Afatinib plus Cetuximab Delays Resistance Compared to Single-Agent Erlotinib or Afatinib in Mouse Models of TKI-Naïve EGFR L858R-Induced Lung Adenocarcinoma

Valentina Pirazzoli1, Deborah Ayeni2,3, Catherine B. Meador4, Basavaraju G. Sanganahalli5, Fahmee Hyder5,6, Elisa de Stanchina7, Sarah B. Goldberg8, William Pao4,9,10, and Katerina Politi1,3,8

Abstract

Purpose: The EGFR tyrosine kinase inhibitors (TKIs), erlotinib and afatinib, have transformed the treatment of advanced EGFR-mutant lung adenocarcinoma. However, almost all patients who respond develop acquired resistance on average approximately 1 year after starting therapy. Resistance is commonly due to a secondary mutation in EGFR (EGFR<sup>T790M</sup>). We previously found that the combination of the EGFR TKI afatinib and the EGFR antibody cetuximab could overcome EGFR<sup>T790M</sup>-mediated resistance in preclinical models. This combination has shown a 29% response rate in a clinical trial in patients with acquired resistance to first-generation TKIs. An outstanding question is whether this regimen is beneficial when used as first-line therapy.

Experimental Design: Using mouse models of EGFR-mutant lung cancer, we tested whether the combination of afatinib plus cetuximab delivered upfront to mice with TKI-naïve EGFR<sup>E538K</sup>-induced lung adenocarcinomas delayed tumor relapse and drug-resistance compared with single-agent TKIs.

Results: Afatinib plus cetuximab markedly delayed the time to relapse and incidence of drug-resistant tumors, which occurred in only 63.6% of the mice in contrast to erlotinib or afatinib treatment where 100% of mice developed resistance. Mechanisms of tumor escape observed in afatinib plus cetuximab resistant tumors include the EGFR<sup>T790M</sup> mutation and Kras mutations. Experiments in cell lines and xenografts confirmed that the afatinib plus cetuximab combination does not suppress the emergence of EGFR<sup>T790M</sup>-mutant lung cancer and indicate that clinical trial development in this area is warranted.

Conclusions: These results highlight the potential of afatinib plus cetuximab as an effective treatment strategy for patients with TKI-naïve EGFR-mutant lung cancer and indicate that clinical trial development in this area is warranted. Clin Cancer Res; 22(2); 426–35. ©2015 AACR.
Afasitib + Cetuximab First-line in EGFR-Mutant Lung Cancer

Translational Relevance

The first-generation tyrosine kinase inhibitors (TKIs) erlotinib and gefitinib have improved progression-free survival in patients with lung adenocarcinomas harboring activating mutations in the EGFR gene. Despite the effectiveness of these compounds, patients inevitably develop progressive disease after a median of approximately 1 year of starting treatment. Effective strategies to delay the emergence of this resistance are needed. Here, we report on a preclinical study that demonstrates how the combination of the EGFR TKI afatinib and the EGFR antibody cetuximab decreases the incidence and delays drug resistance in transgenic mouse models of EGFR-mutant lung cancer. This work lays the foundation for a clinical trial of the combination of afatinib and cetuximab in patients with TKI-naïve EGFR-mutant lung cancer.

New-generation mutant-specific TKIs, such as AZD9291 and CO-1686, are showing clinical activity especially in the setting of T790M-positive disease (7–10).

A previous study conducted in transgenic mice with EGFR_L858R/T790M-induced lung adenocarcinomas demonstrated that the combination of a second-generation TKI afatinib with the anti-EGFR antibody cetuximab can overcome T790M-mediated resistance, while neither drug alone is effective (11). On the basis of these data, a phase IB/II clinical trial of this drug combination was conducted in patients that developed progressive disease after erlotinib or gefitinib. A 29% objective response rate was observed with a median duration of radiographic response of 5.7 months (12).

Given the promising results in the resistance setting as well as data in the first-line setting presented in this article, a randomized phase II/III trial of afatinib plus cetuximab versus afatinib alone in treatment-naïve patients with advanced EGFR-mutant lung cancer is ongoing led by the South West Oncology Group (SWOG).

