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Mechanisms of Acquired Crizotinib Resistance in ALK-Rearranged Lung Cancers

Ryohei Katayama[1,2,*], **Alice T. Shaw**[1,2,3,*], **Tahsin M. Khan**[1,3], **Mari Mino-Kenudson**[2,4], **Benjamin J. Solomon**[5], **Balazs Halmos**[6], **Nicholas A. Jessop**[1], **John C. Wain**[1,7], **Alan Tien Yeo**[1], **Cyril Benes**[1,2], **Lisa Drew**[8], **Jamal Carlos Saeh**[8], **Katherine Crosby**[9], **Lecia V. Sequist**[1,2], **A. John Iafrate**[2,4], and **Jeffrey A. Engelman**[1,2,†]

[1]Massachusetts General Hospital Cancer Center, Boston, MA 02129, USA

[2]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA

[3]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[4]Department of Pathology, Massachusetts General Hospital, Boston, MA 02114, USA

[5]Peter MacCallum Cancer Center, East Melbourne, Victoria 3002, Australia

[6]Columbia University Medical Center, New York City, NY 10032, USA

[7]Department of Surgery, Harvard Medical School, Boston, MA 02115, USA

[8]AstraZeneca R&D Boston, Waltham, MA 02451, USA

[9]Cell Signaling Technology Inc., Danvers, MA 01923, USA

Abstract

Most anaplastic lymphoma kinase (*ALK*)–positive non–small cell lung cancers (NSCLCs) are highly responsive to treatment with ALK tyrosine kinase inhibitors (TKIs). However, patients

†To whom correspondence should be addressed. jengelman@partners.org.
*These authors contributed equally to this work.

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with these cancers invariably relapse, typically within 1 year, because of the development of drug resistance. Herein, we report findings from a series of lung cancer patients ($n = 18$) with acquired resistance to the ALK TKI crizotinib. In about one-fourth of patients, we identified a diverse array of secondary mutations distributed throughout the *ALK* TK domain, including new resistance mutations located in the solvent-exposed region of the adenosine triphosphate–binding pocket, as well as amplification of the *ALK* fusion gene. Next-generation ALK inhibitors, developed to overcome crizotinib resistance, had differing potencies against specific resistance mutations. In addition to secondary *ALK* mutations and *ALK* gene amplification, we also identified aberrant activation of other kinases including marked amplification of *KIT* and increased autophosphorylation of epidermal growth factor receptor in drug-resistant tumors from patients. In a subset of patients, we found evidence of multiple resistance mechanisms developing simultaneously. These results highlight the unique features of TKI resistance in *ALK*-positive NSCLCs and provide the rationale for pursuing combinatorial therapeutics that are tailored to the precise resistance mechanisms identified in patients who relapse on crizotinib treatment.

INTRODUCTION

Chromosomal rearrangements involving the *ALK* gene, which encodes the anaplastic lymphoma kinase, occur in a variety of human malignancies, including non–small cell lung cancer (NSCLC), anaplastic large cell lymphoma (ALCL), and inflammatory myofibroblastic tumor (IMT) (1). These rearrangements lead to the expression of *ALK* fusion genes, in which the fusion partner mediates ligand-independent oligomerization of ALK, resulting in constitutive ALK kinase activation. In addition, *ALK* can be amplified or mutated in pediatric neuroblastoma, leading to oncogenic activation (2-4).

The most common *ALK* fusion oncogene in NSCLC is echinoderm microtubule-associated protein-like 4 (*EML4*)–*ALK*. First reported in 2007 (5, 6), *EML4-ALK* is present in 3 to 5% of NSCLC patients. These patients tend to have distinctive clinical features, including young age of onset, absence of smoking history, and adenocarcinoma histology (7, 8). Although the frequency of *ALK* rearrangements in the overall population of NSCLC patients is only ~4%, this represents ~8000 patients in the United States each year and ~40,000 patients worldwide each year. Indeed, *ALK*-rearranged NSCLC affects more people each year than many other kinase-driven malignancies including chronic myelogenous leukemia.

