanisms of TKI resistance distinct from further mutations in EGFR (10), including oncogene addiction to other kinases. Such “bypass” RTK signaling is a well-documented mechanism of EGFR TKI resistance as evidenced by compensatory activation of MET, HER2, AXL, IGF1R, and FGFR in the context of EGFR TKI-acquired resistance (12–17). Identifying bypass pathways responsible for mediating TKI resistance may provide novel targets needed for therapeutic intervention.

EPHA2 is overexpressed in lung cancer, correlating to poor patient outcomes (18–20). EPHA2 belongs to the largest family of RTKs, the EPH RTKs, which have been implicated in the regulation of a wide array of pathological conditions, including cancer (21). Upon binding to their ligands, EPHRINS, EPH RTKs oligomerize and are capable of activating multiple downstream signaling pathways, including RAS/MAPK, PI3K/akt, and RHO/RAC (21). We previously reported that targeting EPHA2 in ERBB2-driven murine mammary tumor models resulted in impaired

Introductions

Lung cancer remains the leading cause of cancer-related deaths in the United States despite a significant number of advancements in the molecular diagnosis and treatment of this disease (1). One of the most extensively studied molecular subset in lung cancer is those harboring activating mutations in the epidermal growth factor receptor (EGFR) gene. These mutations, most commonly a point mutation in exon 21 (L858R) or a deletion in exon 19 (LREA; ref. 2), are activating mutations that result in enhanced

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S. Wang and L. Tan contributed equally to this article.

Corresponding Author: Jin Chen, Vanderbilt University School of Medicine, 2706 MCN, 1161 21st Avenue South, Nashville, TN 37232. Phone: 615-343-3819; Fax: 615-343-8648; E-mail: jin.chen@vanderbilt.edu
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tumor initiation and metastatic progression, and that heightened levels of EPHA2 were sufficient to mediate resistance to ERBB2 TKI therapy in human breast cancer cell lines (22, 23). In lung cancer, genetic and pharmacologic inhibition of EPHA2 results in increased tumor cell death in vitro and decreased tumor burden in vivo (24). However, the role of EPHA2 in resistance to EGFR TKIs in lung cancer remains undefined.

Because targeted inhibition of EPHA2 has proven useful in lung cancer subtypes with constitutive MAPK signaling and because EPHA2 expression positively correlates to TKI resistance of a known ERBB family member in breast cancer, we hypothesized that it would be an effective target for the treatment of EGFR TKI-resistant lung cancer. In this study, we found that EPHA2 is overexpressed in erlotinib-resistant lung cancer cells compared with erlotinib-sensitive lung cancer cells. Genetic ablation of EPHA2 in EGFR T790M mutant, erlotinib-resistant cells led to both increased apoptosis and decreased proliferation. Gene targeting of EphA2 in an inducible, genetically engineered mouse model of EGFR TKI resistance led to decreased tumor growth and progression. Treatment of EGFR TKI-resistant cells with an ATP-competitive, small-molecule TKI of EPHA2 ALW-II-41-27 decreased cell viability in vitro and tumor growth in vivo. Collectively, these studies demonstrate the promise and utility of targeting EPHA2 in EGFR TKI-resistant lung cancer.

Materials and Methods

Microarray analysis

Data from 58 matched lung tumor specimens and adjacent normal lung (116 total samples) with annotated mutation status were downloaded from Gene Expression Omnibus (GSE32863; ref. 25). Normalized gene expression data for EPHA2 were extracted and compared between normal and tumor tissue in all patients or by the presence or absence of the EGFR genotype. A paired-sample Student t test was used to compare normal versus tumor for each group, using patient-specific matching.

For microarray experiments, RNA was extracted from erlotinib-sensitive and erlotinib-resistant cell lines in the absence of erlotinib for 72 hours (26). Microarray profiling was performed using U133 Plus chips (Affymetrix). Normalized expression data were analyzed in R3.1.1. Hierarchical clustering was performed using the complete linkage algorithm. Distances for clustering were calculated using Matlab 2012a (The MathWorks Inc.), and 5% FBS. All mouse experiments were conducted under Institutional Review Board (IRB)-approved protocols (Vanderbilt University IRB# 050644). Written informed consent was obtained from all patients. All samples were de-identified, and protected health information was reviewed according to the Health Insurance Portability and Accountability Act guidelines. Paired patient tumor samples before and after TKI treatment were stained for EPHA2 and analyzed in R3.1.1. Hierarchical clustering was performed using the complete linkage algorithm. Distances for clustering were calculated using Matlab 2012a (The MathWorks Inc.), and protected health information was reviewed according to the Health Insurance Portability and Accountability Act guidelines.

