Published in final edited form as:

Cancer Res. 2016 January 15; 76(2): 305–318. doi:10.1158/0008-5472.CAN-15-0717.

EPHA2 blockade overcomes acquired resistance to EGFR kinase inhibitors in lung cancer

Katherine R. Amato¹, Shan Wang^{2,*}, Li Tan^{3,4,*}, Andrew K. Hastings⁵, Wenqiang Song², Christine M. Lovly^{1,6}, Catherine B. Meador¹, Fei Ye⁷, Pengcheng Lu⁷, Justin M. Balko¹, Daniel C. Colvin⁸, Justin M. Cates^{5,6}, William Pao^{1,6}, Nathanael S. Gray^{3,4}, and Jin Chen^{1,2,6,9,10,#}

¹Department of Cancer Biology, Vanderbilt University, Nashville, Tennessee, USA

²Division of Rheumatology and Immunology, Vanderbilt University, Nashville, Tennessee, USA

³Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts, USA

⁴Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts, USA

⁵Department of Pathology, Microbiology, and Immunology, Vanderbilt University, Nashville, Tennessee, USA

⁶Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, Tennessee, USA

⁷Department of Biostatistics, Vanderbilt University, Nashville, Tennessee, USA

⁸Vanderbilt University Institute of Imaging Science, Vanderbilt University, Nashville, Tennessee, USA

⁹Department of Cell and Developmental Biology, Vanderbilt University, Nashville, Tennessee, USA

¹⁰Veterans Affairs Medical Center, Tennessee Valley Healthcare System, Nashville, Tennessee, USA

Abstract

Despite the success of treating *EGFR* mutant lung cancer patients with EGFR tyrosine kinase inhibitors (TKIs), 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 cells decreased S6K1-mediated

[#]Correspondence should be addressed to: Jin Chen, M.D., Ph.D., Professor of Medicine and Cancer Biology, T-3207E, Medical Center North, Vanderbilt University School of Medicine, 1161 21st Avenue South, Nashville, TN 37232, jin.chen@vanderbilt.edu, Telephone: 615-343-3819, FAX: 615-343-8648.

^{*}These authors contributed equally to this work

Conflict of Interest: The authors have declared that no conflict of interest exists.

phosphorylation of cell death agonist BAD, resulting in reduced tumor cell proliferation and increased apoptosis. Furthermore, pharmacologic inhibition of EPHA2 by the small molecule inhibitor, ALW-II-41-27, decreased both survival and proliferation of erlotinib resistant tumor cells and inhibited tumor growth in vivo. ALW-II-41-27 was also effective in decreasing viability of cells with acquired resistance to the third generation EGFR TKI, AZD9291. Collectively, these data define a role for EPHA2 in the maintenance of cell survival of TKI resistant, *EGFR* mutant lung cancer and indicate that EPHA2 may serve as a useful therapeutic target in TKI resistant tumors.

Keywords

EPHA2; acquired resistance; non-small cell lung cancer

Introduction

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) (2), are activating mutations that result in enhanced EGFR kinase activity (3) as well as exquisite sensitivity to first-generation EGFR-specific tyrosine kinase inhibitors (TKIs), 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 (9) and third (10) generation 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 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 to 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 tyrosine kinase inhibitor 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) (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's 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 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 as $1-r$, where r represents the correlation coefficient value. All tests are significant at two-sided 5% level, false-discovery-rate (FDR); corrected p-values were reported for multiple comparisons.

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 (HIPAA) guidelines. Paired patient tumor samples before and after TKI treatment were stained for EPHA2 expression by immunohistochemistry (IHC), as described in the IHC

section below. Of the three patient samples that displayed elevated EPHA2 expression in post-relapse tumor sections, one patient harbored an *EGFR*^{T790M} mutation (Patient #14). The mutation status of Patient #12 is unknown. *EGFR*^{T790M} was not detected in Patient #1, nor were mutations in BRAF, PI3K, KRAS, or NRAS. Other mechanisms of resistance (such as *MET* amplification or AXL) were not tested.

Cells and cell culture

EGFR mutant PC-9 and HCC827 (E746-A750) and PC-9/ER, PC-9/ERC15, PC-9/ERC16, and HCC827/ER (E746-A750; T790M) NSCLC cells were provided by Dr. William Pao (Vanderbilt University). Erlotinib resistant cells were derived as described and characterized (27). AZD9291 resistant cells were derived as described and characterized (28). Cell lines were authenticated and sequenced for signature mutations (27). *EGFR* TKI resistant lines were maintained in the presence of 1μM erlotinib (Cell Signaling Technology) throughout the study (refreshed every 72 hours) although experiments were routinely conducted after a 72 hour drug holiday. ALW-II-41-27 and NG-25 were generously provided by Nathanael Gray (Dana-Farber Cancer Institute, Harvard Medical School). ALW-II-41-27 was determined to have >99.7% purity by LCMS analysis. 293T cells used for lentivirus production were purchased from ATCC.