In cell line experiments and genetically engineered mouse models, *EML4-ALK* is a potent oncogenic “driver” (9, 10). Cancer cells harboring this rearrangement become dependent on or “addicted” to ALK and hence are highly sensitive to ALK kinase inhibition (11). In these cancers, ALK is the sole regulator of critical growth and survival pathways, including the canonical phosphatidylinositol 3-kinase (PI3K)–AKT and mitogen-activated or extracellular signal–regulated protein kinase kinase (MEK)–extracellular signal–regulated kinase (ERK) pathways, and inhibition of ALK leads to suppression of these pathways and induction of cell growth arrest and apoptosis (10, 12). Consistent with the preclinical studies, patients with advanced *ALK*-positive NSCLC are exquisitely sensitive to ALK-targeted therapies (13). In an early-phase study of the ALK tyrosine kinase inhibitor (TKI) crizotinib, the objective response rate (ORR) was 56% and the median progression-free survival (PFS) was 10 months (14). On the basis of its demonstrated efficacy and safety in phase 1 and 2 studies, crizotinib was recently granted accelerated approval by the Food and Drug Administration (FDA) for the treatment of advanced, *ALK*-positive NSCLC.

Although most patients with *ALK*-positive NSCLC derive substantial clinical benefit from crizotinib, the benefit is relatively short-lived because of the development of acquired resistance. Acquired resistance has emerged as the major hurdle preventing ALK inhibitors, and targeted therapies in general, from having a truly transformative impact on patients. To

date, only two case reports have been published describing the identification of secondary resistance mutations in crizotinib-resistant NSCLC (15, 16). One patient's tumor harbored two nonoverlapping mutations within the *ALK* tyrosine kinase (TK) domain, the gatekeeper L1196M substitution [which is analogous to T790M in epidermal growth factor receptor (EGFR) and T315I in ABL] and C1156Y (15), whereas the second patient's tumor harbored a single L1152R mutation (16). Recently, in cell line experiments, EGFR activation has also been shown to mediate crizotinib resistance (16), suggesting that EGFR may be co-opted to bypass ALK inhibition. Whether bypass mechanisms such as EGFR are activated in patients who have relapsed on crizotinib is unknown.

Here, we present findings from a series of patients with acquired crizotinib resistance. Among 18 patients who underwent biopsy after relapsing on crizotinib, we identified 4 (22%) with mutations within the ALK TK domain, including 3 new mutations and an additional case with *ALK* fusion gene amplification. In vitro biochemical and cell line studies reveal that these mutations confer differential sensitivity to second-generation ALK TKIs, several of which have entered early-phase clinical development to overcome crizotinib resistance. One of the mutations is highly resistant to all of the inhibitors examined. In addition, in about one-half of patient samples, we discovered evidence of alternative TK activation involving not only EGFR but also KIT through *KIT* gene amplification. In cell line experiments, aberrant expression of these receptor tyrosine kinases (RTKs) can mediate crizotinib resistance, and inhibition of these RTKs resensitizes the cancer cells to crizotinib. This suggests a potential role for combinatorial therapeutics in overcoming crizotinib resistance in the clinic. This work highlights the importance of identifying the precise mechanism of TKI resistance in each patient to tailor therapeutic strategies and ultimately improve clinical outcomes in patients with acquired TKI resistance.

RESULTS

Secondary mutations within the *ALK* TK domain in a subset of crizotinib-resistant cancers

To identify mechanisms of crizotinib resistance that develop in patients, we biopsied resistant tumors from 18 patients with advanced *ALK*-positive NSCLC who had relapsed on crizotinib. Consistent with other studies of acquired resistance to targeted therapies, all 18 patients with ALK-positive NSCLC had initially responded to crizotinib as indicated by an improvement in disease burden on computed tomography scans (17-20). The duration of crizotinib therapy ranged from 4 to 34 months, with a median of 10.5 months (Table 1). Most of the patients (15 of 18) underwent repeat biopsy while still on crizotinib or within 1 month of discontinuing crizotinib (Table 1). Tumor specimens were derived from core biopsies or resections, mediastinal lymph node samples, or malignant pleural effusions. All 18 drug-resistant tumor samples had adenocarcinoma histology. *ALK* rearrangement in the resistant lesion was confirmed by fluorescence in situ hybridization (FISH) in all resistant specimens except for three because of limited tissue (Table 1). In 1 of the 15 cases examined, *ALK* FISH revealed high-level gene amplification (Fig. 1A). No *ALK* resistance mutations were found in this specimen, so it appears that high-level amplification of the wild-type *ALK* fusion gene is sufficient to cause resistance. This finding is consistent with our previous observation in H3122 cells that amplification of wild-type *EML4-ALK* causes resistance to crizotinib (12).

