

A Novel ALK Secondary Mutation and EGFR Signaling Cause Resistance to ALK Kinase Inhibitors

Takaaki Sasaki^{1,4}, Jussi Koivunen^{1,4}, Atsuko Ogino^{1,4}, Masahiko Yanagita^{1,4}, Sarah Nikiforow^{1,4,6}, Wei Zheng^{2,3}, Christopher Lathan^{1,4,6}, J. Paul Marcoux^{1,4,6}, Jinyan Du⁷, Katsuhiko Okuda^{1,4}, Marzia Capelletti^{1,4}, Takeshi Shimamura^{1,4,6}, Dalia Ercan^{1,4}, Magda Stumpfova^{1,4}, Yun Xiao⁵, Stanislaw Weremowicz⁵, Mohit Butaney^{1,4}, Stephanie Heon^{1,4,6}, Keith Wilner⁸, James G. Christensen⁸, Michel J. Eck^{2,3}, Kwok-Kin Wong^{1,4,6}, Neal Lindeman⁵, Nathanael S. Gray^{2,3}, Scott J. Rodig⁵, and Pasi A. Jänne^{1,4,6}

Abstract

Anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors (TKI), including crizotinib, are effective treatments in preclinical models and in cancer patients with ALK-translocated cancers. However, their efficacy will ultimately be limited by the development of acquired drug resistance. Here we report two mechanisms of ALK TKI resistance identified from a crizotinib-treated non-small cell lung cancer (NSCLC) patient and in a cell line generated from the resistant tumor (DFCI076) as well as from studying a resistant version of the ALK TKI (TAE684)-sensitive H3122 cell line. The crizotinib-resistant DFCI076 cell line harbored a unique L1152R ALK secondary mutation and was also resistant to the structurally unrelated ALK TKI TAE684. Although the DFCI076 cell line was still partially dependent on ALK for survival, it also contained concurrent coactivation of epidermal growth factor receptor (EGFR) signaling. In contrast, the TAE684-resistant (TR3) H3122 cell line did not contain an ALK secondary mutation but instead harbored coactivation of EGFR signaling. Dual inhibition of both ALK and EGFR was the most effective therapeutic strategy for the DFCI076 and H3122 TR3 cell lines. We further identified a subset (3/50; 6%) of treatment naive NSCLC patients with ALK rearrangements that also had concurrent EGFR activating mutations. Our studies identify resistance mechanisms to ALK TKIs mediated by both ALK and by a bypass signaling pathway mediated by EGFR. These mechanisms can occur independently, or in the same cancer, suggesting that the combination of both ALK and EGFR inhibitors may represent an effective therapy for these subsets of NSCLC patients. *Cancer Res*; 71(18); 6051–60. ©2011 AACR.

Introduction

The emerging impact of targeted therapies as cancer treatments has led to a therapeutic paradigm shift in the field of oncology. Several kinase inhibitors have been identified as effective clinical therapies for a broad range of cancers and, specifically, in those in which the target of the kinase inhibitor has undergone a gain of function genomic alteration (1, 2). However, the clinical success of treatment with kinase inhibitors

is uniformly limited by the development of acquired drug resistance. Two common mechanisms of acquired drug resistance have been identified. These include secondary mutations in the target of the kinase itself, which abrogate the inhibitory activity of the drug, and activation of alternative signaling pathways that bypass the continued requirement for inhibition of the original target (3–5). The understanding of the mechanistic bases for drug resistance will continue to inform the development of strategies to overcome or prevent clinical drug resistance, thereby providing a greater therapeutic benefit for cancer patients (6, 7).

Chromosomal rearrangements in the anaplastic lymphoma kinase (ALK) gene have been detected in anaplastic large cell lymphoma (ALCL), inflammatory myofibroblastic tumor (IMT), and in non-small cell lung cancer (NSCLC; refs. 8–10). In NSCLC, ALK rearrangements have been detected in 3% to 13% of patients, are more common in never-smokers and in those with adenocarcinoma (11). In addition, they are often mutually exclusive with other oncogenic alterations detected in NSCLC, including epidermal growth factor receptor (EGFR) mutations. ALK kinase inhibitors are effective therapies in both preclinical *in vitro* and *in vivo* models and in NSCLC patients harboring ALK rearrangements (2, 12, 13). In the phase I clinical trial of crizotinib, a radiographic tumor

Authors' Affiliations: Departments of ¹Medical Oncology, ²Cancer Biology, ³Biological Chemistry and Molecular Pharmacology, ⁴Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute; Departments of ⁵Pathology and ⁶Medicine, Brigham and Women's Hospital, Boston; ⁷The Broad Institute, Cambridge, Massachusetts; and ⁸Pfizer Global Research and Development, Department of Research Pharmacology, La Jolla, California

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Corresponding Author: Pasi A. Jänne, Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, D820, 450 Brookline Avenue, Boston, MA 02215. Phone: 617-632-6076; Fax: 617-582-7683; E-mail: pjanne@partners.org

doi: 10.1158/0008-5472.CAN-11-1340

©2011 American Association for Cancer Research.

response rate of 55% was observed in *ALK* rearranged NSCLC patients (2). This agent is currently in phase III clinical development in this genomically defined patient population.

Recent studies have also identified and studied crizotinib resistance mechanisms. To date 3 secondary mutations, all identified from crizotinib-treated NSCLC or IMT patients, have been reported (14, 15). These mutations either involve the "gatekeeper" residue (L1196) or sites away from crizotinib binding (F1174L and C1156Y; refs. 14, 15). The mechanistic basis for how the different mutations lead to crizotinib resistance is not fully understood. The L1196 mutation may create a steric hindrance for crizotinib binding, whereas the F1174L mutation likely promotes the active conformation of *ALK*, thus disfavoring crizotinib binding which preferentially binds the inactive conformation of *ALK* (14). Continued studies of these and other resistance mechanisms will be critical to the design of subsequent treatments for NSCLC patients with *ALK* rearrangements.

In this study, using cell line models of *ALK* inhibitor resistance, either derived from a crizotinib-resistant patient or generated *in vitro*, we uncover additional mechanisms of *ALK* kinase inhibitor resistance. Our findings underscore the complexity of drug resistance mechanisms and the therapeutic challenges of treating multiple concurrent resistance mechanisms.

Materials and Methods

Patients

Patients were either identified from the Thoracic Oncology Program at DFCI ($n = 3$) or were treated in a clinical trial (NCT00932893) with crizotinib that was sponsored by Pfizer, Inc ($n = 1$). Tumor biopsies were obtained under an Institutional Review Board approved protocol. All patients provided written informed consent.

ALK and EGFR genomic analyses

The *ALK* kinase domain was sequenced from all of the available specimens. The PCR primers and conditions are available upon request. *ALK* FISH was carried out using the break apart probe (Vysis LSI *ALK* Dual Color; Abbott Molecular) as previously described (14, 16). *EGFR* mutation detection was done in a CLIA certified laboratory using previously described methods (17).