We hypothesize that patients may derive an even greater benefit from upfront treatment with the combination of afatinib plus cetuximab, with the goal of delaying the development of resistance. Here, we investigated the therapeutic effect of first-line afatinib plus cetuximab combination therapy versus erlotinib or afatinib alone in a mouse model of lung cancer driven by EGFR_L858R, which we previously developed (13).

Materials and Methods

Transgenic mice

TetO-EGFR_L858R mice and CCSP-rTAla mice were previously described (13). Doxycycline was administered by feeding mice with doxycycline-impregnated food pellets (625 ppm; Harlan Tekland). Erlotinib and cetuximab (obtained from the Organic Synthesis Core Facility at MSKCC, NY) were suspended in 0.5% (w/v) methylcellulose. Erlotinib was administered intraperitoneally (i.p., 25 mg/kg, 5 days a week) while afatinib was administered orally (per os, 25 mg/kg, 5 days a week). Cetuximab (Erbitux; Bristol-Myers Squibb and Eli Lilly Pharmaceuticals) was administered intraperitoneally (i.p., 1 mg twice a week). Our initial intent was to compare afatinib plus cetuximab to erlotinib, therefore mice were randomized to treatment with these agents. Afatinib treatment was included later as we began to develop the concept of the first-line trial of afatinib versus afatinib plus cetuximab. At the end of the study, mice were euthanized by CO2 asphyxiation. All animals were kept in pathogen-free housing under guidelines approved by the Yale University Institutional Animal Care and Use Committee (IACUC).

Magnetic resonance imaging

All procedures were performed in accordance with protocols approved by the Yale University IACUC and in agreement with the NIH Guide for the Care and Use of Laboratory Animals. Respiratory gated, gradient-echo MR images of mice were collected with a 4T (31-cm bore) small-animal Bruker horizontal-bore spectrometer (Bruker AVANCE). All data were collected using a T2*-weighted contrast using a custom-built 4 cm diameter 1H Bollinger coil. Prior to the imaging experiments, mice were anesthetized with isoflurane and were maintained on isoflurane/O2 (2–2.5% v/v) throughout data collection. Animal core-body temperature was maintained at 37 ± 1°C by circulation of warm air through the bore of the magnet. Although during the MR imaging, the respiration rates for all mice were regular, MR data collection was synchronized with animal respiration using an MR compatible small-animal monitoring and gating system (SA Instruments, Inc.), which allowed MRI acquisition in the same phase of the breathing cycle. All the MR images were collected during postexpiratory periods with the following MR parameters: field of view = 2.56 × 2.56 × 1.80 cm^2, image matrix = 256 × 128 × 24, repetition time = 100 ms, echo time = 4.5 ms, flip angle = 30 degree, 2 averages. These scan parameters were chosen to maximize the contrast between healthy lung tissue and tumor. Following a treatment period, mice in different treatment groups were scanned repeatedly over weeks off drug to evaluate the presence of recurrent tumors. Tumor volume was quantified by calculating the area of visible lung opacities present in each image sequence per mouse using Biolmage Suite 3.01 (14).

Sequencing

Freshly harvested tumors and adjacent normal lung tissue were pulverized in liquid nitrogen and RNA was extracted using the RNeasy platform (Qiagen, #74104). RNA was then treated with DNase 1 (RNase-Free DNA Set, Qiagen #79254). cDNA was synthesized using the Superscript III First-Strand cDNA Synthesis Kit (Invitrogen, #18080-051). The cDNA was used as template to amplify the EGFR transgene and Kras cDNA. PCR products were sequenced by Sanger sequencing and sequence tracings were manually reviewed in the forward and reverse directions. The presence of the EGFR_L858R mutation was confirmed with the following primers: EGFR-2445F: 5′-gggattctgggtatctgta-3′, EGFR-2502R: 5′-catggtgggagggagcc-3′. The presence of the EGFR_T790M mutation was evaluated using the following primers: EGFR-2074F: 5′-cttacacccgagggaga-3′, EGFR-2502R: 5′-caccaaggaaccttcct-3′. The presence of Kras mutations were evaluated using the following primers: Kras-2074F: 5′-aagaggctgtgc-3′, Kras-2074R: 5′-ccctcccagttctcatgta-3′. Mutations in genes encoding the mouse Pi3K catalytic subunit alpha (Pik3ca) and beta (Pik3cb) were investigated using the following oligos: Pik3ca-F1: 5′-gcaacggcagaaaaatc-3′, Pik3ca-R1: 5′-ttcaggagc-gaaga-3′, Pik3ca-F3: 5′-tgcgcaagcaaa-3′, Pik3cb-R2: 5′-gggagcacgtggagaa-3′.
Cell lines and in vitro EGFR<sub>T790M</sub> selection