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Tumor biopsy samples

All patient tumor biopsy samples were obtained under Institutional Review Board (IRB)-approved protocols (Vanderbilt University IRB# 050644). Written informed consent was obtained from all patients. All samples were de-identified, and protected health information was reviewed according to the Health Insurance Portability and Accountability Act guidelines. Paired patient tumor samples before and after TKI treatment were stained for EPHA2 and analyzed in R3.1.1. Hierarchical clustering was performed using the complete linkage algorithm. Distances for clustering were calculated as 1 − r, where r represents the correlation coefficient value. All tests are significant at the two-sided 5% level, false discovery rate; corrected P values were reported for multiple comparisons.

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Figure 1. EPHA2 expression in erlotinib-sensitive and erlotinib-resistant EGFR-mutant lung cancer. A, comparison of EPHA2 expression in tumor and adjacent normal tissue from 58 paired lung adenocarcinoma patient samples (25). ALL, all tumor samples; EGFR, tumor samples harboring EGFR mutations; N, normal (green); T, tumor (red). **P < 0.005, paired Student t test. B, PC-9 erlotinib-resistant cells (PC-9/ER), erlotinib-resistant clones (PC-9/ERC15 and PC-9/ERC16), and HCC827 erlotinib-resistant cells (HCC827/ER) were assessed for resistance to erlotinib. **P < 0.005, two-way ANOVA with Bonferroni post hoc analysis. C, heatmap showing the mRNA expression profiles of EPH RTKs and EPHRIN ligands in erlotinib-sensitive PC-9 cells and PC-9 cells with acquired resistance to erlotinib (PC-9/ER). D, EPHA2 protein expression levels were assessed via Western blot analysis in erlotinib-resistant cell lines (ER) and afatinib-resistant cell lines (BR) harboring EGFR T790M and their parental cells. E, EPHA2 expression and phosphorylation in PC-9/ERC16 cells were measured over time after erlotinib was removed from the culture media. Phosphorylation of ERK was also assessed over this time course. F, IHC showed increased EPHA2 levels in EGFR TKI-resistant tumor samples compared with pretreatment tumor specimens from three patients. Scale bar, 50 μm.
Immunohistochemistry

IHC staining on tumor sections was performed as described previously (32–34), using antibodies against EPHA2 (Life Technologies; #347400), EGFR L858R (Cell Signaling Technology, #3197S), proliferating cell nuclear antigen (PCNA; BD Pharminogen, #555567), and Von Willebrand factor (vWF; DakoCytomation, #A0082). PCNA$^+$ staining was quantified as the average percentage of PCNA$^+$ nuclei relative to total nuclei (proliferation index). Apoptosis assays were performed using the Apoptag Red In Situ Apoptosis Detection Kit as per the manufacturer’s protocol (Millipore). TUNEL$^+$ staining was quantified as the percentage of TUNEL$^+$ nuclei relative to total nuclei (apoptotic index). Tumor vessels were quantified by assessing the vWF$^+$ vessels (pixels). Four fields of at least 5 independent tumors per genotype or treatment condition were assessed for all staining quantification. Biotin goat anti-rabbit (BD Pharmingen), anti-rabbit Cy3 (Jackson Immunoresearch), retrievagen A (pH 6.0; BD Pharmingen, #550524), streptavidin peroxidase reagents (BD Pharmingen, #51-75477E), and the liquid 3,3'-diaminobenzidine tetrahydrochloride substrate kit (Zymed Laboratories) were used for IHC. Cytoseal XYL (Richard Allan Scientific) or ProLong Gold antifade reagent with DAPI (Life Technologies) were used to mount slides.

Tumor xenografts

HCC827/ER (2.5 × 10$^6$) or PC-9/ERC16 (1 × 10$^6$) cells were injected with Matrigel into the hind flanks of 6-week-old athymic nude mice (Foxn1nu; Harlan). Once tumors reached approximately 150 mm$^3$, mice were randomized by body weight and tumor volume into treatment groups (n = 5 per group) to receive 15 mg/kg of either erlotinib, ALW-II-41-27, or the vehicle alone (10% 1-methyl-2-pyrrolidinone and 90% PEG 300) twice daily via intraperitoneal injection. Tumors were measured daily with digital calipers, and tumor volumes were calculated by using the following formula: volume = length × width$^2$ × 0.52. Additionally, to monitor the toxicity of the given drugs, body weight was measured daily.

Results

EPHA2 is overexpressed in EGFR-mutant lung cancer with further overexpression upon development of acquired resistance to EGFR TKIs

EPHA2 is overexpressed in lung cancer patient tumor samples irrespective of the histological subtype (19). To investigate whether EPHA2 expression correlates with EGFR mutation status in...
Analysis of a dataset of 58 matched normal and lung adenocarcinoma tissue samples, we found that EPHA2 expression was significantly higher (P = 0.003) in tumor tissue compared to adjacent normal tissue, consistent with previous studies. In patients whose tumors tested positive for an EGFR mutation, EPHA2 expression was also markedly increased compared to adjacent normal tissue.