Cell lines and expression constructs

The NSCLC cell lines H3122 (*EML4-ALK* variant 1 E13:A20) and DFCI-032 (*EML4-ALK* variant 1 E13:A20), A549, HCC827 (*EGFR* del E746_A750) have been previously published (13). The H3122 cells were obtained from the NIH and confirmed by fingerprinting using the Power Plex 1.2 system (Promega) in October 2010. The DFCI076 (*EML4-ALK* variant 3 (E6:A20) cell was established at Dana-Farber Cancer Institute from pleural effusion from a patient who had developed acquired resistance to crizotinib. The DFCI076 cells were cultured in RPMI 1640 (GIBCO) supplemented with 10% FBS, 100 units/mL penicillin, 100 mg/mL streptomycin, and 1 mmol/L sodium pyruvate (RPMI 10% medium).

The *EML4-ALK* (Variant 1) cDNA from the H3122 cell line and the *EGFR-del* (*EGFR delE746_A750*) cDNA were cloned into pDNR-Dual (BD Biosciences) as described previously (14). To generate *EML4-ALK* mutants, L1152R, L1196M, C1156Y, or F1174L mutations were introduced using site-directed mutagenesis (Agilent) with mutant specific primers according to the manufacturer's instructions and as previously described (14). All constructs were confirmed by DNA sequencing. Retroviral infection and culture of Ba/F3 cell were done using previously described methods (18). Polyclonal cell lines were established by puromycin selection and subsequently cultured in the absence of interleukin-3 (IL-3). Uninfected Ba/F3 cells or cell lines expressing green fluorescent protein (GFP) were used as controls.

Cell proliferation and growth assays

Crizotinib and the pan-ERBB inhibitor PF299804 were provided by Pfizer. TAE684 and BMS-536,924 were synthesized as previously described (19, 20). Recombinant human EGF (PHG0314) was purchased from Invitrogen. Growth and inhibition of growth was assessed by MTS assay according to previously established methods (18). All experimental points were set up in 6 to 12 wells and all experiments were repeated at least 3 times. For clonogenic assays, cells were plated in triplicate on the 6-well plates and subject to drug exposure for 14 days, the colonies were fixed and stained with 0.5% crystal violet in 25% methanol, and the numbers of colonies were counted.

ALK and EGFR shRNA constructs and lentiviral infection

ALK and *EGFR* short hairpin RNA (shRNA) constructs cloned into the pLKO.1 puro vector as previously described (21). A vector containing a nontargeting (NT) shRNA and GFP shRNA was used as a control. Lentivirus production, titrations, and infections were done as in ref. 21. The specific shRNA sequences are available upon request.

Generation of *in vitro* drug-resistant H3122 cells

To generate drug-resistant cells, the H3122 cells were exposed to increasing concentrations of TAE684, similar to our previously described methods (3, 21). TAE684 concentrations were increased stepwise from 1 to 100 nmol/L when the cells resumed growth kinetics, similar to untreated parental cells. To confirm the emergence of a resistant clone, MTS assays were done following growth at each concentration. The *in vitro* drug-resistant cells were subcloned by single-cell isolation.

Antibodies and Western blotting

Cell lysis, Western blotting, and immunoblotting was done as previously described (18). Anti-*ALK* (DF53), anti-phospho-*EGFR*, anti-total-*EGFR*, anti-*EGFR* L858R, anti-*EGFR* delE746_A750, anti-PARP, anti- α -tubulin, anti-phospho-Akt (Ser-473), and anti-total-Akt, were obtained from Cell Signaling Technology. Total ERK1/2 and phospho-ERK1/2 (pT185/pY187) antibodies were from Invitrogen. Immunoprecipitations were done using anti-Flag-M2 agarose (Sigma-Aldrich Co.). The receptor tyrosine kinase (RTK) array was

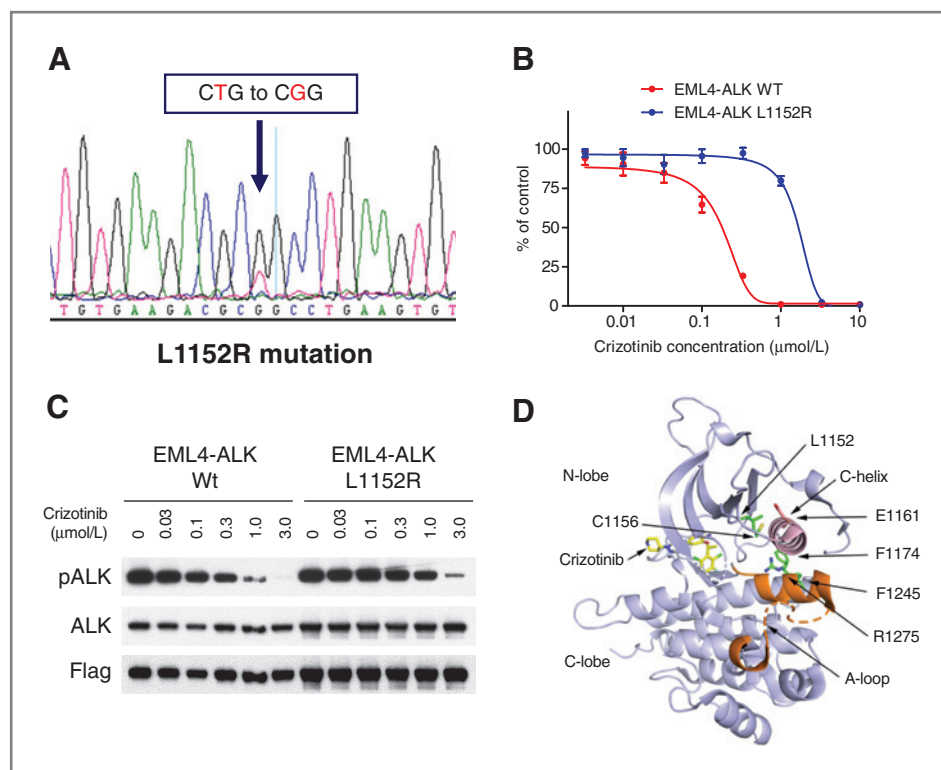


Figure 1. The ALK mutation L1152R causes the ALK tyrosine kinase inhibitor resistance. **A**, sequence tracing from posttreatment tumor specimens. There is a T to G mutation in codon 3455 in exon 23 resulting in the L1152R mutation. **B**, Ba/F3 cells were treated with crizotinib at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. There is a significant effect of the L1152R mutation at 300 nmol/L ($P < 0.001$). **C**, Ba/F3 cells with indicated genotypes were treated with increasing concentrations of crizotinib for 6 hours. Cell extracts were immunoprecipitated with an anti-FLAG antibody followed by immunoblotting to detect the indicated proteins. **D**, ribbon diagram depicting the crystal structure of ALK kinase in complex with crizotinib. The side chains of residues that are sites of resistance mutations, including the L1152R mutation described here, are shown in green. Note that L1152 and C1156 are not in contact with the ATP-binding cleft but are adjacent to each other and to the C-helix (pink). Both the L1152R and C1156Y mutations could introduce hydrogen bond interactions with E1161 on the C-helix. All of the resistance mutations identified to date cluster around the conformationally sensitive C-helix and activation loop, suggesting that they may affect kinase activity and inhibitor binding through alterations in the structure or stability of these elements. The activation loop (A-loop, colored orange) is partially disordered in this structure as denoted by the dashed line. Figure is drawn from PDB ID 2XP2.