Human lung adenocarcinoma cell lines, PC-9 and PC-9/BRc1 were cultured in RPMI + 10% fetal bovine serum (FBS) and supplemented with 1% penicillin/streptomycin, 1% hygromycin B (Invitrogen) 100 U/ml penicillin/streptomycin (Corning). PC-9 cells were obtained from the Varms Laboratory and maintained in the Pao laboratory since 2004. The isogenic afatinib-resistant cell line PC-9/BRc1 was derived from parental PC-9 cells as previously described (15). Both cell lines were routinely tested for mycoplasma contamination and authentication was performed by confirming the presence of the predicted EGFR kinase domain mutations and using STR profiling (GenePrint 10 System) at the Yale University DNA Analysis Facility in July 2015.

Aliquots of cell mixtures containing 75% PC-9 parental cells (T790M-negative) and 25% PC-9/BRc1 cells (T790M-positive) were prepared and plated in separate dishes. One aliquot was saved as the pretreatment sample to empirically determine the starting T790M allele frequency. Cell mixtures were harvested following 7 days of treatment with EGFR inhibitors. Drugs were refreshed every 72 hours. Genomic DNA was extracted using the DNeasy kit (Qiagen, #69504) and was subjected to SNPPhost sequencing for T790M (16). T790M allele frequency was determined by measuring the relative heights of T790M mutant versus wild-type <i>EGFR</i> peaks [(mutant peak height) / (mutant + wild-type peak height)].

Quantitative PCR

Genomic DNA from pulverized tumors and adjacent normal lung was extracted using the Wizard genomic purification kit (Promega, #A1120). Quantitative PCR was performed with TaqMan copy number assays (Applied Biosystems) using the Viia7 Real Time PCR System (Applied Biosystems). Ten nanograms of genomic DNA were used in the reaction. Amplification was carried out for 40 cycles (10 minutes at 95°C, 15 seconds at 95°C, 1 minute at 60°C). Quadruplicate C<sub>T</sub> values were averaged and normalized to genomic DNA from the tail of a C57BL/6J mouse. A TaqMan copy number reference assay for mouse Tcr (Applied Biosystems) was used for all the reactions.

Results

Afatinib plus cetuximab combination therapy delays relapse compared with erlotinib or afatinib alone

As a first step in testing whether the afatinib plus cetuximab combination was more effective than single-agent TKIs in the initial treatment of EGFR-mutant lung cancer, we measured tumor relapse following treatment with the different regimens. CCSp- <i>raf</i>/TatO-EGFR<sup>E854K</sup> tumor-bearing mice were treated with afatinib plus cetuximab, afatinib, or erlotinib for 4 weeks, after which treatment was interrupted (Fig. 1). Doxycycline treatment, to induce transgene expression was initiated at weaning and maintained throughout the life of the mice. To evaluate response to each treatment, tumor volume was quantified by MRI at the beginning and at the end of the 4 weeks of treatment. MR images taken at the end of the treatment period showed dramatic responses in all three treatment groups: the median tumor volume change on erlotinib,
Afatinib, and afatinib plus cetuximab was /C0 100% (Supplementary Fig. S1A and S1B; Supplementary Table S1).