Given the known role of EPHA2 in promoting lung cancer growth and survival, we investigated if EPHA2 is upregulated in EGFR-mutant lung cancer cells with EGFR T790M-mediated, acquired resistance to erlotinib. The erlotinib-resistant, EGFR-mutant lung cancer cell lines PC-9/ER and HCC827/ER were generated after completion of a drug escalation protocol. Microarray analysis of PC-9 and PC-9/ER cells revealed that three EPH receptors, EPHA2, EPHB2, and EPHB4, were overexpressed in the erlotinib-resistant PC-9/ER cells, compared to erlotinib-sensitive PC-9 parental cells. To validate these findings, we assessed EPHA2 protein levels in the same isogenic, paired cell lines (PC-9 and PC-9/ER) as well as an independent cell line pair, HCC827 and HCC827/ER. EPHA2 was overexpressed in all of the erlotinib-resistant cell lines, as well as in two independent single-cell EGFR T790M-containing clones derived from the PC-9/ER cell line, confirming observations from our gene expression analysis. Interestingly, we found that...

Figure 3.
Loss of EphA2 results in decreased tumor burden and increased survival in a TKI-resistant EGFR L858R+T790M transgenic model. A, lungs of EGFR L858R+T790M mice from wild-type (EphA2+/+) or EphA2 knockout (EphA2−/−) were collected, and total lung wet weight was measured at 10 and 15 weeks of age to assess the additional mass contributed to the lungs by the tumor burden. Average lung weight ± SEM is shown (n = 10 per time point per genotype). **, P ≤ 0.005; two-way ANOVA with Bonferroni post hoc analysis (n.s., not statistically significant). B, wild-type and EphA2-deficient mice were subjected to MRI analysis at 15 and 20 weeks of age. T2-weighted MRI images were taken in the axial plane with slice thickness of 1 mm. Representative images at 15 and 20 weeks are shown. White arrows indicate tumor tissue (H, heart; S, spine). C, tumor volumes were quantified as a composite of 10 serial MRI slices of the lung per mouse using Matlab software and were graphed as an average tumor volume (mm³) ± SEM (n ≥ 5 mice per genotype). D, Kaplan-Meier survival curves for EGFR L858R+T790M mice with or without EphA2. Mutant EGFR gene expression was induced by doxycycline (DOX) at 3 weeks of age. **, P < 0.005.
Figure 4.

EPHA2 deficiency results in decreased proliferation and increased apoptosis in EGFR<sup>L858R+T790M</sup> tumors. A, hematoxylin and eosin (H&E)-stained lung sections (20 weeks) from EGFR<sup>L858R+T790M</sup> EphA2<sup>+/+</sup> and EGFR<sup>L858R+T790M</sup> EphA2<sup>-/-</sup> mice in the presence or absence of doxycycline (DOX). Scale bar, 200 μm. Loss of EPHA2 protein expression and the presence of EGFR<sup>L858R+T790M</sup>-mutant protein expression were confirmed by IHC. Scale bar, 50 μm. B, apoptosis in tumor sections was measured by the TUNEL assay. TUNEL<sup>+</sup> nuclei (red) are indicated with arrowheads. Scale bar, 30 μm. C, apoptosis was quantified as a percentage of TUNEL<sup>+</sup> nuclei relative to the total nuclei. An apoptosis index is presented as the average percentage of TUNEL<sup>+</sup> nuclei per total nuclei ± SEM (n = 5 per genotype). D, tumor cell proliferation was assessed by PCNA IHC. Arrowheads, representative proliferating nuclei. Scale bar, 50 μm. E, proliferation was quantified by assessing the total number of PCNA<sup>+</sup> nuclei (brown) compared with the total nuclei. A proliferation index was calculated as a proportion of PCNA<sup>+</sup> nuclei per total nuclei ± SEM. F, the presence of tumor microvessels was assessed by vWF immunofluorescence staining (red, indicated by arrowheads). Scale bar, 50 μm. G, microvessels in the tumor were quantified by measuring vWF<sup>+</sup> pixels in each tumor field ± SEM (n = 5 per genotype). **, P < 0.05; ***, P < 0.01, Student t test.
EPHA2 could be regulated by the presence of erlotinib, and EPHA2 expression and phosphorylation increased in a time-dependent manner after erlotinib withdrawal in PC-9/ERC16 cells (Fig. 1E), consistent with a previous observation that EPHA2 expression is regulated by MAPK signaling (35). In addition to mRNA and protein expression levels, we assessed the cellular localization of EPHA2 in the context of acquired resistance to erlotinib. As judged by confocal immunofluorescence, the presence of EPHA2 on the cell surface was not altered by sensitivity to erlotinib (Supplementary Fig. S1).