purchased from R&D Systems and used according to the manufacturer's recommended conditions. ALK immunohistochemistry (IHC) was done using the mouse monoclonal anti-human ALK (clone: 5A4; Abcam) as previously described (16). EGFR IHC was done as previously described (22).

Phosphotyrosine profiling

Phosphotyrosine profiling was done using a bead based assay using previously described methods (23). Parental and drug-resistant cells were treated with either vehicle or drug for 6 hours followed by cell lysis prior to analysis.

Detection of EGFR ligands

EGF, amphiregulin, TGF- α , HB-EGF, and epiregulin were measured in cell culture medium using an ELISA, were done according to the manufacturer's recommended procedures (Quantikine; R&D Systems) and as described previously (24). All samples were run in triplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

Cell-cycle analysis and apoptosis assays

A total of 1×10^6 cells treated with each of the compound at indicated concentration or dimethyl sulfoxide were washed in PBS, fixed in 70% ethanol, incubated with RNase in PBS, and resuspended in 500 μ L propidium iodide. The percentage of cells in G₁, S-phase, and G₂-M phases were analyzed by flow cytometry. Apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using the APO-BRDU Kit (BD Biosciences) according to the manufacturer's instructions.

Results

The novel somatic ALK L1152R mutation results in drug resistance to ALK inhibitors

To identify additional mechanisms of resistance to crizotinib, we first studied a NSCLC patient with an ALK rearrangement [*EML4-ALK* variant 3 (E6;A20)] who had developed clinical acquired resistance to crizotinib, following a brief radiographic response after 3 months of treatment. Sequencing of the ALK gene from the clinically progressing tumor

Table 1. Summary of *in vitro* growth assays

Cell line	Vector	Crizotinib IC ₅₀ (nmol/L)	TAE684 IC ₅₀ (nmol/L)
Ba/F3	EML4-ALK	68 ± 4	3 ± 1
Ba/F3	EML4-ALK L1152R	853 ± 27	66 ± 6
H3122	GFP	88	
H3122	EML4-ALK	70	
H3122	EML4-ALK L1152R	260	
H3122	EML4-ALK C1156Y	240	
H3122	EML4-ALK L1196M	240	
H3122	EML4-ALK F1174L	620	
DFCI076		1,008 ± 37	296 ± 29
DFCI076	+PF299804 (1 μmol/L)	314 ± 37	
H3122		100	7 ± 2
H3122 TR3		2,564 ± 107	291 ± 18

(pleural effusion) revealed the presence of a novel mutation (Fig. 1A). This mutation resulted in a change from a leucine to an arginine at position 1152 (L1152R). The recurrent tumor was wild-type for *EGFR* and *KRAS* (data not shown). This mutation was not detected in the tumor of the patient obtained prior to crizotinib treatment (data not shown).

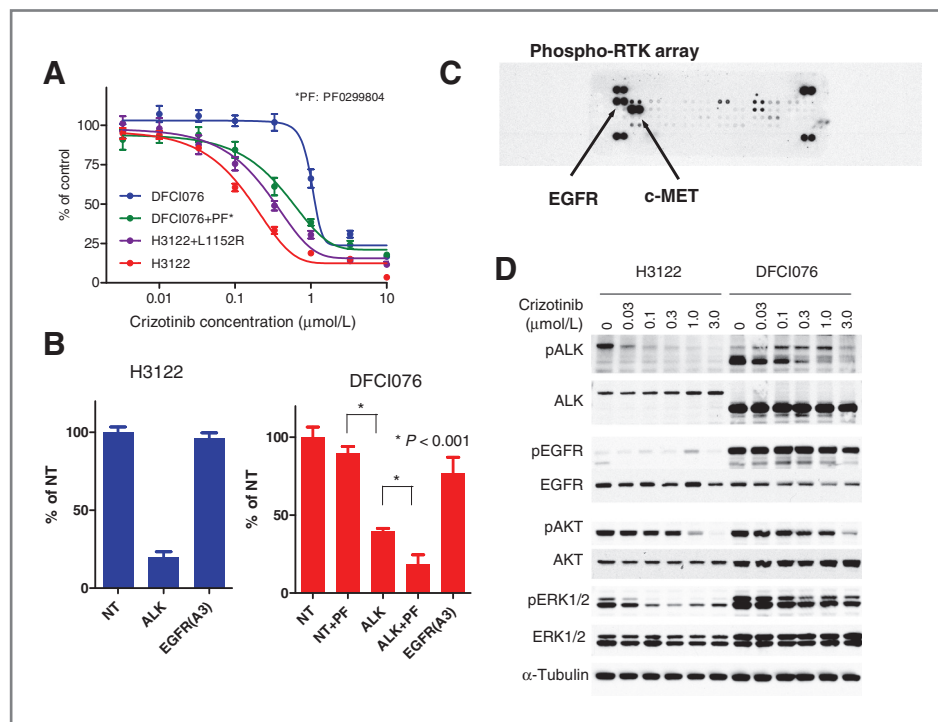
We evaluated the biological impact of the L1152R mutation by introducing it into *EML4-ALK* and generating Ba/F3 cells. Both *EML4-ALK* and *EML4-ALK*L1152R led to IL-3 independent growth of Ba/F3 cells (Supplementary Fig. S1A). The *EML4-ALK* L1152R cells were significantly more resistant than the parental cells to both crizotinib (Fig. 1B) and ALK inhibitor TAE684 (Supplementary Fig. S1B). The L1152R mutation diminished crizotinib-mediated inhibition of downstream AKT and ERK 1/2 phosphorylation (Supplementary Fig. S1C). Consistent with these findings on growth, greater concentrations of crizotinib were required to inhibit ALK phosphorylation in the *EML4-ALK* L1152R cells compared with those with *EML4-ALK* alone (Fig. 1C). Furthermore, to compare the impact of resistance in endogenous *EML4-ALK* NSCLC cells, the L1152R and previously identified resistance mutations (C1156Y, L1196M, and F1174L) were stably expressed in H3122 cells and the cells were examined for crizotinib resistance (14, 15). All of the resistance mutations, C1156Y, L1196M, L1152R, and F1174L, resulted in the significant elevation of IC₅₀ compared with the control cells (GFP or *EML4-ALK* wild-type), but there were no significant difference among the C1156Y, L1196M, and L1152R mutations (Supplementary Fig. S1D and Table 1). Analogous to the known resistance mutation C1152Y, examination of the published crystal structure of ALK in an inactive conformation reveals that the L1152R mutation is not in direct contact with the ATP-binding pocket, where both crizotinib and TAE684 are expected to bind (Fig. 1D, ref. 25). The currently available structures do not reveal a clear mechanistic basis as to how L1152R may mediate ALK inhibitor resistance.