We determined whether any of the resistant cancers had developed mutations in *ALK* that might underlie their resistance phenotype. We extracted total nucleic acid from each resistant case and sequenced exons 20 to 28 corresponding to the *ALK* TK domain. In seven cases, fresh-frozen samples were available, and the entire kinase domain of ALK or the entire *EML4-ALK* was amplified from complementary DNA (cDNA) and sequenced. Among the 18 crizotinib-resistant patients, we identified 4 (22%) with resistance mutations:

3 missense mutations (L1196M, G1202R, and S1206Y) and an amino acid (threonine) insertion mutation (1151Tins) (fig. S1, A and B). We confirmed the presence of these mutations by subcloning the amplified polymerase chain reaction (PCR) products into the pCR4 vector and sequencing individual bacterial colonies (fig. S1, A and B).

To determine whether the *ALK* mutations arose de novo in the resistant specimens, we obtained pre-crizotinib treatment specimens from three of the four patients with resistance mutations (MGH011, MGH020, and MGH021). We prepared total nucleic acid from each pre-crizotinib sample and sequenced the ALK TK domain by standard Sanger sequencing. None of the *ALK* mutations discovered in the resistant samples were identified in the corresponding pre-crizotinib specimens. To further corroborate these findings, we used an allele-specific PCR assay to examine whether the pre-crizotinib sample of patient MGH020 harbored low levels of L1196M. We previously developed this assay to detect the gatekeeper L1196M mutation and showed that this assay is highly sensitive with a detection limit of 0.3% or less (12). Using this assay, we identified L1196M in the resistant MGH020 specimen and in a cell line harboring EML4-ALK L1196M (H3122 CR1), but not in the pre-crizotinib sample corresponding to MGH020 (fig. S1C). These results demonstrate that the *ALK* mutations identified in the setting of crizotinib resistance were not prevalent in the tumors before crizotinib therapy.

On the basis of the crystal structure of ALK (21), all four identified mutations are clustered near the adenosine triphosphate (ATP)–binding pocket of ALK (Fig. 1B). The L1196M amino acid substitution is equivalent to gatekeeper mutations observed in EGFR (T790M) and BCR-ABL (T315I) that confer resistance to gefitinib and imatinib, respectively. This mutation was previously reported in a patient who had relapsed on crizotinib and in a cell line model of resistance (12, 15). Among the three new mutations, the G1202R mutation is analogous to an imatinib-resistant BCR-ABL mutation (G340W), which was identified by random mutagenesis screening (22) but has not been reported in patient samples. The other two mutations (1151T insertion and S1206Y) appear to be unique to ALK and crizotinib resistance. Both G1202R and S1206Y are located in the solvent-exposed region of the kinase domain (that is, the solvent front) abutting the crizotinib-binding site (Fig. 1B), and it is likely that they diminish the affinity of crizotinib for the mutant ALK. In contrast, the 1151Tins residue is predicted to lie farther away at the loop of the N terminus of α helix C (Fig. 1B). This position is not adjacent to the crizotinib-binding site, and previous studies suggest that this mutation may affect the affinity of ALK for ATP (see Discussion). This mutation causes very high level resistance to crizotinib (see below).