Finally, we assessed EPHA2 expression in samples from patients with EGFR mutations pre- and post-development of resistance to EGFR TKIs. In 4 samples with matched pretreatment and post-relapse tumor sections, we detected higher EPHA2 protein levels by IHC than by IgG control stained adjacent sections in 3 of the post-relapse tumor samples (Fig. 1F and Supplementary Fig. S2). Overall, we determined that EPHA2 is overexpressed in EGFR-mutant lung cancer cells harboring EGFR<sup>T790M</sup>-mediated resistance to erlotinib compared with EGFR-mutant lung cancer cells with sensitivity to erlotinib.

Figure 5. EPHA2 regulates cell signaling that promotes proliferation and survival. A, erlotinib-sensitive and erlotinib-resistant cell lines were transduced with lentiviruses containing either shEPHA2 or a pLKO.1 vector control. The resulting cell populations were selected in 15 μg/mL puromycin for 5 days. Proliferation was measured by quantifying incorporation of BrdU into the cellular DNA using the BrdU Cell Proliferation Assay kit. Experiments were repeated three times, and data are presented as a proliferation index relative to the shControl/C6 SEM. **, P < 0.01, ***, P < 0.005, two-way ANOVA with Bonferroni post hoc analysis. B, apoptosis was measured by quantifying histone-associated DNA fragments using a Cell Death ELISA kit (Roche). Experiments were repeated three times, and data are presented as an apoptotic index relative to the shControl/C6 SEM. **, P < 0.01, ***, P < 0.005; two-way ANOVA with Bonferroni post hoc analysis; n.s. not statistically significant. C, Western blot analysis for cleaved (CL) caspase-3 or PARP in lysates from erlotinib-sensitive or erlotinib-resistant cells that had been treated with control (shControl) or EPHA2-specific (shEPHA2) shRNA. Lysates were collected 72 hours after puromycin selection. D, signaling analysis on cell lysates from two pairs of isogenic erlotinib-sensitive and erlotinib-resistant cell lines were treated with siControl or siEPHA2 for 72 hours. Shown are representative immunoblots in which signaling molecules were detected with the indicated antibodies.
EGFR inhibitors, suggesting a possible correlation between EPHA2 expression and EGFR TKI sensitivity both in vitro and in the clinical setting.

**EPHA2 promotes the cell viability of erlotinib-resistant lung cancer**

To determine if EPHA2 was required for cellular survival in EGFR TKI-resistant lung cancer, we knocked down the expression of EPHA2 using a lentiviral-based shRNA strategy in four erlotinib-resistant and two erlotinib-sensitive lung cancer cell lines. Both of the two independent shRNAs against EPHA2 silenced EPHA2 protein expression and reduced cell viability when tested 3 days after puromycin selection (Fig. 2A and B). Although shEPHA2 reduced cell viability in both erlotinib-sensitive and erlotinib-resistant cell lines, erlotinib-resistant cells displayed a greater dependence on EPHA2 for cell survival than erlotinib-sensitive cell lines. For example, 72 hours after puromycin selection, EPHA2-deficient, erlotinib-resistant PC-9/ER and HCC827/ER cells displayed 20% and 40% cell viability, respectively, while erlotinib-sensitive PC-9 and HCC827 cells maintained 45% and 90% cell viability (Fig. 2C). We next performed a time course to monitor cell viability after EPHA2 knockdown in these cells. The results showed that by 5 days after puromycin selection, EPHA2-deficient, erlotinib-resistant cells displayed a further reduction in the number of viable tumor cells, in some cases with only 10% overall cell viability (Fig. 2D). These data suggest that TKI-resistant lung cancer cells are dependent upon EPHA2 RTK for survival.

To determine the contribution of EPHA2 kinase activity in maintaining cell viability of erlotinib-resistant lung cancer, we knocked down endogenous EPHA2 and rescued cells with either wild-type EPHA2 or EPHA2ΔDE39N, a kinase-dead mutant (36). We observed that wild-type EPHA2, but not EPHA2ΔDE39N, was sufficient to restore cell viability to the control level (Supplementary Fig. S3), indicating that EPHA2 kinase activity is required for maintaining viability of erlotinib-resistant lung cancer cells.