A NSCLC cell line harboring the L1152R mutation is ALK and EGFR dependent

We successfully established a cell line, DFCI076, from the pleural effusion of the patient harboring the *ALK* L1152R

mutation. Similar to the Ba/F3 cells harboring the L1152R mutation, the DFCI076 cells were resistant to both crizotinib and TAE684 (Fig. 2A and Supplementary Fig. S2A). However, these cells were still dependent on ALK for their growth as downregulation of *ALK* using an *ALK*-specific shRNA resulted in significant growth inhibition compared with either a NT- or an *EGFR*-specific shRNA (Fig. 2B, Supplementary Fig. S2B and C). Similarly, the *ALK* shRNA, but not the *EGFR* shRNA, was effective in the crizotinib and TAE684-sensitive H3122 cell line (Fig. 2B). However, the degree of growth inhibition by the *ALK* shRNA was not as dramatic in the DFCI076 cells compared with the H3122 cells. This prompted us to evaluate whether the DFCI076 cells might contain other concurrent resistance mechanisms. We assessed the activation status of multiple RTKs using the human phospho-RTK arrays as in our prior study (3). Using this approach, we observed strong *EGFR* and *MET* phosphorylation in the DFCI076 cells (Fig. 2C). The DFCI076 cells did not contain an *EGFR* mutation or an *EGFR* amplification (data not shown) but secreted the *EGFR* ligand amphiregulin (Supplementary Fig. S2D). Although crizotinib is a potent *MET* inhibitor and successfully inhibited phospho-*MET*, it does not inhibit *EGFR* activity and, even at high concentrations, did not lead to downregulation of pAKT and pERK1/2 to the extent observed in H3122 cells (Fig. 2D). Combined inhibition of *ALK* (using a shRNA) and *EGFR*, using the pan-ERBB inhibitor PF299804, was significantly more effective ($P < 0.001$) than either strategy alone in the DFCI076 cells (Fig. 2B). In addition, the growth curve of DFCI076 cells treated with both PF299804 and crizotinib was similar to the H3122 cells engineered to express the L1152R mutation and subjected to crizotinib treatment (Fig. 2A). Collectively, these findings suggest that although the DFCI076 cells remain largely *ALK* dependent for their growth, concurrent *EGFR* inhibition may provide additive growth inhibition. These findings are similar to our prior studies of the DFCI032 cell line generated from a NSCLC patient with *EML4-ALK* who was never treated with an *ALK* inhibitor (13). We further confirmed that the DFCI032 cells were sensitive to the combination of the *ALK* shRNA and PF299804 (Supplementary Fig. S2E and F).

Figure 2. DFCI076 cells are codependent on ALK and EGFR. A, the H3122 or DFCI 076 cells were treated with crizotinib, alone or concurrently with 1 $\mu\text{mol/L}$ PF299804, at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. B, downregulation of *ALK* or *EGFR* using shRNAs in H3122 and DFCI076 cells. Further growth inhibition is observed with the addition of 1 $\mu\text{mol/L}$ PF299804. Cell viability is measured relative to the NT control shRNA. C, a phospho-RTK array reveals that the DFCI076 cells contain strong phosphorylation of both EGFR and MET. Cells were grown in medium and the cell lysates were hybridized to a phospho-RTK array. Hybridization signals at the corners serve as controls. D, H3122 and DFCI076 cells are treated with indicated concentrations of crizotinib for 6 hours. Immunoblotting was used to detect the indicated proteins.



ALK inhibitor-resistant H3122 cells contain activation of EGFR signaling

To identify additional mechanisms of resistance to ALK kinase inhibitors, we generated a TAE684-resistant version of the *EML4-ALK* H3122 NSCLC cell line. We have used a similar approach to identify known and previously unknown EGFR kinase inhibitor resistance mechanisms (3, 21). After 6 months of gradually increasing TAE684, we were able to isolate cells that proliferated in 100 nmol/L of TAE684. In our prior studies, we showed that 100 nmol/L of TAE684 inhibited ALK signaling and significantly decreased cell viability in H3122 cells, but this concentration was not generally toxic in non-*ALK* rearranged NSCLC cell lines (13). We subcloned the TAE-resistant (TR) cells from single cells (H3122 TR3) and these cells were resistant to both TAE684 and crizotinib (Fig. 3A, Supplementary Fig. S3A). DNA fingerprinting confirmed that the H3122 TR3 cells were derived from the H3122 parental cells (data not shown). We sequenced the entire ALK kinase domain from the H3122 TR3 cells and did not detect any secondary ALK mutations (data not shown). To determine whether the H3122 TR3 cells were still ALK dependent for their growth, we downregulated ALK using an *ALK*-specific shRNA (Fig. 3B). However, unlike the parental H3122 cells, the H3122 TR3 cells were only minimally growth inhibited by ALK downregulation (Fig. 3B). We further evaluated the ALK locus using FISH. Although all of the H3122 cells contained the *EML4-ALK* inversion, this was only detected in a small fraction (5%) of the H3122 TR3 (Supplementary Fig. S3B and C). The cells that retained the inversion also harbored a concurrent amplification in the ALK locus (Supplementary Fig. S3C).

Together, these findings suggest that the H3122 TR3 cells have evolved to lose their ALK dependence for growth.

To further characterize the H3122 TR3 cells, we carried out phospho-RTK arrays in both the parental and drug-resistant cells with and without TAE684 treatment (Fig. 3C). Compared with the parental cells, the H3122 TR cells contained greater EGFR, IGF1R, and MET phosphorylation and these proteins remained persistently phosphorylated despite TAE684 treatment (Fig. 3C). We also used a previously described quantitative bead based phospho-tyrosine assay to specifically study these 3 proteins in further detail (23). Consistent with the genomic findings, ALK phosphorylation was greater in the H3122 compared with the H3122 TR3 cells (Fig. 3D). TAE684 still effectively inhibited ALK phosphorylation in both cell lines. In contrast and consistent with the RTK array, EGFR phosphorylation was markedly elevated in the H3122 TR3 cells (Fig. 3D). This was inhibited by the EGFR kinase inhibitor gefitinib but not TAE684 (Fig. 3D). We also observed phosphorylated ERBB2 and IGF1R in H3122 TR3 clone using this assay (Supplementary Fig. S3D). Of note, the ectopic expression of *ALK* secondary mutations (Supplementary Fig. S1D) did not lead to an increase in EGFR expression in the H3122 cells (data not shown).