After 4 weeks, treatment was halted and mice were monitored by MRI 4, 8, and 12 weeks off drug to evaluate the presence of recurrent tumors (Fig. 1). In some cases, imaging was performed before the 4-week interval was complete as the mice were showing signs of respiratory distress. After 4 weeks off drug, only 15.4% of the afatinib plus cetuximab-treated mice showed measurable recurrent tumors (defined as tumor volume /C21 100 mm³), in contrast to 77.8% of afatinib-treated and 100% of erlotinib-treated mice. This percentage increased to 84.6% after 8 weeks off drug in the afatinib plus cetuximab group and reached 100% after 12 weeks off-drug (Fig. 1). By 8 weeks off drug, tumors in all mice treated with afatinib as a single agent had recurred. The median tumor burden pretreatment in the afatinib plus cetuximab group was similar to that in the erlotinib and afatinib (erlotinib 225.7 mm³, afatinib 320.7 mm³, afatinib plus cetuximab 206.7 mm³) groups, indicating that the longer time to relapse was not a function of the initial tumor volume (Supplementary Fig. S1; Supplementary Table S1). These data show that afatinib plus cetuximab delays tumor re-emergence by 2-fold in our mouse model of EGFR-mutant lung cancer when compared with erlotinib and afatinib, indicating that the drug combination is more potent than the single agents in eradicating tumor cells.

Generation of afatinib plus cetuximab-resistant lung adenocarcinomas

To investigate whether long-term afatinib plus cetuximab treatment of EGFR<sup>L858R</sup>-mutant lung adenocarcinomas in mice leads to the emergence of drug-resistant tumors, we subjected mice with EGFR<sup>L858R</sup>-induced lung adenocarcinomas to an intermittent drug-dosing schedule, previously used to successfully generate erlotinib-resistant tumors (17). Mice were treated with erlotinib, afatinib, or afatinib plus cetuximab for 4 weeks (see Materials and Methods for details), after which treatment was discontinued until the detection of recurrent tumors by MRI (defined as tumors that grew in the presence of drugs). Upon recurrence, treatment was reinitiated for another 4 weeks. This on/off drug treatment cycle was repeated until the emergence of resistance or for a maximum of 5 times (Fig. 2A; Supplementary Tables S2 and S3). All seven mice intermittently treated with erlotinib developed resistance within 4 drug cycles, confirming previously published results (ref. 17; Fig. 2B). The emergence of drug resistance was also observed in all 5 mice treated with afatinib (Fig. 2B). Consistent with these data, our results indicate that afatinib induces resistance and does not delay the onset of resistance compared with erlotinib (Fig. 2B and C). The emergence of resistance to afatinib plus cetuximab was evaluated in 13 CCSP-rtTA<sup>−</sup>; TetO-EGFR<sup>L858R</sup> mice. The afatinib plus cetuximab drug combination was well tolerated by mice treated with the intermittent dosing protocol as evidenced by their weight patterns compared with TKI treatment alone (Supplementary Fig. S2). Six out of 13 mice that went through at least 3 cycles of afatinib plus cetuximab did not develop resistant disease (Fig. 2B; Supplementary Table S3). Four out of 11 mice that went through at least 4 cycles of afatinib plus cetuximab, did not develop resistant disease (Fig. 2B; Supplementary Table S3). Therefore, in contrast to erlotinib and afatinib, resistance to afatinib plus cetuximab occurred only in 63.6% (7/11) of mice that went through the same minimum number of drug cycles. As afatinib plus cetuximab delayed tumor relapse, each off-drug cycle...
was extended in this cohort of mice. Therefore, in the afatinib plus cetuximab-treated mice, the overall time for resistant tumors to emerge was longer (>2-fold) compared with those treated with single agent (Fig. 2C). In summary, our data reveal that drug resistance is delayed and occurs with decreased incidence in mice treated with afatinib plus cetuximab compared with single-agent TKIs.

**EGFR*<sup>T790M</sup>* and *Kras* mutations are found in afatinib plus cetuximab-resistant lung adenocarcinomas**

To investigate the mechanisms of resistance in tumors that grew out following an initial response to EGFR inhibitor treatment, drug-resistant tumors generated using either continuous (data not shown) or intermittent dosing of the drugs were collected for molecular analysis and histopathologic evaluation. In some cases, multiple resistant tumors per mouse were observed by MRI and at the time of necropsy. However, in most cases only one nodule was large enough for molecular studies. We first evaluated whether EGFR*<sup>T790M</sup>* could account for drug resistance in all three groups of treatment. For this purpose, we extracted RNA from the resistant tumors and the adjacent normal lung and generated cDNA that was used to sequence the TK domain of EGFR. All the erlotinib (*n* = 4) and the afatinib (*n* = 6) resistant tumors analyzed contained the cytosine to thymine
point mutation at position 2369, leading to the T790M amino acid substitution. Interestingly, the same mutation was detected in only 7 out of 13 afatinib plus cetuximab-resistant tumors studied (53.8%; Table 1; Supplementary Fig. S3A). All matched adjacent normal lungs from the same animals were negative for \textit{EGFR}^{T790M} (Table 1; Supplementary Fig. S3A). Expression of the \textit{EGFR}^{L858R} mutant was confirmed in all the resistant tumors and adjacent lungs as expected (Supplementary Fig. S3B).