**EPHA2 promotes tumor growth in an inducible transgenic model of EGFR<sup>L858R/T790M</sup>-mutant lung cancer in vivo**

To assess the contribution of EPHA2 to EGFR TKI-resistant lung cancer in vivo, we crossed EphA2-deficient animals with an inducible EGFR<sup>L858R/T790M</sup>-mutant lung cancer transgenic model (29, 31). In this model, expression of the mutant EGFRI (TestO-EGFR<sup>L858R/T790M</sup>, CCSP–rtTA) is induced upon doxycycline administration and resulting tumors are resistant to erlotinib. To assess tumor burden in lungs of EGFR<sup>L858R/T790M</sup>/EPH2<sup>+/+</sup> and EGFR<sup>L858R/T790M</sup>/EPH2<sup>−/−</sup> mice, we measured the lung wet weight over a time course, as described previously (24, 37). A significant reduction in lung weight was observed in doxycycline-treated, EGFR<sup>L858R/T790M</sup>/EPH2<sup>−/−</sup> mice compared with doxycycline-treated, EGFR<sup>L858R/T790M</sup>/EPH2<sup>+/+</sup> mice (Fig. 3A). Because no changes in lung weight were observed between EGFR<sup>L858R/T790M</sup>/EPH2<sup>−/−</sup> and EGFR<sup>L858R/T790M</sup>/EPH2<sup>+/+</sup> mice that were not fed doxycycline, we attribute the differences seen in mice fed doxycycline to a reduction in tumor burden. To further quantify tumor burden, we monitored mice by MRI when tumors had developed in both groups at 10 weeks of age and again at 15 weeks of age (Fig. 3B). As expected, tumors did not develop in any of the mice not fed doxycycline. Quantification of the MRI images revealed that doxycycline-fed EGFR<sup>L858R/T790M</sup>/EPH2<sup>−/−</sup> mice had a lower overall tumor burden than EGFR<sup>L858R/T790M</sup>/EPH2<sup>+/+</sup> mouse counterparts, which became more evident as the mice aged (Fig. 3C). EPHA2 deficiency also correlated to significantly longer overall survival in this model of TKI-resistant EGFR<sup>L858R/T790M</sup>-mutant lung cancer. EPHA2<sup>−/−</sup> mice did not survive past 25 weeks of age, whereas more than 25% of the EPHA2<sup>+/+</sup> mice survived longer than 1 year on doxycycline (Fig. 3D).

Histological analysis of the lungs confirmed the presence of tumors and EGFR<sup>L858R/T790M</sup> expression in doxycycline-treated animals as well as the absence of EPHA2 expression in EPHA2 knockout animals (Fig. 4A). Western blot analysis confirmed EPHA2 expression in the EPHA2<sup>−/−</sup> animals and a complete lack of EPHA2 protein expression in mice with a targeted deletion of EPHA2, as measured by anti-EPHA2 antibodies against either the N-terminal or C-terminal regions of the EPHA2 protein (Supplementary Fig. S4). In doxycycline-fed, tumor-bearing mice, relative levels of apoptosis, proliferation, and tumor microvessels were quantified. Apoptosis was significantly higher in the tumors of EGFR<sup>L858R/T790M</sup>/EPH2<sup>−/−</sup> mice, than in tumors of EGFR<sup>L858R/T790M</sup>/EPH2<sup>+/+</sup> mice, as measured by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (Fig. 4B and C). Tumor cell proliferation was measured by staining tumor sections for PCNA (Fig. 4D). Proliferation was significantly decreased in EGFR<sup>L858R/T790M</sup>/EPH2<sup>−/−</sup> tumors than in tumors of mice with wild-type levels of EPHA2 (Fig. 4E). Because previous murine studies in breast cancer have indicated that EPHA2 can function to support tumor vasculature (33), we assessed tumor microvessels by VWF IF. No significant differences in VWF staining were seen in the tumor tissue between EGFR<sup>L858R/T790M</sup>/EPH2<sup>+/+</sup> and EGFR<sup>L858R/T790M</sup>/EPH2<sup>−/−</sup> mice (Fig. 4F and G). Together, these data indicate that EPHA2
is required for the maintenance and progression of EGFR TKI-resistant lung cancer in their intrinsic setting and microenvironment, such that deletion of EphA2 limited proliferation and induced apoptosis in this tumor model.

**EPHA2 regulates cell viability in erlotinib-resistant cells through upregulation of proliferation and inhibition of apoptosis**

To dissect the mechanism by which EPHA2 is required for cell viability in erlotinib-resistant lung cancer cells, we quantified both proliferation and apoptosis after silencing EPHA2 (shEPHA2) by using a BrdU incorporation assay and a Cell Death ELISA, respectively. Upon EPHA2 knockdown, we observed a decrease in proliferation of approximately 74% compared with shControl cells, while cell lines sensitive to erlotinib displayed only a 22% decrease in proliferation (Fig. 5A). Consistent with this effect, loss of EPHA2 in erlotinib-resistant cells resulted in a 3.4-fold increase in cellular apoptosis, compared with an only 2-fold increase in cells undergoing apoptosis from EPHA2 knockdown in erlotinib-sensitive cells (Fig. 5B). Knockdown of EPHA2 also increased cleavage of caspase-3 and PARP (Fig. 5C), confirming elevated apoptosis in erlotinib-resistant cells. Signaling analysis from two independent cell lines that contain EGERT790M-mediated erlotinib resistance (PC-9/ERC16 and HCC827/ER) revealed that loss of EPHA2 decreased phosphorylation of p90-RSK, S6 kinase 1, and the prosapoptotic BH3-only protein BAD, whereas other effector proteins did not appear to be affected by EPHA2 loss (Fig. 5D). The phenotype observed is not due to off-target effects, as two independent siRNAs against EPHA2 recapitulated signaling defects seen in the pooled siEPHA2 knockdown (Supplementary Fig. S5). These results consistently suggest a mechanism by which EPHA2 expression maintains cell viability in cells with acquired resistance to erlotinib by promoting both survival and proliferation pathways.