Next, we examined whether activated EGFR had a functional role in the H3122 TR3 cells. We first downregulated *EGFR* using 2 different (A3 and D) *EGFR* shRNAs. Compared with a control shRNA, EGFR knockdown led to significant ($P < 0.05$) decrease in cell proliferation by day 6 in the H3122 TR3 but not the parental cell line (Fig. 3E). This observation was mirrored in a colony formation assay in which treatment with PF299804 resulted in a significant

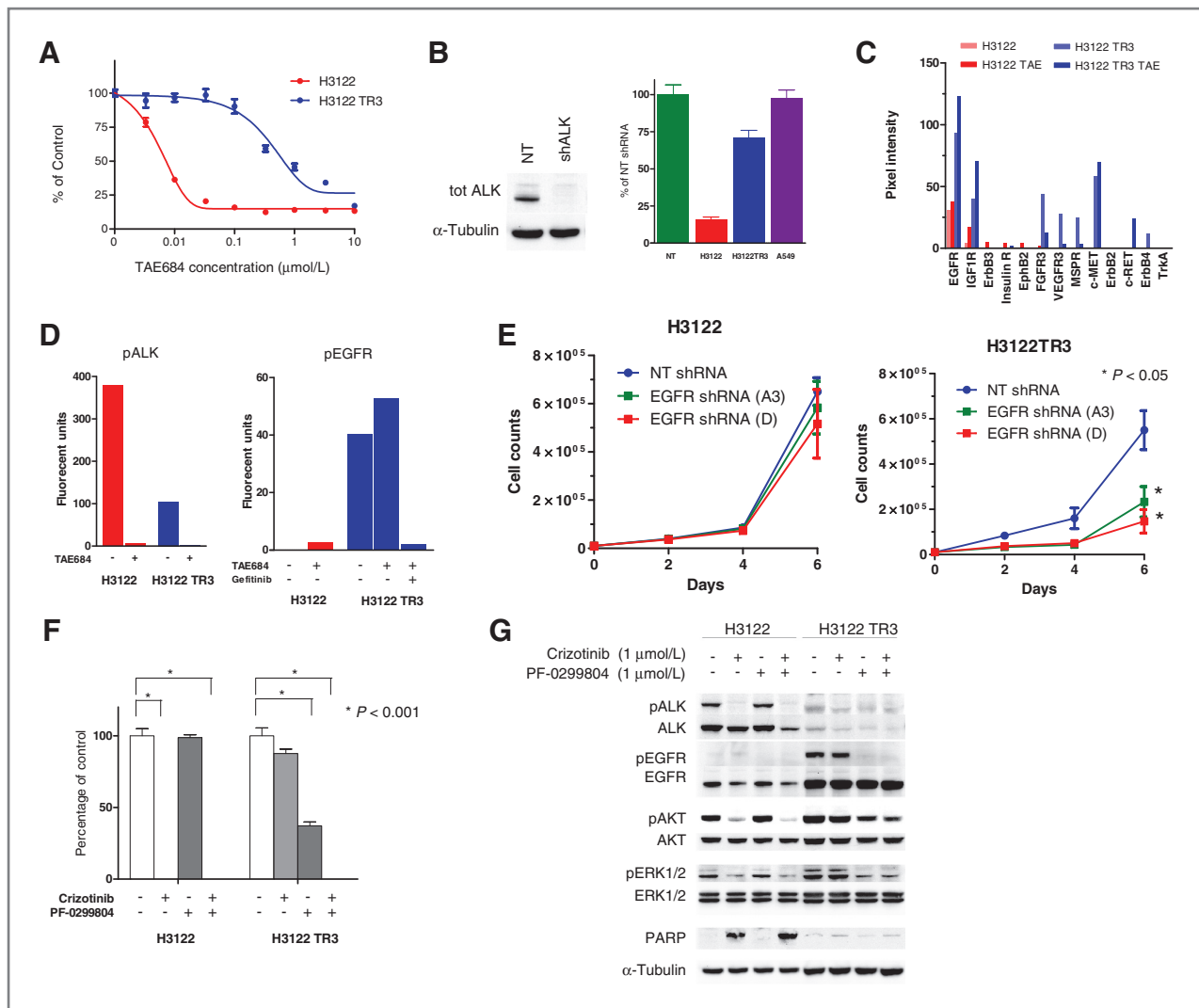


Figure 3. H3122 TR3 cells are ALK inhibitor resistant and contain coactivation of EGFR signaling. **A**, H3122 and TR3 cells were treated with indicated concentrations of TAE684. Viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. **B**, ALK shRNA has minimal effect on H3122 TR3 cell viability. Cell viability is measured relative to NT control. H3122 and A549 cells serve as positive and negative controls, respectively. Successful ALK knockdown in H3122 TR3 cells is confirmed by Western blotting. **C**, summary of RTK array from H3122 and H3122 TR3 cells with and without TAE684 (100 nM/L; 6 hours) treatment. The protein lysates were exposed to the RTK array (R&D). Each spot of membrane were calculated as signal intensity and shown in the bar graph. **D**, ALK and EGFR phosphorylation in H3122 and H3122 TR3 cells with and without TAE684 and gefitinib treatment. Phosphorylation was measured using a bead based assay (methods). **E**, proliferation of H3122 TR3 but not H3122 cells is effected following EGFR knockdown using 2 different shRNAs. **F**, results of colony formation assay after 14 days treatment with indicated compounds with both H3122 and H3122 TR3. The combination of crizotinib (1 μ M/L) and PF299804 (1 μ M/L) effectively inhibited colony formation in H3122 TR3 cells. **G**, H3122 and H3122 TR3 cells were treated with indicated compounds for 6 hours, and immunoblotting was used to detect the indicated proteins. For the PARP blot, cells were treated for 24 hours.

decrease ($P < 0.001$) in H3122 TR3 but not H3122 colonies (Fig. 3F) compared with untreated cells. The combination of the pan-ERBB inhibitor PF299804 and crizotinib was most effective in the H3122 TR3 cells leading to complete inhibition of colony formation. However, the effects of PF299804 and crizotinib were mostly cytostatic as judged by only minimal changes in cleaved PARP and by using a TUNEL assay (Fig. 3G and Supplementary Fig. S3E). There was no effect on growth using the combination of an IGF1R kinase inhibitor BMS-536,924 and crizotinib in the H3122 TR3 cells

despite harboring evidence of IGF1R activation (Supplementary Fig. S3F).