These data suggest that afatinib plus cetuximab treatment does not suppress the emergence of \textit{EGFR}^{T790M} even though it delays its emergence. To further explore this possibility, we performed experiments in cell culture, in which PC-9 cells (T790M negative) and TKI-resistant PC-9/BRc1 cells (T790M positive) were mixed in a 3:1 ratio. The cells were then treated with afatinib, cetuximab, or afatinib plus cetuximab for 1 week, after which the T790M allele frequency was determined.

examine \textit{EGFR}^{T790M} selection in a xenograft model by injecting a 3:1 ratio of PC-9 cells and PC-9/BRc1 cells into the flanks of immunodeficient mice and treating animals with afatinib plus cetuximab for 10 days. By comparing T790M allele frequency pre- and post-treatment, we again found that afatinib plus cetuximab selected for T790M in vivo (Fig. 3B). Interestingly, in these mixing experiments, the abundance of T790M in the afatinib plus cetuximab treatments did not differ from the single-agent studies, in contrast to results in the transgenic mice. This can potentially be explained by this experimental design in which cells with the T790M mutation are mixed with cells without it facilitating the emergence of T790M compared with transgenic mice where the mutation must arise spontaneously. In summary, our data are consistent with the notion that an increase in T790M allele frequency may mediate resistance to treatment with afatinib plus cetuximab in a subset of cases.

We further explored mechanisms of resistance to afatinib plus cetuximab in the remainder of T790M-negative drug-resistant tumors. As resistance to cetuximab treatment has been reported to occur via \textit{KRAS} mutations in metastatic colorectal cancer (18), we checked whether mutations in this gene could be associated with resistance to afatinib plus cetuximab, especially in those tumors without \textit{EGFR}^{T790M} mutations. For this purpose, we sequenced endogenous \textit{Kras} in all the resistant tumors and normal adjacent lung. Interestingly, 5 resistant tumors that were negative for the T790M mutation had acquired a point mutation that changed the glycine amino acid at position 12. We detected a guanine-to-thymine transversion at position 35, leading to G12D, a guanine-to-thymine transversion at position 35, leading to G12V, and three guanine-to-adenosine transition at position 35, leading to G12D (Table 1; Supplementary Fig. S3C). Importantly, these tumors with \textit{Kras} mutations retained production of the EGFR L858R protein (Fig. 3C).

As \textit{MET}, \textit{ERBB2}, or \textit{EGFR} amplification are also associated with resistance to EGFR TKIs (19–21), we looked at alterations in copy number of these genes in the resistant tumors and normal adjacent lung. None of resistant tumors showed amplification of \textit{Met}, \textit{ErbB2}, or \textit{Egf} (Supplementary Fig. S3D). However, variations in the levels of expression of \textit{EGFR} were found in one afatinib-resistant tumor that displayed a >3fold-increased expression level, and two afatinib plus cetuximab-resistant tumors that had approximately 3-fold increased level of expression of the human transgene (Supplementary Fig. S3E). Increased \textit{EGFR} copy number has also been described in human EGFR-mutant TKI-resistant lung adenocarcinomas (21, 22).