All lung cancer cell lines were cultured in RPMI-1640 medium (Corning/Cellgro) supplemented with L-glutamine (2mM), 10% fetal bovine serum (FBS) (Thermo Scientific, HyClone Laboratories Inc.), penicillin (100U/mL), and streptomycin (100μg/mL). 293T cells were grown in DMEM (Corning/Cellgro) supplemented with L-glutamine (2mM), and 10% FBS. All cells were grown in a humidified incubator with 5% CO₂ at 37°C.

Murine tumor studies

TetO-*EGFR*^{L858R+T790M} and *CCSP-rtTA* mice were provided by Dr. William Pao (Vanderbilt University) and have been described previously (29,30). *EphA2* targeted mice have been described previously (31), and were referred to herein as *EphA2*^{-/-} mice because characterization of N-terminal (Bethyl Laboratories, A302-024A) and C-terminal (Santa Cruz, #sc-924) EPHA2 expression revealed that neither the full length nor a truncated form of EPHA2 is expressed in these mice (Supplemental Figure 4). Genotypes were confirmed twice for each animal by analyzing genomic DNA isolated from both tail and ear tissues. TetO-*EGFR*^{L858R+T790M} primers were 5'-ACTGTCCAGCCCACCTGTGT-3' and 5'-GCCTGCGACGGCGGCATCTGC-3'. *CCSP-rtTA* primers were 5'-ACTGCCCATTGCCCCAACAC-3' and 5'-AAAATCTTGCCAGCTTTCCCC-3'. *EphA2* primers were 5'-GGGTGCCAAAGTAGAACTGCG-3' (forward), 5'-GACAGAATAAAACGCACGGGTG-3' (Neo), and 5'-TTCAGCCAAGCCTATGTAGAAAGC-3' (reverse). Doxycycline was administered at the time of weaning (3 weeks old) by feeding the mice doxycycline containing food pellets (625 ppm) (Harland-Tekland) to induce lung specific expression of mutant *EGFR*^{L858R+T790M}.

Lungs removed for gross and histological analysis were first perfused with 1× PBS followed by 10% buffered formalin (Fisher). After 24 hours of fixation in formalin lungs were weighed to determine a total wet weight. MRI imaging analysis was performed at both 10

and 15 weeks of age following the initiation of doxycycline administration at weaning. Tumor volumes were calculated using Matlab 2012a (The MathWorks Inc.) as described previously (24).

All animal experiments were conducted under guidelines approved by the AAALAC and Vanderbilt University Institutional Animal Care and Use Committee.

Immunohistochemistry

Immunohistochemical staining (IHC) on tumor sections was performed as described previously (32–34), using antibodies against EPHA2 (Life Technologies; #347400), EGFR^{L858R} (Cell Signaling Technology, #3197S), PCNA (BD Pharmingen, #555567), and 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 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), retrieval agent 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 XYZ (Richard Allan Scientific) or ProLong Gold antifade reagent with DAPI (Life Technologies) were used to mount slides.

Tumor Xenografts

HCC827/ER (2.5×10⁶) or PC-9/ERC16 (1×10⁶) cells were injected with Matrigel into the hind flanks of 6-week-old athymic nude mice (Foxn1^{nu}) (Harlan). Once tumors reached approximately 150 mm³, mice were randomized by body weight and tumor volume into treatment groups (n=5 per group) to receive 15mg/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 formula; volume = length x width² x 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 non-small cell lung cancer (NSCLC), we analyzed a dataset of 58 matched normal and lung adenocarcinoma tissue samples of which *EGFR* mutation status was known. In all patients (n=58), levels of EPHA2 expression were significantly higher (p=0.003) in the tumor tissue compared to the adjacent normal lung tissue (Figure 1A), consistent with findings from previous studies (18–20). In patients whose tumors tested

positive for an *EGFR* mutation, EPHA2 expression was also markedly increased compared to adjacent normal tissue (Figure 1A).

Given the known role of EPHA2 in promoting lung cancer growth and survival (19,24) and its contribution to drug resistance in breast cancer (23), 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 post completion of a drug escalation protocol (27). The resulting resistant cell lines tolerated erlotinib concentrations more than ten times the IC₅₀ of their parental, TKI sensitive counterparts (Figure 1B). 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 (Figure 1C). 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 erlotinib sensitive and resistant cell line pair, HCC827 and HCC827/ER. EPHA2 was overexpressed in all of the erlotinib resistant cell lines (Figure 1D), as well as in two independent single-cell *EGFR*^{T790M} containing clones derived from the PC-9/ER cell line (Figure 1D), confirming observations from our gene expression analysis. Interestingly, we found that EPHA2 could be regulated by the presence of erlotinib, and EPHA2 expression and phosphorylation increased in a time-dependent manner post erlotinib withdrawal in PC-9/ERC16 cells (Figure 1E), consistent with a previous observation that EPHA2 expression is regulated by MAPK signaling (35). In addition to mRNA and protein expression levels, we also 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 (Supplemental Figure 1).