We further determined how EGFR was activated in the H3122 TR3 cells. We did not identify evidence of an *EGFR* mutation or amplification as detected by FISH (data not shown). However, the supernatant of the H3122 TR3 cells contained significantly ($P < 0.05$) greater amounts of known EGFR ligands including amphiregulin and EGF (Supplementary Fig. S3G), suggesting that the mechanism of EGFR activation in these cells is through a ligand-mediated autocrine activation.

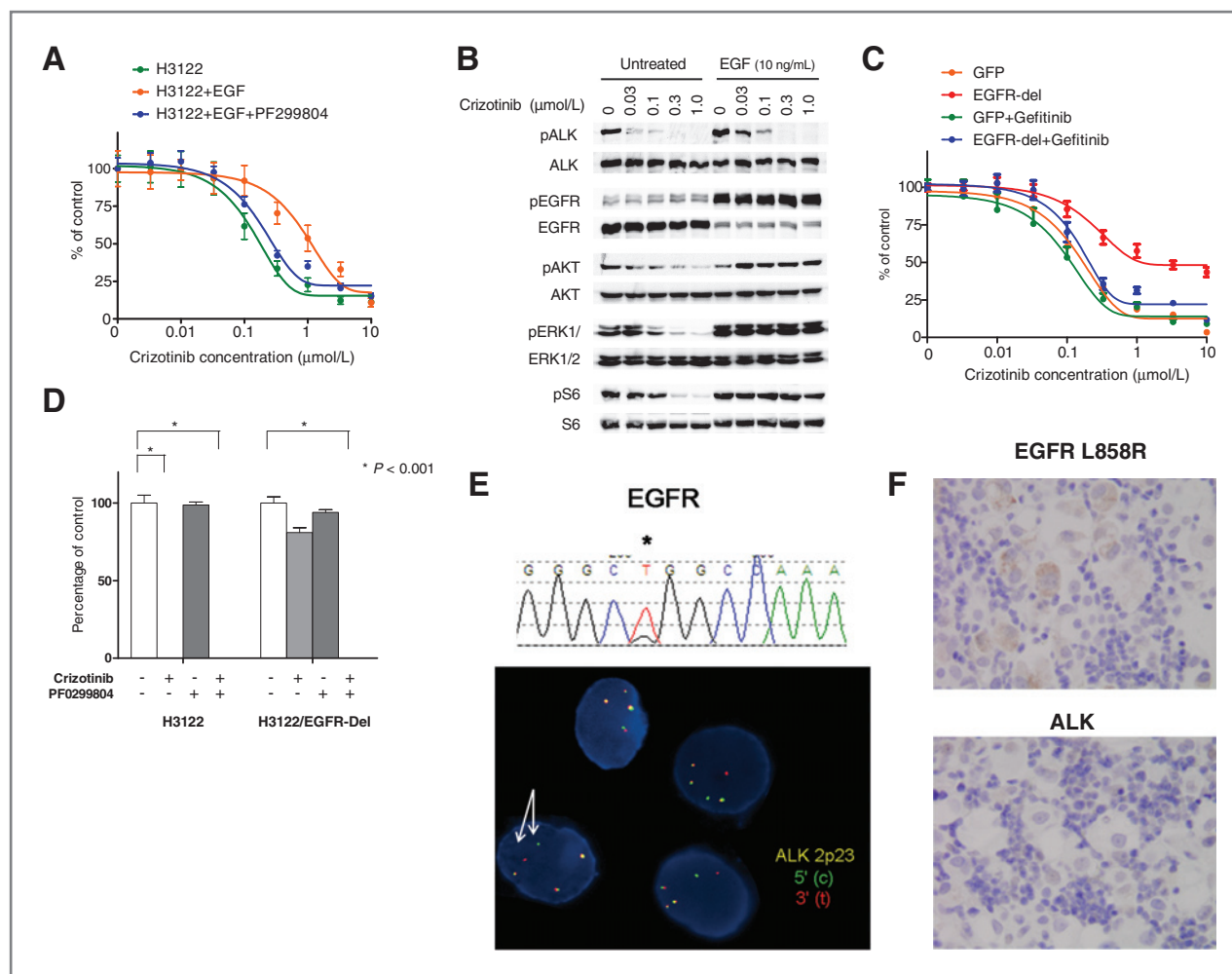


Figure 4. Concurrent ALK and EGFR signaling leads to drug resistance. **A**, H3122 cells were treated with crizotinib at the indicated concentrations in the presence or absence of EGF (10 ng/mL) and PF299804 (1 μ mol/L). Viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. **B**, H3122 cells were treated with crizotinib for 6 hours at indicated concentrations in the absence or presence of EGF (10 ng/mL). Immunoblotting was used to detect the indicated proteins. **C**, H3122 cells stably expressing GFP or EGFR E746_A750 were treated with crizotinib in the absence or presence of gefitinib (1 μ mol/L). Viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. **D**, results of colony formation assay after 14 days treatment with indicated compounds using H3122 and H3122 EGFR E746_A750. The combination of crizotinib and PF299804 effectively inhibited colony formation. **E**, EGFR sequence tracing (left) and ALK FISH analysis (right) from patient 1. Asterisk, T to G mutation resulting in L858R; arrows, split red and green FISH signals. **F**, immunohistochemistry for EGFR L858R (left) and ALK (right) from patient 1.

Activation of EGFR signaling induces resistance to crizotinib

Our studies from the ALK inhibitor-resistant DFCI032, DFCI 076, and H3122 TR3 cells suggest a role for EGFR signaling in mediating crizotinib resistance. To formally evaluate this hypothesis, we activated EGFR signaling in H3122 cells using EGFR ligands and by oncogenic forms of EGFR and determined the effects on crizotinib sensitivity. We observed that exogenous EGF was indeed sufficient to promote resistance to ALK inhibition, and resistance could be reversed using a combination of ALK and EGFR inhibitors (Fig. 4A). In the presence of EGF, crizotinib was still able to inhibit ALK phosphorylation but not AKT, S6, and ERK 1/2 phosphorylation (Fig. 4B). Similarly, introduction of EGFR E746_A750 into H3122 cells promoted crizotinib resistance (Fig. 4C) which is

reversed by the concurrent administration of the EGFR inhibitor gefitinib or PF299804 (Fig. 4D and Supplementary Fig. S4A). Analogously *EML4-ALK* promotes gefitinib resistance in the HCC827 EGFR mutant NSCLC cell line (Supplementary Fig. S4B), which was reversed by concurrent treatment with TAE684 (Supplementary Fig. S4C).