To directly determine whether these mutations confer resistance to crizotinib, we engineered Ba/F3 cells to express *EML4-ALK* harboring each mutation (fig. S2A) and examined cell survival after treatment with crizotinib. As previously reported (12, 15), the L1196M gatekeeper mutation confers high-level resistance to crizotinib (Fig. 1C). We also found that both G1202R and S1206Y solvent front mutations and the 1151Tins mutation also confer resistance to crizotinib (Fig. 1C). We next examined the effect of crizotinib on ALK phosphorylation in the presence or absence of each resistance mutation. Consistent with the Ba/F3 results, crizotinib was less effective at suppressing the phosphorylation of EML4-ALK harboring any of the four resistant mutations (Fig. 1D). Although these mutations were sufficient to cause resistance to crizotinib, these *ALK* mutations were associated with variable degrees of crizotinib resistance. In particular, of the four mutations, S1206Y conferred the least resistance to crizotinib, whereas L1196M, G1202R, and 1151Tins conferred higher-level crizotinib resistance (Fig. 1, C and D). These differences in resistance to crizotinib may have eventual ramifications on the clinical course of patients after the development of resistance, as well as the success of future strategies attempting to overcome resistance by increasing the plasma concentration of crizotinib in patients.

Sensitivity of wild-type and mutant EML4-ALK to next-generation ALK inhibitors and hsp90 inhibition

Several new ALK TKIs are currently under development and are in early-phase clinical studies. These next-generation ALK inhibitors are structurally distinct from crizotinib, and enthusiasm for their development is stoked, in part, by hopes that they can overcome crizotinib resistance mediated by secondary *ALK* mutation. Thus, we wished to examine the potency of these next-generation ALK inhibitors against these resistance mutants. For these assays, we used the Ba/F3 cells expressing EML4-ALK harboring one of the four resistance mutations identified in patient samples. As controls, we also tested Ba/F3 cells expressing the wild-type (that is, crizotinib-sensitive) EML4-ALK as well as parental, interleukin-3 (IL-3)–dependent Ba/F3 cells. Given that the IL-3–dependent Ba/F3 cells do not express ALK or rely on ALK activation for growth, any inhibitory activity in these control cells is due to off-target toxicity of the tested compound.

We focused on three ALK inhibitors: the tool compound NVP-TAE684, a 5-chloro-2,4-diaminophenylpyrimidine (23), and two drugs currently in phase 1 clinical trials for *ALK*-positive cancers (CH5424802 and ASP-3026). CH5424802 was recently shown to be a potent inhibitor of wild-type as well as L1196M mutant ALK (24). ASP-3026 is also relatively potent and selective against wild-type ALK in vitro (tables S1 and S2). As shown in Fig. 1E, NVP-TAE684 demonstrated potent activity against EML4-ALK expressing the L1196M or S1206Y mutation (fig. S2, B and F). However, this activity was about fourfold less than that against wild-type EML4-ALK. By comparison, NVP-TAE684 was substantially less potent against Ba/F3 cells expressing either G1202R or 1151Tins EML4-ALK, and was less effective against control Ba/F3 cells (Fig. 1E and fig. S2, B and F). Ba/F3 lines expressing any mutant form of EML4-ALK were still more than 100-fold more sensitive to NVP-TAE684 than the parental Ba/F3 cells. Figure 1E displays the potency of each drug against each mutant EML4-ALK relative to wild-type EML4-ALK. The absolute IC_{50} (the concentration of a substance required to inhibit the activity of another substance by 50%) values are shown in fig. S2F.

The clinically available ALK inhibitors CH5424802 and ASP-3026 showed distinct selectivity profiles against the ALK resistance mutations. CH5424802 was more active against S1206Y EML4-ALK but was relatively less active against L1196M, G1202R, and 1151Tins EML4-ALK (Fig. 1E and fig. S2, C and F). In contrast, ASP-3026 was not as potent as crizotinib and CH5424802 against wild-type EML4-ALK in the cellular assays (fig. S2, D and F). However, the G1202R resistance mutation reduced the relative potency of ASP-3026 to a lesser extent than the other two ALK inhibitors (only a fourfold shift in IC_{50} compared to wild-type ALK; Fig. 1E and fig. S2, D and F). The 1151Tins mutation led to marked resistance to all of the ALK inhibitors examined. The suppression of phospho-ALK by the different inhibitors across the various mutations was consistent with the potencies observed in the Ba/F3 studies (figs. S2F and S3A). In addition, direct in vitro IC_{50} measurements of CH5424802 and ASP-3026 against the solvent front and gatekeeper mutants were also relatively consistent with results from the cellular studies in Ba/F3 cells (fig. S3B). Together, these results suggest that different ALK resistance mutations may confer different degrees of resistance to next-generation ALK inhibitors. Thus, it is possible that the different ALK inhibitors will show efficacy in the clinic depending on the specific resistance mutation present in individual patients.