**Pharmacologic inhibition of EPHA2 decreases cell survival of erlotinib-resistant lung cancer cells in vitro and tumor growth in vivo**

To assess the value of pharmacologic inhibition of EPHA2 in lung cancer subsets with acquired resistance to first-generation EGFR TKIs, we treated cells with an EphA2 small-molecule inhibitor, ALW-II-41-27, that was recently characterized for EPHA2 target engagement and specificity in the context of lung cancer and melanoma both in vitro and in vivo (24, 38). NG-25, a structural analogue that possesses a similar profile of kinase targets but does not inhibit EPHA2, was used as a control. We first assessed the effects of pharmacologic inhibition of EPHA2 via ALW-II-41-27 on four cell lines with acquired resistance to erlotinib. TKI-resistant cells treated with 1 μmol/L of ALW-II-41-27 displayed a time-dependent decrease in the number of viable tumor cells with an average reduction of cell viability of 60% 72 hours after drug treatment, whereas there was no significant change in the viability of cells treated with NG-25 at the same dose (Fig. 6A). To determine the versatility of EPHA2 inhibition in various contexts of acquired resistance to EGFR TKIs, we assessed the effectiveness of ALW-II-41-27 on cells resistant to the third-generation EGFR inhibitor AZD9291. We found that ALW-II-41-27 inhibited cell viability to a similar extent in cells resistant to AZD9291 as in cells resistant to erlotinib, suggesting that EPHA2 represents a potentially important bypass pathway that could be leveraged in multiple settings of EGFR TKI resistance (Fig. 6B).

To determine whether the reduced cell viability observed upon EPHA2 inhibitor treatment was due to decreased cell proliferation or increased apoptosis, we performed BrdU incorporation and Cell Death ELISA assays. Treatment with 1 μmol/L ALW-II-41-27 decreased cell proliferation (Fig. 6C) and increased apoptosis (Fig. 6D) in erlotinib-resistant cell lines. ALW-II-41-27–induced apoptosis was accompanied by the cleavage of caspase-3 and PARP as well as decreased expression of antiapoptotic proteins BCL-xL and MCL-1 (Fig. 6E). Immunofluorescence studies revealed that EPHA2 is located on the cell surface regardless of sensitivity to erlotinib, but ALW-II-41-27 inhibited ligand-induced EPHA2 endocytosis, consistent with the notion that EPHA2 kinase activity is required for receptor endocytosis (Supplementary Fig. S6). To assess the acute signaling consequences of targeting EPHA2 via ALW-II-41-27 treatment, cell lysates were collected from erlotinib-sensitive and erlotinib-resistant lung cancer cells after treatment with 1 μmol/L ALW-II-41-27 for 6 hours. Signaling studies revealed decreased phosphorylation of EPHA2 (Y588 and S897) and its key effector proteins, such as p90-RSK, S6K1, S6, and BAD (Fig. 6F), recapitulating the effects observed in EPHA2 knockdown experiments. These data suggest that ALW-II-41-27 inhibits EPHA2 signaling pathways necessary to maintain proliferation and survival in erlotinib-resistant EGFRT790M-mutant lung cancer cells.

To assess the utility and efficacy of ALW-II-41-27 on tumors with acquired resistance to erlotinib in vivo, we treated xenografted tumors (HCC827/ER or PC-9/ERC16) with ALW-II-41-27, erlotinib, or the vehicle alone twice a day at 15 mg/kg via intraperitoneal injection. After 14 days of the treatment regimen, ALW-II-41-27 significantly inhibited growth of the erlotinib-resistant tumors (Fig. 7A and B). Toxicity as measured by body weight was not significantly changed by any of the drugs compared with the vehicle over the course of this study (data not shown). Analysis of tumor lysates revealed decreased phosphorylation of EPHA2 (Y588 and S897), p90-RSK, and S6K1 (Fig. 7C and D), consistent with results observed in vitro (Fig. 6F). These data indicate that pharmacologic inhibition of EPHA2 may be advantageous in lung cancers with acquired resistance to erlotinib as inhibition of this receptor is able to mitigate key survival signaling pathways and induce an apoptotic phenotype.