A subset of *EML4-ALK* NSCLC patients harbor concurrent EGFR mutations

Our *in vitro* studies suggest that EGFR signaling can contribute to ALK kinase inhibitor resistance in *EML4-ALK* NSCLC. In addition, we show that a cancer cell line that harbors a concurrent ALK rearrangement and an EGFR mutation would be expected to be resistant to both single-agent ALK and EGFR inhibitors. Acquired drug resistance

Table 2. Characteristics and outcomes of NSCLC patients harboring concurrent *EGFR* mutations and *ALK* rearrangements

Pt.	EGFR mutation	ALK FISH	EGFR IHC	ALK IHC	Erlotinib Therapy	Outcome
1	L858R	+	L858R Ab positive	Negative	Yes	PR; 9 mo
2	Del E746_A750	+	Exon 19 del Ab positive	Negative	Yes	PR; 5 + mo
3	A767_V769dupASV	+	N.D.	Positive	No	N/A

Abbreviations: IHC, immunohistochemistry; PR, partial response; N.D., not done; N/A, not applicable.

mechanisms can sometimes also occur *de novo* and both *EGFR* T790M and *MET* amplification have been described in cancers from *EGFR* tyrosine kinase inhibitor (TKI) naive patients (26). In our prior study, we identified one treatment naive NSCLC patient that harbored a concurrent *EGFR* mutation and *EML4-ALK* (13). However, this tumor was obtained from a patient that had undergone surgery and thus never received systemic therapy. Subsequently, in 50 crizotinib treatment naive NSCLC patients harboring *ALK* rearrangements at DFCI, all detected in a clinical laboratory, we have now identified 3 (6%) patients that also harbor concurrent *EGFR* mutations (Table 2). Two of the three patients have undergone therapy with erlotinib and both have achieved partial responses (Table 2). Evaluation of the pretreatment tumor from patient 1 using the *EGFR* L858R-specific antibody shows *EGFR* staining (Fig 4E), whereas no staining was observed with the *ALK*-specific antibody (Fig 4E) despite the presence of the *ALK* genomic rearrangement (Table 2).

Discussion

ALK TKIs are emerging as effective clinical therapies for cancers containing genetic rearrangements in *ALK* including NSCLC, IMT, and ALCL (8, 10, 27). However, the clinical success of this therapeutic approach is uniformly limited by the development of drug resistance. The mechanistic understanding of drug resistance may help to develop effective subsequent clinical treatments and/or rational combination therapeutic strategies.

In this study, by studying patient-derived tumors and cell lines, we uncover novel *ALK* TKI resistance mechanisms. These include both a secondary mutation (L1152R) in *ALK* and activation of *EGFR* signaling. Importantly, these can occur together in the same tumor (i.e., the DFCI 076 cell line) highlighting both the complexity of drug resistance mechanisms and the therapeutic challenges in developing strategies to overcome clinical drug resistance.

Secondary mutations in kinases are a common mechanism of drug resistance to kinase inhibitors (4, 5, 28, 29). A few distinct categories of mutations have so far been identified. These include secondary mutations that alter drug contact residues, thus creating a steric hindrance for drug binding (30). Alternatively, secondary mutations can promote conformational changes in the kinase and thus disfavoring the binding of a kinase inhibitor (30). The L1152R mutation is not located in the kinase domain. The currently available

crystal structures of *ALK* do not provide a clear explanation of the mechanistic basis of drug resistance imparted by this mutation. Furthermore, despite different *EML4-ALK* variants that have been described to date, there is no evidence to suggest that the mechanisms of acquired resistance will vary on the basis of specific *EML4-ALK* variant (11). Notably the L1152R mutation, unlike the F1174L mutation, is also resistant to TAE684 (14). Thus structurally distinct *ALK* inhibitors are needed to overcome this mutation and several are under preclinical development. Additional studies, including solving the crystal structure for the *ALK* L1152R, will be necessary to obtain further insight into how this mutation causes drug resistance.

Prior studies have generated crizotinib-resistant H3122 cells and detected both evidence of an *ALK* amplification and the presence of the L1196M gatekeeper mutation (31). We also identify *ALK* amplification in a subset of the H3122 TR3 cells (Supplementary Fig. S3B) but not the L1196M mutation. Because TAE684, unlike crizotinib, can effectively inhibit the growth of H3122 *EML4-ALK* L1196M cells, our findings are consistent with the prior studies (31). In fact, they suggest that a more potent *ALK* inhibitor may be able to prevent the emergence of this specific drug resistance mechanism. Whether this will ultimately translate into a clinical benefit (such as a prolongation of progression-free survival) for NSCLC patients can only be determined from clinical trials.

Our studies identify activation of *EGFR* signaling as a bypass signaling mechanisms that contributes to *ALK* inhibitor resistance. Concurrent inhibition of both *EGFR* and *ALK* is therapeutically effective in all of the resistant models. Intriguingly, different models have differing degrees of apparent *EGFR* dependence. The DFCI076 cells are mostly *ALK* dependent (Fig. 2B), whereas the H3122 TR3 cells are more *EGFR* dependent (Fig. 3D) for their growth. The DFCI032 cells are equally codependent with very little effect on growth by only *EGFR* or *ALK* inhibition (Supplementary Fig. S2F and ref. 13). These different examples may be representative of a dynamic process with variable degrees of adaptation to *EGFR* signaling in the presence of *ALK* inhibition. However, we cannot completely exclude the possibility that activation of *EGFR* signaling in these cell lines did not arise in the process of generating the cell lines. Additional evaluation of tumor specimens for changes in *EGFR* phosphorylation obtained from patients that have developed crizotinib resistance will be necessary. Further investigation is also needed to study

changes in EGFR signaling over time to further understand how this adaptive process evolves. Furthermore, whether the process will revert in the absence of ALK inhibition needs to be determined. Such observation may be clinically significant as it would suggest that drug-resistant cancers could regain their sensitivity following a therapeutic holiday from ALK inhibitors. To date we have grown the H3122 TR3 cells 60 passages in the absence of TAE684 and have not observed a reversion to TAE684 sensitivity (data not shown).

It is noteworthy that both *in vitro* and in NSCLC patients, activated EGFR signaling occurs concurrently with *EML4-ALK*. Such cancers could still retain sensitivity to single-agent EGFR or ALK inhibitors if the tumor was heterogenous and contained 2 independent populations: one with *EML4-ALK* and one with an *EGFR* mutation. Alternatively, a tumor could contain both genetic alterations but only expressed one of the mutant proteins. In both instances, such patients may achieve a transient partial response following therapy with either single agent. Our limited studies of crizotinib naive NSCLC patients, with both genetically confirmed *EML4-ALK* and *EGFR* mutations, suggest that ALK is not expressed as both patients treated with erlotinib achieved a clinical response. In contrast, the *in vitro* studies would predict that coexpression of *EML4-ALK* and mutant *EGFR* in the same cells would lead to resistance to both single-agent ALK and EGFR inhibitors. Why some cancers harbor an *ALK* rearrangement which does not lead to ALK expression remains to be determined. It will also be of interest to determine whether the mechanism of erlotinib resistance in our patients, with both an *EGFR*

mutation and *ALK* rearrangement, will involve reactivation of ALK expression.