ALK fusion proteins are known hsp90 (heat shock protein 90) clients, and hsp90 inhibitors have shown impressive activity against EML4-ALK in clinical trials and preclinical studies (25) (12, 26). We therefore determined whether the resistant ALK mutants are sensitive to 17-allylamino-17-demethoxygeldanamycin (17-AAG), an hsp90 inhibitor. We used the Ba/F3 system described above. In cell survival assays, 17-AAG was highly active against all

four mutant forms of EML4-ALK, similar to its potency against wild-type EML4-ALK (Fig. 1E and fig. S2, E and F). 17-AAG also suppressed the growth of parental, non–ALK-expressing Ba/F3 cells at relatively low concentrations (Fig. 1E and fig. S2E). Consistent with the cell survival data, 17-AAG decreased both phospho-ALK and total ALK protein levels in all Ba/F3 lines expressing wild-type or mutant EML4-ALK (fig. S3C). Thus, hsp90 inhibition might represent an alternative therapeutic strategy for overcoming crizotinib resistance mediated by secondary *ALK* mutations, particularly in the case of mutations such as 1151Tins, which confer high-level resistance to all ALK TKIs examined.

EML4-ALK gene mutation and amplification in models of acquired crizotinib resistance

Our interrogation of patient samples for genetic alterations in ALK identified resistance mutations or amplification in only 5 of 18 (28%) cases. Therefore, we aimed to identify additional resistance mechanisms. One successful approach to discovering resistance mechanisms has been to culture sensitive cell lines in increasing concentrations of the kinase inhibitor until resistance emerges. The resistant cell line can then be interrogated to identify the resistance mechanisms, leading to the identification of resistance biomarkers and new strategies to overcome resistance (12, 19, 27, 28). We treated H3122 cells, which express *EML4-ALK* variant 1 and are highly sensitive to crizotinib, with increasing concentrations of crizotinib for more than 4 months. We generated three independent, crizotinib-resistant cell lines from the highly sensitive *EML4-ALK*–expressing H3122 cells. These were designated H3122 CR1 (crizotinib-resistant), CR2, and CR3, and were maintained in 1 μM crizotinib. All three H3122 CR cell lines were as resistant to crizotinib as cancer cell lines without *ALK* rearrangement (IC_{50} > 1 μM, Fig. 2A). As previously reported, H3122 CR1 cells harbor both the gatekeeper L1196M *EML4-ALK* mutation and amplification of the mutated *EML4-ALK* allele (12). In contrast to parental H3122 cells, all the resistant cell lines maintain PI3K-AKT and MEK-ERK signaling in the presence of crizotinib (Fig. 2B and fig. S4A). The H3122 CR1 and CR2 cells maintained ALK phosphorylation in the presence of crizotinib, but the H3122 CR3 cells did not (Fig. 2B). In addition, we noted that both H3122 CR1 and CR2 cells expressed higher levels of total EML4-ALK protein compared with either parental or H3122 CR3 cells (Fig. 2B and fig. S4A). Consistent with those results, quantitative PCR of genomic DNA (gDNA) revealed *EML4-ALK* gene amplification in H3122 CR1 and CR2 cells, but not in parental H3122 or H3122 CR3 cells (fig. S4B, consistent with previously reported results) (12).