**Discussion**

EGFR-mutant lung tumors acquire resistance to TKIs through a variety of mechanisms, including secondary mutations within EGFR at position T790 (8), mutations in EGFR effector proteins (39, 40), histologic transformation (39), and upregulation of parallel RTKs (e.g., MET, HER2, and AXL; refs. 13, 14, 17). Here we have demonstrated that EPHA2 overexpression serves as an additional novel mechanism of drug resistance particularly in EGERF790M-mutant lung cancer. We found that knockdown of EPHA2 resulted in decreased proliferation and increased apoptosis in erlotinib-resistant cells with EGERF790M mutations. Genetic targeting of EPHA2 significantly inhibited EGERF790M-mutant lung tumor progression and prolonged overall survival in vivo. Furthermore, an EPHA2 small molecule inhibitor, ALW-II-41-27, mitigated viability of erlotinib-resistant cells and reduced tumor growth in a xenograft model. These data suggest that pharmacologic inhibition of EPHA2 may represent a viable, alternative strategy for treating EGERF790M-mutant lung cancers harboring resistance to first-line EGFR TKI therapies.
The ability of EPHA2 to maintain cell viability in the context of tumorigenesis has been demonstrated previously. EPHA2 overexpression has been observed to contribute to tumorigenesis in a variety of tissues, including breast, ovary, skin, brain, and lung (20, 41–43). Studies from our laboratory have demonstrated that EPHA2 has a distinct role in tumor promotion in the epithelial component of both breast and lung tumors, as evidenced by targeted inhibition of EPHA2 in murine models of these tumor types (22, 24). Previous studies in breast cancer and glioma have established that EPHA2 signaling is mitigated upon ligand engagement, whereas ligand-independent EPHA2 signaling and cross-talk with other oncogenic pathways serve to promote tumor cell proliferation and motility (22, 36, 44). In lung cancer, the tumor-promoting role of EPHA2 appears to be ligand independent, as exogenous EPHRIN-A1 stimulation inhibits tumor cell proliferation (45). In our study, the EPHA2 inhibitor ALW-II-41-

**Figure 7.** ALW-II-41-27 decreases tumor growth of erlotinib-resistant lung cancer in vivo. A and B, 2.5 × 10^6 HCC827/ER cells or 1 × 10^6 PC-9/ERC16 cells were injected into the dorsal flanks of nude mice subcutaneously. Tumors were allowed to grow to approximately 150 mm^3 before administration of 15 mg/kg ALW-II-41-27, erlotinib, or vehicle alone via intraperitoneal injection twice daily. Tumors were measured every day with a digital caliper, and tumor volumes were calculated (n = 5/treatment group). Data are presented as the mean tumor volume ± SEM. **P ≤ 0.005, two-way ANOVA with Bonferroni post hoc analysis. C and D, signaling analyses on tumor lysates from three independent mice per group were performed. Shown are representative immunoblots in which signaling molecules were detected with the indicated antibodies.
27 reduced the phosphorylation of both Y588 and S897 of the EPHA2 receptor (Fig. 6F), suggesting that both tyrosine and serine phosphorylation may be important in maintaining cell viability in EGFR TKI-resistant tumor cells. As ALW-II-41-27 is a kinase inhibitor, inhibition of EPHA2 kinase activity will result in reduced phosphorylation of the juxtamembrane tyrosine residue Y588. In contrast, reduced levels of phosphorylation at S897 of EPHA2 are likely the result of inhibition of phosphorylation of RSK (46) or AKT (36), both of which were reduced in ALW-II-41-27–treated samples.

Although the EPHA2 receptor has previously been shown to regulate RAS/MAPK signaling in breast cancer cells (22, 35), in EGFR<sup>T790M</sup>–mutant lung cancer, loss of EPHA2 does not appear to significantly affect the activities of ERK, but rather modulates phosphorylation levels of p90-RSK, S6K1 (a known substrate of mTORC1), and the proapoptotic protein BAD. These results are consistent with the recent findings that activation of mTORC1 is associated with acquired resistance of EGFR–mutant lung cancer to combined EGFR inhibition via a TKI and cetuximab (47). mTORC2 has also been implicated in the maintenance of EGFR TKI-resistant lung cancer (48); however, the degree to which mTORC2 plays a role in EPHA2-mediated maintenance of cell viability in erlotinib-resistant cells remains to be determined.

Targeting EPHA2 in EGFR TKI-resistant lung tumors represents a unique opportunity for mitigating cell viability, as our studies have demonstrated that erlotinib-resistant cells are more dependent on EPHA2 for cell viability than erlotinib-sensitive cells (Fig. 5A and B). Knockdown of EPHA2 induced a greater than 3-fold increase in cell death and greater than 3-fold reduction in proliferation in erlotinib-resistant cells relative to their parental, erlotinib-sensitive counterparts. These data indicate a distinct addiction of EGFR<sup>T790M</sup>–mutant lung tumors to EPHA2 for survival, and it may illuminate why RNAi-mediated EPHA2 knockdown experiments as well as pharmacologic inhibition of EPHA2 appear to be remarkably effective without the combination of other inhibitors. It is, however, possible that combination of an EPHA2 inhibitor in this context with inhibitors of other kinases, such as EGFR, MEK, ERK, or IGF-1R, could further diminish cell survival. Additional investigation is needed to assess whether the EPHA2 signaling addiction observed is specific to the erlotinib-mediated development of EGFR<sup>T790M</sup> resistance acquired in the presence of any EGFR TKI.