### Signaling pathway activation in resistant tumors

Blockade of EGFR by targeted therapy results in the inhibition of the MAPK/ERK as well as the PI3K/AKT pathways in tumor cells. By gaining the somatic T790M mutation, such cells maintain activation of these downstream pathways. To confirm this scenario in our samples, we performed immunoblotting on lysates from untreated, drug-sensitive and resistant tumors (Fig. 3C). Indeed, phosphorylated EGFR levels were restored to untreated levels in \textit{EGFR}^{T790M} positive tumors but not in those harboring a \textit{Kras} mutation. Interestingly, the \textit{EGFR}^{T790M} tumors also showed increased ErbB2 phosphorylation that was not detected in the \textit{Kras}-mutant tumors. In the \textit{Kras}-mutant tumors, instead, elevated phospho-Erk levels were found (Fig. 3C). Staining for the mitotic marker phospho-histone H3 on tumor

| Table 1. Summary of resistance mechanisms in murine drug-resistant tumors |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Mouse | Drug treatment | Sample | Primary \textit{EGFR} mutation | Secondary \textit{EGFR} mutation | \textit{Kras} mutation |
| 6501 | E | Normal | L858R | Neg | Neg |
| 8814 | E | Normal | L858R | Neg | Neg |
| 8817 | E | Normal | L858R | Neg | Neg |
| 8818 | E | Normal | L858R | Neg | Neg |
| 9194 | A | Normal | L858R | Neg | Neg |
| 9196 | A | Normal | L858R | Neg | Neg |
| 1100 | A | Normal | L858R | Neg | Neg |
| 4392 | A+C | Normal | L858R | Neg | G2R |
| 4973 | A+C | Normal | L858R | Neg | G2V |
| 597 | A+C | Normal | L858R | Neg | G2D |
| 593 | A+C | Normal | L858R | Neg | G2D |
| 7087 | A+C | Normal | L858R | Neg | G2D |
| 7091 | A+C | Normal | L858R | Neg | G2D |
| 9072 | A+C | Normal | L858R | Neg | G2D |

Abbreviations: E, erlotinib; A, afatinib; A+C, afatinib+cetuximab.

* Mice underwent continuous and not intermittent drug treatment.
sections revealed active proliferation in EGFRT790M positive and Kras-mutant afatinib plus cetuximab-resistant tumors, indicating that regardless of the mechanism these tumors have escaped drug treatment allowing them to proliferate (Fig. 3D).

Lung adenocarcinomas with somatic mutations in EGF are characterized by activation of the PI3K–AKT–mTOR pathway (23) that can be further engaged in settings of resistance to TKIs and thus attenuate apoptosis and enhance proliferation (24). To investigate the contribution of the mTOR pathway to resistance to afatinib plus cetuximab, we performed immunoblotting for pS6, a widely used marker of mTOR activation. After 5 days of treatment with erlotinib or afatinib alone and afatinib plus cetuximab decreased levels of pS6 were observed. We did not observe an increase in the pS6 levels in resistant tumors, consistent with the mutationally driven mechanisms of resistance found (Fig. 3C).

**Discussion**

The emergence of acquired resistance to single-agent EGFR TKI inhibitor treatment is the barrier to achieving long-term benefit from these targeted therapies in patients with EGF-R-mutant lung cancer. Therefore, there is an urgent need for therapeutic regimens that can delay or prevent the emergence of drug resistance. Here
we show that dual targeting of mutant EGFR with the irreversible TKI afatinib and the EGFR antibody cetuximab in the first-line setting reduces the incidence and delays drug resistance in mice with EGFR\(^{L858R-T790M}\)-induced lung adenocarcinomas. Moreover, we investigated mechanisms of resistance in these afatinib plus cetuximab-resistant tumors. Our data highlight the potential of this drug combination for the first-line treatment of patients with EGFR-mutant lung cancer.