Lastly, 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 pre-treatment and post-relapse tumor sections, we detected higher EPHA2 protein levels by immunohistochemistry as compared to IgG control stained adjacent sections in 3 of the post-relapse tumor samples (Figure 1F and Supplemental Figure 2). Overall, we determined that EPHA2 is overexpressed in *EGFR* mutant lung cancer cells harboring *EGFR*^{T790M} mediated resistance to erlotinib compared to *EGFR* mutant lung cancer cells with sensitivity to 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 lenti-viral based shRNA strategy in four erlotinib resistant and two erlotinib sensitive lung cancer cell lines. Both of the two independent shRNAs against *EPHA2* (sh*EPHA2*) silenced EPHA2 protein expression and reduced cell viability when tested three days after puromycin selection (Figure 2A and 2B). Although sh*EPHA2* reduced cell viability in both erlotinib sensitive and resistant cell lines, erlotinib resistant cells displayed a greater dependence on *EPHA2* for cell survival compared to 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 (Figure 2C). We next performed a time course to monitor cell viability after *EPHA2* knockdown in these cells. The results showed that by five days post 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 (Figure 2D). These data suggests 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 *EPHA2*^{D739N}, a kinase dead mutant (36). We observed that wild-type *EPHA2*, but not *EPHA2*^{D739N}, was sufficient to restore cell viability to the control level (Supplemental Figure 3), 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*^{L858R+T790M} 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*^{L858R+T790M} mutant lung cancer transgenic model (29,31). In this model, expression of the mutant *EGFR* (TetO-*EGFR*^{L858R+T790M}, *CCSP-rtTA*) is induced upon doxycycline administration and resulting tumors are resistant to erlotinib. To assess tumor burden in lungs of *EGFR*^{L858R+T790M}/*EphA2*^{+/+} and *EGFR*^{L858R+T790M}/*EphA2*^{-/-} 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*^{L858R+T790M}/*EphA2*^{-/-} mice compared to doxycycline-treated, *EGFR*^{L858R+T790M}/*EphA2*^{+/+} mice (Figure 3A). Because no changes in lung weight were observed between *EGFR*^{L858R+T790M}/*EphA2*^{-/-} and *EGFR*^{L858R+T790M}/*EphA2*^{+/+} 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 imaging when tumors had developed in both groups at 10 weeks of age and again at 15 weeks of age (Figure 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*^{L858R+T790M}/*EphA2*^{-/-} mice had a lower overall tumor burden than *EGFR*^{L858R+T790M}/*EphA2*^{+/+} mouse counterparts, which became more evident as the mice aged (Figure 3C). *EphA2* deficiency also correlated to significantly longer overall survival in this model of TKI resistant *EGFR*^{L858R+T790M} mutant lung cancer. *EphA2*^{+/+} mice did not survive past 25 weeks of age, whereas more than 25% the *EphA2*^{-/-} mice survived longer than 1 year on doxycycline (Figure 3D).