To determine whether H3122 CR2 and CR3 cells might harbor a resistance mutation, we prepared cDNA and examined the entire coding sequence of *EML4-ALK*. In H3122 CR2 cells, we detected the same highly resistant 1151Tins mutation in *EML4-ALK* as was identified in one of our crizotinib-resistant patients (MGH021, fig. S4C). In contrast, no *ALK* mutation was identified in H3122 CR3 cells, consistent with the observation that crizotinib effectively suppressed ALK phosphorylation in this cell line (Fig. 2B). We next determined whether the crizotinib-resistant H3122 cell lines were sensitive to the next-generation ALK inhibitors or 17-AAG. A549, PC9, and HCC827 cell lines are KRAS or EGFR mutant cancers and were included as controls. As shown in Fig. 2C, H3122 CR2 (1151Tins) cells demonstrated high-level resistance to all three ALK inhibitors tested (fig. S5, A to C), similar to Ba/F3 cells expressing the same 1151Tins *EML4-ALK* mutation (Fig. 1D). However, the H3122 CR2 cells were highly susceptible to 17-AAG treatment, similar to the H3122 and H3122 CR1 cells (Fig. 2C and fig. S5D). In contrast, the H3122 CR3 cells, which had no *ALK* mutation, were resistant to all of the ALK inhibitors as well as 17-AAG (IC_{50} similar to that of non–*ALK*-rearranged cancer cell lines) (Fig. 2C and fig. S5D). Thus, 17-AAG seemed effective against the cancer cell lines with ALK resistance mutations, but not against the H3122 CR3 cells that did not have a genetic alteration in *ALK*.

EGFR-mediated resistance to crizotinib in cell lines and patient samples

H3122 CR3 cells do not harbor a secondary *ALK* mutation or *EML4-ALK* gene amplification and were thus resistant to both ALK inhibition and hsp90 inhibition. Crizotinib treatment of this cell line suppressed phosphorylation of ALK to the same extent as in the sensitive parental cells (Fig. 2B). However, despite ALK inhibition, both AKT and ERK activation were maintained in the presence of crizotinib (Fig. 2B), suggesting that these pathways are being maintained by a regulator other than ALK. Studies from other oncogene addiction paradigms suggest that activation of alternative RTKs can lead to resistance to kinase inhibitors (19, 29, 30). To address this possibility, we used phospho-RTK arrays to assess the effect of crizotinib on 42 phospho-RTKs in parental H3122 and CR3 cells. Compared to the parental cells, H3122 CR3 cells contained higher levels of phospho-EGFR and phospho-ERBB3 both before and after crizotinib treatment (Fig. 3A). This finding was confirmed by immunoblotting directly for phospho-EGFR and phospho-ERBB3 (Fig. 3B). We did not detect *EGFR* mutation or gene amplification that might underlie the activation of EGFR in H3122 CR3 cells. However, quantitative reverse transcription–PCR (RT-PCR) revealed up-regulation of EGFR mRNA as well as the EGFR ligand amphiregulin and the ERBB3 ligand NRG1 in the resistant cells (fig. S6). Thus, EGFR activation in H3122 CR3 cells may be due to up-regulation of the receptor itself as well as two ligands, leading to persistent ALK-independent activation of downstream signaling cascades.

To determine whether increased ERBB signaling might underlie the acquired crizotinib resistance of H3122 CR3 cells, we treated cells with crizotinib, gefitinib (an EGFR TKI), or a combination. Whereas H3122 CR3 cells were resistant to either crizotinib or gefitinib alone, the combined treatment suppressed AKT and ERK phosphorylation (Fig. 3B) and led to significant growth suppression (Fig. 3C). However, despite inhibition of downstream signaling pathways, H3122 CR3 cells remained less sensitive to the combination of crizotinib and gefitinib (or erlotinib) than parental H3122 cells treated with crizotinib alone (Fig. 3C). To determine whether the mitigated response may indicate that the H3122 CR3 cells fail to undergo apoptosis in response to combination treatment with crizotinib and gefitinib, we performed annexin V staining of parental and resistant cells. Whereas treatment of parental H3122 cells with crizotinib induced marked apoptosis after 72 hours, treatment of H3122 CR3 cells with crizotinib, gefitinib, or the combination failed to induce apoptosis (Fig. 3D). To investigate the molecular basis for this finding, we examined both protein and mRNA levels of BIM, a key mediator of apoptosis in cancers addicted to kinases (31-35). Whereas BIM protein appeared to be dephosphorylated and up-regulated in H3122 CR3 cells treated with combined crizotinib and gefitinib, the up-regulation of BIM was much less than that observed in parental H3122 cells treated with crizotinib alone (Fig. 3E). Accordingly, BIM mRNA was lower in the H3122 CR3 cells (fig. S7). This is consistent with our recent findings that BIM mRNA may account for different BIM protein levels and the differing potential of oncogene-addicted cancers for undergoing apoptosis (36). Together, these results suggest that, whereas EGFR activation may mediate acquired crizotinib resistance, EGFR activation does not fully explain the acquired resistance phenotype, and combined ALK and EGFR kinase inhibition in crizotinib-resistant disease may not be as effective as crizotinib in treating crizotinib-sensitive disease.