Several new strategies and drugs have been developed in recent years aimed at overcoming resistance to first and second-generation TKIs. The afatinib plus cetuximab combination was originally found to lead to tumor regression in transgenic mice harboring EGFR\(^{L858R-T790M}\)-induced lung adenocarcinomas (11). In a clinical trial of this combination, 29% of patients with TKI-refractory lung adenocarcinomas (both with and without the T790M mutation) responded to these agents (12). One of the disadvantages of this combination, however, is the increased toxicity observed due to inhibition of wild-type EGFR. More recently, third-generation EGFR TKIs, such as AZD9291 and CO1686, which specifically target mutant EGFR, including the EGFR\(^{T790M}\) mutation, have been developed and are showing promise in clinical trials in patients with TKI-resistant EGFR-mutant tumors (7–10). While it is at present unknown how best to sequence these different therapies, emerging preclinical studies suggest that appropriate sequencing of the agents will be important (25). Indeed, resistance to erlotinib, afatinib, and afatinib plus cetuximab can be overcome using the third-generation TKI AZD9291, but the reverse does not occur (25). Together with this observation, our data indicate that afatinib plus cetuximab is superior to afatinib or cetuximab alone when used as first-line therapy, suggesting a potential treatment scenario in which afatinib plus cetuximab are given upfront followed by third-generation TKI treatment if and when resistance emerges. A Cooperative group phase II/III trial of afatinib plus cetuximab versus afatinib alone in patients with TKI-naïve EGFR-mutant lung cancer is underway. Patients with exon 19 deletion mutant tumors exhibit improved survival upon upfront afatinib treatment (26) and whether they benefit differently from combined afatinib plus cetuximab treatment compared with patients with L858R-induced tumors remains to be determined. Of note, our study compares afatinib versus afatinib plus cetuximab in mice harboring the L858R point mutation and not EGFR Exon 19 deletion mutations. Afatinib plus cetuximab can effectively lead to the regression of tumors harboring the EGFR\(^{T790M}\) mutation (11). Prior to this work, however, it was unclear whether this mutation could emerge as a mechanism of resistance to afatinib plus cetuximab upon treatment of TKI-naïve tumors. We show that in mice with EGFR\(^{L858R}\)-induced tumors, long-term treatment with afatinib plus cetuximab can lead to the emergence of EGFR\(^{T790M}\). Further supporting this result, when we mixed cells with and without EGFR\(^{T790M}\) and treated them with afatinib plus cetuximab, cells harboring EGFR\(^{T790M}\) outgrew the EGFR\(^{T790M}\)-negative cells. Our data support the possibility that one of the mechanisms of resistance to this drug combination is the EGFR\(^{T790M}\) mutation, a finding that will be confirmed in the clinical trial of this drug combination. These results, also suggest that additional mediators of sensitivity and resistance to afatinib plus cetuximab are likely to exist since we know that tumors harboring EGFR\(^{T790M}\) mutations can be responsive to this drug combination in mice and humans (11, 12). Previously, we had found mTOR pathway activation as a mechanism of resistance to afatinib plus cetuximab in tumors already harboring an EGFR\(^{T790M}\) mutation (24). Interestingly, in the afatinib plus cetuximab-resistant tumors examined here, we did not observe activation of this pathway above baseline levels (Fig. 3C). This is likely due to the fact that all of the resistant tumors examined in this study either had acquired the EGFR\(^{T790M}\) mutation or a Kras mutation and suggests that the mechanisms of resistance to afatinib plus cetuximab in TKI-naïve and resistant tumors may be different. Importantly, our study points to specific potential resistance mechanisms that should and will be examined in the phase II/III cooperative group trial of afatinib versus afatinib plus cetuximab.

One of the surprising findings from our study was that approximately 50% of afatinib plus cetuximab-resistant tumors in the EGFR\(^{L858R}\) mouse model harbored mutations in Kras. Mutations in Kras are a well-established mechanism of primary resistance to EGFR TKIs (27), but have not been found to emerge following successful TKI treatment (e.g., acquired resistance) in patients (28). Previously, we found that Kras mutations could emerge in transgenic models of EGFR-mutant lung cancer following erlotinib treatment (17). The discrepancy between humans and mice could be due to the fact that Kras mutations can arise spontaneously in aged mouse lungs (29); thus, it is possible that the Kras-mutant tumors arise independently of EGFR mutations and/or drug treatment. Alternatively, we cannot exclude that the afatinib plus cetuximab drug regimen may contribute to the emergence of Kras mutations. Indeed, Kras mutations have been identified in colorectal cancers that have acquired resistance to cetuximab (18). EGFR and Kras mutations are mutually exclusive in human lung cancer, possibly reflecting the lethality of expressing mutations in both genes (30). In the resistant tumors, however, suppression of EGFR activity may be permissive for the survival of Kras-mutant cells. Analysis of samples from the clinical trials of these agents will further shed light on this issue. Although the EGFR\(^{T790M}\) mutation and Kras mutations were the only mechanisms of resistance identified in our study, we expect that a broader array of mechanisms will be found in the context of the planned phase II/III clinical trial. Indeed, plans for the molecular analysis of repeat biopsy specimens at the time of acquired resistance to afatinib or afatinib plus cetuximab in this trial include whole exome sequencing, analysis of receptor tyrosine kinase amplification and protein levels and will provide a comprehensive picture of the mechanistic basis for resistance to these agents in patients with EGFR-mutant lung cancer.