Histological analysis of the lungs confirmed the presence of tumors and *EGFR*^{L858R+T790M} expression in doxycycline treated animals as well as the absence of *EPHA2* expression in *EphA2* knockout animals (Figure 4A). Western blot analysis confirmed *EPHA2* expression in the *EphA2*^{+/+} 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 (Supplemental Figure 4). 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^{L858R+T790M}/EphA2^{-/-}* mice, compared to tumors of *EGFR^{L858R+T790M}/EphA2^{+/+}* mice, as measured by TUNEL staining (Figure 4B and 4C). Tumor cell proliferation was measured by staining tumor sections for proliferating cell nuclear antigen (PCNA) (Figure 4D). Proliferation was significantly decreased in *EGFR^{L858R+T790M}/EphA2^{-/-}* tumors compared to tumors of mice with wild-type levels of EPHA2 (Figure 4E). Because previous murine studies in breast cancer have indicated that EPHA2 can function to support tumor vasculature (33), we assessed tumor microvessels by Von Willebrand factor (vWF) immunohistochemistry. No significant differences in vWF staining were seen in the tumor tissue between *EGFR^{L858R+T790M}/EphA2^{+/+}* and *EGFR^{L858R+T790M}/EphA2^{-/-}* mice (Figure 4F and 4G). 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 up-regulation 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* (sh*EPHA2*) by using a BrdU incorporation assay and a Cell Death ELISA, respectively. Upon *EPHA2* knockdown, we observed a decrease in proliferation of approximately 74 percent compared to sh*Control* cells, while cell lines sensitive to erlotinib displayed only a 22 percent decrease in proliferation (Figure 5A). Consistent with this effect, loss of *EPHA2* in erlotinib resistant cells resulted in a 3.4 fold increase in cellular apoptosis, compared to an only 2 fold increase in cells undergoing apoptosis from *EPHA2* knockdown in erlotinib sensitive cells (Figure 5B). Knockdown of *EPHA2* also increased cleavage of caspase 3 and PARP (Figure 5C), confirming elevated apoptosis in erlotinib resistant cells. Signaling analysis from two independent cell lines that contain *EGFR^{T790M}* mediated erlotinib resistance (PC-9/ERC16 and HCC827/ER) revealed that loss of *EPHA2* decreased phosphorylation of p90-RSK, S6 kinase 1, and the pro-apoptotic BH3-only protein BAD, whereas other effector proteins were not appear to be affected by *EPHA2* loss (Figure 5D). The phenotype observed is not due to off-target effects, as two independent siRNAs against *EPHA2* recapitulated signaling defects seen in the pooled si*EPHA2* knockdown (Supplemental Figure 5). 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 pharmacological 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 analog that possesses a similar profile of kinase targets but does not inhibit EPHA2, was used as a control. We first assessed the effects of pharmacological inhibition of EPHA2 via ALW-II-41-27 on four cell lines with acquired resistance to erlotinib. TKI resistant cells treated with 1 μ M 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% at 72 hours after drug treatment, whereas there was no significant change in the viability of cells treated with NG-25 at the same dose (Figure 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 (Figure 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 μ M ALW-II-41-27 decreased cell proliferation (Figure 6C) and increased apoptosis (Figure 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 anti-apoptotic proteins BCL-xL and MCL-1 (Figure 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 (Supplemental Figure 6). To assess the acute signaling consequences of targeting EPHA2 via ALW-II-41-27 treatment, cell lysates were collected from erlotinib sensitive and resistant lung cancer cells after treatment with 1 μ M 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 (Figure 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 *EGFR* 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 15mg/kg via intraperitoneal injection. After 14 days of the treatment regimen, ALW-II-41-27 significantly inhibited growth of the erlotinib resistant tumors (Figure 7A and 7B). Toxicity as measured by body weight was not significantly changed by any of the drugs compared to 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 (Figure 7C and 7D), consistent with results observed *in vitro* (Figure 6F). These data indicate that pharmacological 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) (13,14,17). Here we have demonstrated that *EPHA2* overexpression serves as an additional novel mechanism of drug resistance particularly in *EGFR*^{T790M} mutant lung cancer. We found that knockdown of *EPHA2* resulted in decreased proliferation and increased apoptosis in erlotinib resistant cells with *EGFR*^{T790M} mutations. Genetic targeting of *EphA2* significantly inhibited *EGFR*^{L858R+T790M} 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 pharmacological inhibition of *EPHA2* may represent a viable, alternative strategy for treating *EGFR*^{T790M} 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 lab 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-41-27, reduced the phosphorylation of both Y588 and S897 of the *EPHA2* receptor (Figure 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*^{T790M} 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 pro-apoptotic 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 (Figure 5A and 5B). 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*^{T790M} 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*^{T790M} or if it is a universal feature of *EGFR*^{T790M} resistance acquired in the presence of any EGFR TKI. Preliminary data from our lab indicates 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 to cells sensitive to these inhibitors and are highly sensitive to EPHA2 inhibition (Figure 1D, Figure 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 is currently under investigation.

Although strategies to overcome *EGFR*^{T790M} mediated TKI resistance are rapidly evolving, including but not limited to the development of *EGFR*^{T790M} 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*^{T790M} disease (27,28). We have observed that EPHA2 expression in *EGFR*^{T790M} mutant cells increases in a time-dependent fashion after being withdrawn from erlotinib (Figure 1E). Interestingly, there have been several clinical reports of patients with *EGFR*^{T790M} mutant lung tumors that exhibited a flare of tumor growth post 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*^{T790M} 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 (Figure 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 re-respond to first-line EGFR TKIs. Thus, it may be possible in the future to treat *EGFR*^{T790M} mutant 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 pharmacological inhibitor, ALW-II-41-27, has 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*^{T790M} 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 rationale for optimizing the sequence of treatment with existing first-generation EGFR inhibitors to maximize patient benefit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Support: This work was supported by the Department of Veterans Affairs through a VA Merit Award (J. Chen), the Department of Defense (W81XWH-14-1-0181) (J. Chen), NIH grants R01 CA95004, CA177681 (J. Chen), CA173469 (N. Gray), CA121210 (C. Lovly), P01 CA129243 (C. Lovly), U54 CA143798 (C. Lovly), F31 CA167878 (K. Amato), a Damon Runyon Clinical Investigator Award (C. Lovly), a LUNgevity Career Development Award (C. Lovly), a Melley Family Scholarship (C. Meador), a pilot project grant from the VICC Thoracic Center (J. Chen), and funds from Astra Zeneca (C. Lovly). Confocal experiments were performed through the use of the VUMC Cell Imaging Shared Resource, supported by NIH grants CA68485, DK20593, DK58404, DK59637 and EY08126. This work was also supported by the NCI Cancer Center Support Grant (P30 CA068485) utilizing the Translational Pathology, Flow Cytometry, and Small Animal Imaging Shared Resources.