Preliminary data from our laboratory indicate that cells with acquired resistance to two, unique second-generation EGFR inhibitors, afatinib or XL-647, and a third-generation EGFR inhibitor, AZD9291, also display overexpression of EPHA2 compared with cells sensitive to these inhibitors and are highly sensitive to EPHA2 inhibition (Figs. 1D and Fig. 6B, and data not shown). Studies to characterize the role of EPHA2 in viability maintenance of EGFR-mutant cells with acquired resistance to second- and third-generation EGFR TKIs are currently in progress.

Although strategies to overcome EGFR<sup>T790M</sup>–mediated TKI resistance are rapidly evolving, including but not limited to the development of EGFR<sup>T790M</sup>–mutant–specific EGFR inhibitors (10) and the combination of second-generation EGFR TKIs with antibodies against EGFR such as cetuximab (11), persistent treatment of a single target (e.g., EGFR) may make tumors more likely to engage in alternative, non–EGFR–related bypass escape mechanisms (10). Recent studies indicate that optimizing the dose and sequence of TKI treatment may be an essential component in the effective treatment of EGFR<sup>T790M</sup> disease (27, 28). We have observed that EPHA2 expression in EGFR<sup>T790M</sup>–mutant cells increases in a time-dependent fashion after being withdrawn from erlotinib (Fig. 1E). Interestingly, there have been several clinical reports of patients with EGFR<sup>T790M</sup>–mutant lung tumors that exhibited a flare of tumor growth after TKI withdrawal (49, 50). This could be due in part to the surge in EPHA2 expression we observe upon EGFR TKI withdrawal. It is quite possible that EPHA2 inhibition may be most efficacious in EGFR<sup>T790M</sup>–mutant tumors during an EGFR TKI ‘holiday,’ when EPHA2 levels are at their highest. Because EPHA2 inhibition preferentially eliminates EGFR TKI-resistant cells over EGFR TKI-sensitive cells (Fig. 5A), it is reasonable to hypothesize that after a regimen of EPHA2 inhibition the tumor may be repopulated with more EGFR TKI-sensitive cells and may reexpress to first-line EGFR TKIs. Thus, it may be possible in the future to treat EGFR TKI-resistant tumors with cycles of sequential EGFR TKIs followed by EPHA2 inhibitors with the ultimate goal of eradicating both the EGFR TKI-sensitive and TKI-resistant disease. Although the specificity and functional importance of the EPHA2 pharmacologic inhibitor ALW-II-41-27 have already been characterized previously in the context of lung cancer (24), further compound iterations are in development to enhance target specificity and pharmacodynamics in vivo.

In summary, we show that EPHA2 overexpression is required for survival of erlotinib-resistant lung cancer, and that both genetic and pharmacologic inhibition of EPHA2 results in decreased survival and proliferation of cells with EGFR<sup>T790M</sup>–mediated, erlotinib resistance. These studies not only present evidence for the utility of EPHA2 inhibitors in the treatment of erlotinib-resistant tumors, but also provide a rationale for optimizing the sequence of treatment with existing first-generation EGFR inhibitors to maximize patient benefit.

Disclosure of Potential Conflicts of Interest
C.M. Lovly has received speakers bureau honoraria from Abbot Molecular, Harrison and Star, Novartis, and Qiagen and is a consultant/advisory board member for Novartis, Pfizer, and Sequenom. W. Pao has ownership interest (including patents) in Molecular MD. J. Chen has received speakers bureau honoraria from Wayne State University, Ohio State University, and Oklahoma Medical Research Foundation. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: K.R. Amato, J. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.R. Amato, S. Wang, L. Tan, A.K. Hastings, W. Song, C.M. Lovly, C.B. Meador, D.C. Colvin, N.S. Gray
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.R. Amato, S. Wang, F. Ye, P. Lu, J.M. Ballo, W. Pao
Writing, review, and/or revision of the manuscript: K.R. Amato, S. Wang, L. Tan, C.M. Lovly, J.M. Ballo, D.C. Colvin, W. Pao, N.S. Gray, J. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.R. Amato, C.B. Meador, D.C. Colvin, J. Chen
Study supervision: K.R. Amato, J. Chen

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References


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Katherine R. Amato, Shan Wang, Li Tan, et al.


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