In conclusion, further investigation of the afatinib plus cetuximab combination therapy for patients with untreated EGFR-mutant lung cancer warrants investigation, despite the potential for added toxicity, and may represent an alternative to single-agent TKI treatment that could delay the emergence of drug resistance.

Disclosure of Potential Conflicts of Interest

S.B. Goldberg reports receiving a commercial research grant from AstraZeneca and is a consultant/advisory board member for Clovis. W. Pao is an employee of Roche and reports receiving royalties from MolecularMD. K. Politi reports receiving commercial research grants from AstraZeneca and Kolltan; has ownership interest (including patents) in Molecular MD; and is a consultant/advisory board member for Takeda. No potential conflicts of interest were disclosed by the other authors.

www.aacjrournals.org

Clin Cancer Res; 22(2) January 15, 2016 433

Published OnlineFirst September 4, 2015; DOI: 10.1158/1078-0432.CCR-15-0620

Cetuximab First-line in EGFR-Mutant Lung Cancer

Afatinib + Cetuximab First-line in EGFR-Mutant Lung Cancer

S.B. Goldberg reports receiving a commercial research grant from AstraZeneca and is a consultant/advisory board member for Clovis. W. Pao is an employee of Roche and reports receiving royalties from MolecularMD. K. Politi reports receiving commercial research grants from AstraZeneca and Kolltan; has ownership interest (including patents) in Molecular MD; and is a consultant/advisory board member for Takeda. No potential conflicts of interest were disclosed by the other authors.
Conception and design: V. Pirazzoli, E. de Stanchina, W. Pao, K. Politi
Development of methodology: V. Pirazzoli, B. Sanganahalli, W. Pao, K. Politi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Pirazzoli, D. Ayeni, C.B. Meador, B. Sanganahalli, E. de Stanchina, W. Pao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Pirazzoli, D. Ayeni, B. Sanganahalli, F. Hyder, S.B. Goldberg, W. Pao, K. Politi
Writing, review, and/or revision of the manuscript: V. Pirazzoli, D. Ayeni, C.B. Meador, B. Sanganahalli, F. Hyder, S.B. Goldberg, W. Pao, K. Politi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Pirazzoli, W. Pao, K. Politi
Study supervision: W. Pao, K. Politi

Acknowledgments

The authors thank Mary Ann Melnick for expert technical assistance and critical reading of the manuscript.

References


Grant Support

This work was funded by NIH/NCI grant R01CA120247 (to K. Politi), R01CA122110 (to W. Pao and K. Politi), P50CA196530-01 (K. Politi and S.B. Goldberg), R01CA41012 (to F. H), P30NS052519 (to F. H), P30CA68485 (to W. Pao), P30CA08748 (to E. de Stanchina), the American Italian Cancer Foundation (to V. Pirazzoli), Uniting Against Lung Cancer (to K. Politi), and Yale University. C.B. Meador was supported by Public Health Service Award T32 GM07347 from the National Institute of General Medical Studies for the Vanderbilt Medical-Scientist Training Program and the VICC Melly Family Scholarship. Sarah Goldberg is funded by the Department of Defense, the Hope Foundation, and AstraZeneca. D. Ayeni was supported by an NSF Predoctoral fellowship (DGE-1122492).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 13, 2015; revised August 14, 2015; accepted August 18, 2015; published OnlineFirst September 4, 2015.


Afatinib plus Cetuximab Delays Resistance Compared to Single-Agent Erlotinib or Afatinib in Mouse Models of TKI-Naïve EGFR L858R-Induced Lung Adenocarcinoma

Valentina Pirazzoli, Deborah Ayeni, Catherine B. Meador, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-15-0620

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/10/02/1078-0432.CCR-15-0620.DC1

Cited articles
This article cites 30 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/22/2/426.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/22/2/426.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.