References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA: a cancer journal for clinicians*. 2014; 64(1):9–29. [PubMed: 24399786]
2. Ladanyi M, Pao W. Lung adenocarcinoma: guiding EGFR-targeted therapy and beyond. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc.* 2008; 21(Suppl 2):S16–22.
3. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science*. 2004; 305(5687):1163–7. [PubMed: 15284455]
4. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *The New England journal of medicine*. 2004; 350(21):2129–39. [PubMed: 15118073]
5. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004; 304(5676):1497–500. [PubMed: 15118125]
6. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, et al. EGF receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101(36):13306–11. [PubMed: 15329413]
7. Riely GJ, Pao W, Pham D, Li AR, Rizvi N, Venkatraman ES, et al. Clinical course of patients with non-small cell lung cancer and epidermal growth factor receptor exon 19 and exon 21 mutations treated with gefitinib or erlotinib. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2006; 12(3 Pt 1):839–44. [PubMed: 16467097]
8. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS medicine*. 2005; 2(3):e73. [PubMed: 15737014]
9. Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102(21):7665–70. [PubMed: 15897464]

10. Cross DA, Ashton SE, Ghiorghiu S, Eberlein C, Nebhan CA, Spitzler PJ, et al. AZD9291, an Irreversible EGFR TKI, Overcomes T790M-Mediated Resistance to EGFR Inhibitors in Lung Cancer. *Cancer discovery*. 2014; 4(9):1046–61. [PubMed: 24893891]
11. Regales L, Gong Y, Shen R, de Stanchina E, Vivanco I, Goel A, et al. Dual targeting of EGFR can overcome a major drug resistance mutation in mouse models of EGFR mutant lung cancer. *The Journal of clinical investigation*. 2009; 119(10):3000–10. [PubMed: 19759520]
12. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2013; 19(8):2240–7. [PubMed: 23470965]
13. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science*. 2007; 316(5827):1039–43. [PubMed: 17463250]
14. Takezawa K, Pirazzoli V, Arcila ME, Nebhan CA, Song X, de Stanchina E, et al. HER2 amplification: a potential mechanism of acquired resistance to EGFR inhibition in EGFR-mutant lung cancers that lack the second-site EGFR T790M mutation. *Cancer discovery*. 2012; 2(10):922–33. [PubMed: 22956644]
15. Cortot AB, Repellin CE, Shimamura T, Capelletti M, Zejnullahu K, Ercan D, et al. Resistance to irreversible EGF receptor tyrosine kinase inhibitors through a multistep mechanism involving the IGF1R pathway. *Cancer research*. 2013; 73(2):834–43. [PubMed: 23172312]
16. Terai H, Soejima K, Yasuda H, Nakayama S, Hamamoto J, Arai D, et al. Activation of the FGF2-FGFR1 autocrine pathway: a novel mechanism of acquired resistance to gefitinib in NSCLC. *Molecular cancer research: MCR*. 2013; 11(7):759–67. [PubMed: 23536707]
17. Zhang Z, Lee JC, Lin L, Olivas V, Au V, LaFramboise T, et al. Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. *Nature genetics*. 2012; 44(8):852–60. [PubMed: 22751098]
18. Kinch MS, Moore MB, Harpole DH Jr. Predictive value of the EphA2 receptor tyrosine kinase in lung cancer recurrence and survival. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2003; 9(2):613–8. [PubMed: 12576426]
19. Faoro L, Singleton PA, Cervantes GM, Lennon FE, Choong NW, Kanteti R, et al. EphA2 mutation in lung squamous cell carcinoma promotes increased cell survival, cell invasion, focal adhesions, and mammalian target of rapamycin activation. *The Journal of biological chemistry*. 2010; 285(24):18575–85. [PubMed: 20360610]
20. Brannan JM, Dong W, Prudkin L, Behrens C, Lotan R, Bekele BN, et al. Expression of the receptor tyrosine kinase EphA2 is increased in smokers and predicts poor survival in non-small cell lung cancer. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2009; 15(13):4423–30. [PubMed: 19531623]
21. Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. *Nature reviews Cancer*. 2010; 10(3):165–80. [PubMed: 20179713]
22. Brantley-Sieders DM, Zhuang G, Hicks D, Fang WB, Hwang Y, Cates JM, et al. The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling. *The Journal of clinical investigation*. 2008; 118(1):64–78. [PubMed: 18079969]
23. Zhuang G, Brantley-Sieders DM, Vaught D, Yu J, Xie L, Wells S, et al. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. *Cancer research*. 2010; 70(1):299–308. [PubMed: 20028874]
24. Amato KR, Wang S, Hastings AK, Youngblood VM, Santapuram PR, Chen H, et al. Genetic and pharmacologic inhibition of EPHA2 promotes apoptosis in NSCLC. *The Journal of clinical investigation*. 2014; 124(5):2037–49. [PubMed: 24713656]
25. Selamat SA, Chung BS, Girard L, Zhang W, Zhang Y, Campan M, et al. Genome-scale analysis of DNA methylation in lung adenocarcinoma and integration with mRNA expression. *Genome research*. 2012; 22(7):1197–211. [PubMed: 22613842]
26. Chmielecki J, Pietanza MC, Aftab D, Shen R, Zhao Z, Chen X, et al. EGFR-mutant lung adenocarcinomas treated first-line with the novel EGFR inhibitor, XL647, can subsequently retain