Currently there is an ongoing phase I clinical trial of crizotinib and PF299804 (NCT01121575) originally designed to evaluate the therapeutic benefit of inhibiting MET (crizotinib is a potent MET inhibitor) and EGFR T790M in erlotinib-resistant *EGFR* mutant NSCLC patients. However, our studies suggest that combination of crizotinib and PF299804 may represent a rational therapeutic strategy for at least a subset of *EML4-ALK* NSCLC patients that develop acquired crizotinib resistance.

Disclosure of Potential Conflicts of Interest

M.J. Eck: commercial research support, Novartis. P.A. Jänne: consultant/advisory board and clinical trial support, Pfizer. The other authors disclosed no potential conflicts of interest.

Acknowledgments

The authors thank Pfizer, Inc. for the clinical trial support.

Grant Support

This study was supported by NIH R01CA136851 (N.S. Gray and P.A. Jänne), R01CA135257 (P.A. Jänne), The Cammarata Family Foundation Research Fund (P.A. Jänne), and by the NCI Lung SPORE P50CA090578 (P.A. Jänne).

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 April 18, 2011; revised June 22, 2011; accepted July 6, 2011; published OnlineFirst July 26, 2011.

References

- Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–57.
- Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693–703.
- 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;316:1039–43.
- Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kochev O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352:786–92.
- Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 2001;293:876–80.
- Kantarjian H, Shah NP, Hochhaus A, Cortes J, Shah S, Ayala M, et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 2010;362:2260–70.
- Saglio G, Kim DW, Issaragrisil S, Le Coutre P, Etienne G, Lobo C, et al. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. *N Engl J Med* 2010;362:2251–9.
- Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming *EML4-ALK* fusion gene in non-small-cell lung cancer. *Nature* 2007;448:561–67.
- Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, et al. Fusion of a kinase gene, *ALK*, to a nucleolar protein gene, *NPM*, in non-Hodgkin's lymphoma. *Science* 1994;263:1281–4.
- Lawrence B, Perez-Atayde A, Hibbard MK, Rubin BP, Dal Cin P, Pinkus JL, et al. TPM3-*ALK* and TPM4-*ALK* oncogenes in inflammatory myofibroblastic tumors. *Am J Pathol* 2000;157:377–84.
- Sasaki T, Rodig SJ, Chirieac LR, Janne PA. The biology and treatment of *EML4-ALK* non-small cell lung cancer. *Eur J Cancer* 2010;46:1773–80.
- Soda M, Takada S, Takeuchi K, Choi YL, Enomoto M, Ueno T, et al. A mouse model for *EML4-ALK*-positive lung cancer. *Proc Natl Acad Sci U S A* 2008;105:19893–7.
- Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes AJ, et al. *EML4-ALK* fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. *Clin Cancer Res* 2008;14:4275–83.
- Sasaki T, Okuda K, Zheng W, Butrynski J, Capelletti M, Wang L, et al. The neuroblastoma-associated F1174L *ALK* mutation causes resistance to an ALK kinase inhibitor in *ALK*-translocated cancers. *Cancer Res* 2010;70:10038–43.
- Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, et al. *EML4-ALK* mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med* 2010;363:1734–9.
- Mino-Kenudson M, Chirieac LR, Law K, Hornick JL, Lindeman N, Mark EJ, et al. A novel, highly sensitive antibody allows for the routine detection of *ALK*-rearranged lung adenocarcinomas by standard immunohistochemistry. *Clin Cancer Res* 2010;16:1561–71.
- 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;304:1497–500.
- Zhou W, Ercan D, Chen L, Yun CH, Li D, Capelletti M, et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nature* 2009;462:1070–4.
- McDermott U, Iafrate AJ, Gray NS, Shioda T, Classon M, Maheswaran S, et al. Genomic alterations of anaplastic lymphoma kinase may sensitize tumors to anaplastic lymphoma kinase inhibitors. *Cancer Res* 2008;68:3389–95.

20. Galkin AV, Melnick JS, Kim S, Hood TL, Li N, Li L, et al. Identification of NVP-TAE684, a potent, selective, and efficacious inhibitor of NPM-ALK. *Proc Natl Acad Sci U S A* 2007;104:270–5.
21. Ercan D, Zejnullahu K, Yonesaka K, Xiao Y, Capelletti M, Rogers A, et al. Amplification of EGFR T790M causes resistance to an irreversible EGFR inhibitor. *Oncogene* 2010;29:2346–56.
22. Yu J, Kane S, Wu J, Benedettini E, Li D, Reeves C, et al. Mutation-specific antibodies for the detection of EGFR mutations in non-small-cell lung cancer. *Clin Cancer Res* 2009;15:3023–8.
23. Du J, Bernasconi P, Clauser KR, Mani DR, Finn SP, Beroukhi R, et al. Bead-based profiling of tyrosine kinase phosphorylation identifies SRC as a potential target for glioblastoma therapy. *Nat Biotechnol* 2009;27:77–83.
24. Yonesaka K, Zejnullahu K, Lindeman N, Homes AJ, Jackman DM, Zhao F, et al. Autocrine production of amphiregulin predicts sensitivity to both gefitinib and cetuximab in EGFR wild-type cancers. *Clin Cancer Res* 2008;14:6963–73.
25. Bossi RT, Saccardo MB, Ardini E, Menichincheri M, Rusconi L, Magnaghi P, et al. Crystal structures of anaplastic lymphoma kinase in complex with ATP competitive inhibitors. *Biochemistry* 2010;49:6813–25.
26. Sequist LV, Martins RG, Spigel D, Grunberg SM, Spira A, Janne PA, et al. First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic EGFR mutations. *J Clin Oncol* 2008;26:2442–9.
27. Gambacorti-Passerini C, Messa C, Pogliani EM. Crizotinib in anaplastic large-cell lymphoma. *N Engl J Med* 2011;364:775–6.
28. 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 Med* 2005;2:1–11.
29. Heinrich MC, Corless CL, Blanke CD, Demetri GD, Joensuu H, Roberts PJ, et al. Molecular correlates of imatinib resistance in gastrointestinal stromal tumors. *J Clin Oncol* 2006;24:4764–74.
30. Sherbenou DW, Druker BJ. Applying the discovery of the Philadelphia chromosome. *J Clin Invest* 2007;117:2067–74.
31. Katayama R, Khan TM, Benes C, Lifshits E, Ebi H, Rivera VM, et al. Therapeutic strategies to overcome crizotinib resistance in non-small cell lung cancers harboring the fusion oncogene EML4-ALK. *Proc Natl Acad Sci U S A* 2011;108:7535–40.