- moderate sensitivity to erlotinib. *Journal of thoracic oncology: official publication of the International Association for the Study of Lung Cancer*. 2012; 7(2):434–42.
27. Chmielecki J, Foo J, Oxnard GR, Hutchinson K, Ohashi K, Somwar R, et al. Optimization of dosing for EGFR-mutant non-small cell lung cancer with evolutionary cancer modeling. *Science translational medicine*. 2011; 3(90):90ra59.
 28. Meador CB, Jin H, de Stanchina E, Nebhan CA, Pirazzoli V, Wang L, et al. Optimizing the Sequence of Anti-EGFR-Targeted Therapy in EGFR-Mutant Lung Cancer. *Molecular cancer therapeutics*. 2014
 29. Regales L, Balak MN, Gong Y, Politi K, Sawai A, Le C, et al. Development of new mouse lung tumor models expressing EGFR T790M mutants associated with clinical resistance to kinase inhibitors. *PloS one*. 2007; 2(8):e810. [PubMed: 17726540]
 30. Tichelaar JW, Lu W, Whitsett JA. Conditional expression of fibroblast growth factor-7 in the developing and mature lung. *The Journal of biological chemistry*. 2000; 275(16):11858–64. [PubMed: 10766812]
 31. Brantley-Sieders DM, Caughron J, Hicks D, Pozzi A, Ruiz JC, Chen J. EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation. *Journal of cell science*. 2004; 117(Pt 10):2037–49. [PubMed: 15054110]
 32. Brantley DM, Cheng N, Thompson EJ, Lin Q, Brekken RA, Thorpe PE, et al. Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. *Oncogene*. 2002; 21(46):7011–26. [PubMed: 12370823]
 33. Brantley-Sieders DM, Fang WB, Hicks DJ, Zhuang G, Shyr Y, Chen J. Impaired tumor microenvironment in EphA2-deficient mice inhibits tumor angiogenesis and metastatic progression. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 2005; 19(13):1884–6. [PubMed: 16166198]
 34. Politi K, Zakowski MF, Fan PD, Schonfeld EA, Pao W, Varmus HE. Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. *Genes & development*. 2006; 20(11):1496–510. [PubMed: 16705038]
 35. Macrae M, Neve RM, Rodriguez-Viciana P, Haqq C, Yeh J, Chen C, et al. A conditional feedback loop regulates Ras activity through EphA2. *Cancer cell*. 2005; 8(2):111–8. [PubMed: 16098464]
 36. Miao H, Li DQ, Mukherjee A, Guo H, Petty A, Cutter J, et al. EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. *Cancer cell*. 2009; 16(1):9–20. [PubMed: 19573808]
 37. Fisher GH, Wellen SL, Klimstra D, Lenczowski JM, Tichelaar JW, Lizak MJ, et al. Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. *Genes & development*. 2001; 15(24):3249–62. [PubMed: 11751631]
 38. Miao B, Ji Z, Tan L, Taylor M, Zhang J, Choi HG, et al. EphA2 is a Mediator of Vemurafenib Resistance and a Novel Therapeutic Target in Melanoma. *Cancer discovery*. 2014
 39. Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Science translational medicine*. 2011; 3(75):75ra26.
 40. Ohashi K, Sequist LV, Arcila ME, Moran T, Chmielecki J, Lin YL, et al. Lung cancers with acquired resistance to EGFR inhibitors occasionally harbor BRAF gene mutations but lack mutations in KRAS, NRAS, or MEK1. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109(31):E2127–33. [PubMed: 22773810]
 41. Brantley-Sieders DM, Jiang A, Sarma K, Badu-Nkansah A, Walter DL, Shyr Y, et al. Eph/ephrin profiling in human breast cancer reveals significant associations between expression level and clinical outcome. *PloS one*. 2011; 6(9):e24426. [PubMed: 21935409]
 42. Udayakumar D, Zhang G, Ji Z, Njauw CN, Mroz P, Tsao H. EphA2 is a critical oncogene in melanoma. *Oncogene*. 2011; 30(50):4921–9. [PubMed: 21666714]
 43. Li X, Wang L, Gu JW, Li B, Liu WP, Wang YG, et al. Up-regulation of EphA2 and down-regulation of EphrinA1 are associated with the aggressive phenotype and poor prognosis of

- malignant glioma. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2010; 31(5):477–88. [PubMed: 20571968]
44. Miao H, Gale NW, Guo H, Qian J, Petty A, Kaspar J, et al. EphA2 promotes infiltrative invasion of glioma stem cells in vivo through cross-talk with Akt and regulates stem cell properties. *Oncogene*. 2015; 34(5):558–67. [PubMed: 24488013]
45. Brannan JM, Sen B, Saigal B, Prudkin L, Behrens C, Solis L, et al. EphA2 in the early pathogenesis and progression of non-small cell lung cancer. *Cancer prevention research*. 2009; 2(12):1039–49. [PubMed: 19934338]
46. Zhou Y, Yamada N, Tanaka T, Hori T, Yokoyama S, Hayakawa Y, et al. Crucial roles of RSK in cell motility by catalysing serine phosphorylation of EphA2. *Nature communications*. 2015; 6:7679.
47. Pirazzoli V, Nebhan C, Song X, Wurtz A, Walther Z, Cai G, et al. Acquired resistance of EGFR-mutant lung adenocarcinomas to afatinib plus cetuximab is associated with activation of mTORC1. *Cell reports*. 2014; 7(4):999–1008. [PubMed: 24813888]
48. Fei SJ, Zhang XC, Dong S, Cheng H, Zhang YF, Huang L, et al. Targeting mTOR to overcome epidermal growth factor receptor tyrosine kinase inhibitor resistance in non-small cell lung cancer cells. *PloS one*. 2013; 8(7):e69104. [PubMed: 23874880]
49. Chaft JE, Oxnard GR, Sima CS, Kris MG, Miller VA, Riely GJ. Disease flare after tyrosine kinase inhibitor discontinuation in patients with EGFR-mutant lung cancer and acquired resistance to erlotinib or gefitinib: implications for clinical trial design. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2011; 17(19):6298–303. [PubMed: 21856766]
50. Becker A, Crombag L, Heideman DA, Thunnissen FB, van Wijk AW, Postmus PE, et al. Retreatment with erlotinib: Regain of TKI sensitivity following a drug holiday for patients with NSCLC who initially responded to EGFR-TKI treatment. *European journal of cancer*. 2011; 47(17):2603–6. [PubMed: 21784628]

Figure 1. EPHA2 expression in erlotinib sensitive and 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's 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 was measured over time after erlotinib was removed from the culture media. Phosphorylation of ERK was also assessed over this time course. **(F)** Immunohistochemistry showed increased EPHA2 levels in EGFR TKI resistant tumor samples compared to pre-treatment tumor specimens from 3 patients. Scale bar: 50μm.

Figure 2. EPHA2 is required for cell viability in erlotinib resistant lung cancer cells

Erlotinib sensitive and resistant cell lines were transduced with lentiviruses containing either *shEPHA2* or a pLKO.1 vector control. The resulting cell populations were selected in 1.5µg/mL puromycin for 5 days. Shown are immunoblots for EPHA2 expression in PC-9 cells and PC-9 cells with acquired resistance to erlotinib (PC-9/ER) and erlotinib resistant clonal cell populations (PC-9/ERC15 and PC-9/ERC16) (A), as well as HCC827 cells and HCC827 cells with acquired resistance to erlotinib (HCC827/ER) 3 days after completion of puromycin selection (B). β-Tubulin expression was used as a loading control. (C) Erlotinib sensitive and resistant cell lines were treated as in (A&B), and cell viability was analyzed by the MTT assay at 72 hours after puromycin selection. Experiments were repeated three times with 6 replicates per experiment. Data is averaged for the *shEPHA2*#1 and *shEPHA2*#2 and percentage of cell viability is presented as the mean viability of *shEPHA2* knockdown cells relative to that of the *shControl* cells ± SEM. *p 0.05, **p 0.005, one way ANOVA with Bonferroni post hoc analysis. (D) Cells were treated as in C, and cell viability was assessed over 5 days. Experiments were repeated three times, and the mean *shEPHA2* knockdown relative to the vector control cells ± SEM was presented. **p 0.005, one way ANOVA with Bonferroni post hoc analysis.

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 $\pm$ SEM is shown ($n = 10$ per time point per genotype). ** $p < 0.005$; two way ANOVA with Bonferroni post hoc analysis. n.s. not 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 1mm. 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 the Matlab software and were graphed as an average tumor volume (mm³) $\pm$ SEM (n at least 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^{L858R+T790M}* tumors

(A) H&E stained lung sections (20 weeks) from *EGFR^{L858R+T790M}EphA2^{+/+}* and *EGFR^{L858R+T790M}EphA2^{-/-}* mice in the presence or absence of doxycycline (DOX). Scale bar: 200µm. Loss of EPHA2 protein expression and the presence of *EGFR^{L858R+T790M}* mutant protein expression were confirmed by immunohistochemistry. Scale bar: 50µm. (B) Apoptosis in tumor sections was measured by the TUNEL assay. TUNEL positive nuclei (red) are indicated with arrowheads. Scale bar: 30µm. (C) Apoptosis was quantified as a percentage of TUNEL positive nuclei relative to the total nuclei. An apoptosis index is presented as the average percentage of TUNEL positive nuclei per total nuclei ± SEM. (n = 5 per genotype). (D) Tumor cell proliferation was assessed by PCNA immunohistochemistry. Arrowheads indicate representative proliferating nuclei. Scale bar: 50µm. (E) Proliferation was quantified by assessing the total number of PCNA positive nuclei (brown) compared with the total nuclei. A proliferation index was calculated as a proportion of PCNA positive nuclei per total nuclei ± SEM. (F) The presence of tumor microvessels was assessed by vWF immunofluorescence staining (red, indicated by

arrowheads). Scale bar: 50 μ m. **(G)** Microvessels in the tumor were quantified by measuring vWF positive pixels in each tumor field $\pm$ SEM (n = 5 per genotype). *p<0.05, **p<0.01, Student's *t* test.

Figure 5. EPHA2 regulates cell signaling that promotes proliferation and survival

(A) Erlotinib sensitive and resistant cell lines were transduced with lentiviruses containing either shEPHA2 or a pLKO.1 vector control. The resulting cell populations were selected in 1.5 μ g/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 3 times, and data are presented as a proliferation index relative to the shControl $\pm$ SEM. *p 0.05; **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 3 times, and data are presented as an apoptotic index relative to the shControl $\pm$ SEM. *p 0.05; **p 0.005; two way ANOVA with Bonferroni post hoc analysis. n.s. not significant (C) Western blot analysis for cleaved (Cl.) caspase 3 or PARP in lysates from erlotinib sensitive or resistant cells that had been treated with control (shControl) or EPHA2 specific (shEPHA2) shRNA. Lysates were collected 72 hours post-puromycin selection. (D) Signaling analysis on cell lysates from two

pairs of isogenic erlotinib sensitive and resistant cell lines were treated with si*Control* or si*EPHA2* for 72 hours. Shown are representative immunoblots in which signaling molecules were detected with the indicated antibodies.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. A selective EPHA2 small molecule inhibitor decreases cell survival of erlotinib resistant lung cancer cells in vitro

(A) Four cell lines with acquired resistance to erlotinib were treated with ALW-II-41-27, NG-25, erlotinib, or DMSO for 72 hours, and cell viability was assessed by the MTT assay. Shown are percentages of cell viability in drug treatment groups relative to a DMSO control group. **p 0.005, two way ANOVA with Bonferroni post hoc analysis. (B) Two cell lines with acquired resistance to AZD9291 were treated with ALW-II-41-27, NG-25, erlotinib, AZD9291, or DMSO for 72 hours, and cell viability was assessed by the MTT assay. Shown are the percentages of cell viability in drug treatment groups relative to a DMSO control group. *p 0.05; **p 0.005, two way ANOVA with Bonferroni post hoc analysis. (C) Cells were treated with 1μM ALW-II-41-27, NG-25, or erlotinib for 72 hours, and BrdU was added 2 hours prior to the commencement of the assay. Proliferation was measured by quantifying incorporation of BrdU into the cellular DNA using the BrdU Cell Proliferation Assay kit. Experiments were repeated 3 times, and data are presented as a proliferation index relative to DMSO ± SEM. *p 0.05; **p 0.005, two way ANOVA with Bonferroni post hoc analysis. (D) Cells were treated as in C and apoptosis was measured by quantifying histone-associated DNA fragments using a Cell Death ELISA kit. Experiments were repeated 3 times, and data are presented as an apoptotic index relative to the DMSO ± SEM. **p 0.005, two way ANOVA with Bonferroni post hoc analysis. (E) Immunoblotting for

proteins involved in apoptosis from lysates of cells treated with 1 μ M ALW-II-41-27, 1 μ M erlotinib, or DMSO for 24 hours. **(F)** Pairs of erlotinib sensitive and resistant cells were treated with 1 μ M ALW-II-41-27, 1 μ M erlotinib, or DMSO for 6 hours. Phosphorylation of signaling molecules was determined by western blot analysis using anti-phospho and anti-total protein antibodies as indicated.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

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 $\sim 150 \text{ mm}^3$ before administration of 15mg/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. ($n = 5/\text{treatment group}$). Data is presented as the mean tumor volume $\pm$ 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.