These in vitro findings spurred us to determine whether there is evidence for EGFR activation as a resistance mechanism in patient specimens. We therefore examined the resistant tumors from the 18 *ALK*-positive patients who had relapsed on crizotinib (Table 1). On the basis of immunohistochemical (IHC) staining for phospho-EGFR, we detected EGFR activation (1+ to 2+ staining) in all but one of the resistant specimens with sufficient tissue for IHC analysis (Table 1). In nine cases, we were able to compare the resistant tumor specimen with the original diagnostic specimen obtained before crizotinib therapy. In four of

the nine cases, we detected increased EGFR activation in the resistant compared with the corresponding sensitive sample (Fig. 3F), supporting a possible role for EGFR in mediating crizotinib resistance. In addition, one of the four cases with evidence of EGFR activation (MGH021) also had a secondary *ALK* mutation. Thus, more than one mechanism of resistance may contribute to the development of crizotinib resistance in a single patient, recapitulating the heterogeneity of resistance mechanisms observed in the H3122 cell line models.

Unexpectedly, we detected EGFR activation in all but one of the pretreatment specimens (Fig. 3F and Table 1). None of these cases was found to harbor an activating *EGFR* mutation. Indeed, in contrast to a recent study that reported coexisting *ALK* rearrangement and *EGFR* mutation in 3 of 50 crizotinib-naïve patients (16), we identified no cases of overlap among 103 *ALK*-positive patients and 214 *EGFR* mutation–positive patients. Thus, EGFR activation in the setting of crizotinib-naïve, *ALK*-positive NSCLC is not likely to be the result of *EGFR* mutation.

Given that we observed EGFR phosphorylation in a significant proportion of the treatment-naïve cancer specimens, we wished to determine whether EGFR activation may be functionally important in crizotinib-sensitive disease and perhaps may mitigate initial responsiveness to crizotinib. In H3122 cells, the addition of an EGFR inhibitor did not increase the potency of crizotinib (Fig. 3C). However, we studied another cell line, MGH006, which had been derived from a crizotinib-naïve patient with advanced *ALK*-positive NSCLC that we reported previously (25). In cell survival assays, MGH006 cells were sensitive to crizotinib (fig. S8A), but they were less sensitive than the H3122 cells (relative IC_{50}, 4.1 to 1). Similar to resistant H3122 CR3 cells, MGH006 cells expressed high levels of phosphorylated and total EGFR protein (Fig. 3B and fig. S8B). Compared with crizotinib alone, treatment of MGH006 cells with the combination of crizotinib and gefitinib led to marked suppression of downstream AKT and ERK phosphorylation (fig. S8B). In particular, suppression of the ERK signaling pathway required concomitant inhibition of ALK and EGFR in this cell line. Combined ALK and EGFR inhibition also led to more potent growth suppression and marked induction of apoptosis (fig. S8, A and C). Together, these results suggest that even in crizotinib-naïve patients, EGFR activity may contribute to maintenance of downstream signaling, thereby diminishing the efficacy of single-agent crizotinib.

Crizotinib resistance mediated by KIT amplification and stromal SCF

In 6 of the 18 crizotinib-resistant specimens, we had sufficient tissue to screen for mutations in 14 cancer-related genes using a highly sensitive, multiplexed genotyping platform referred to as Snapshot (37). In the other 12 samples, the nucleic acid was exhausted in our investigations to identify *ALK* resistance mutations. Although no mutations were observed in those six cases, one sample, MGH0NZ, was negative for *KIT* mutation by Snapshot and standard Sanger sequencing, but the sequence peak on the raw Snapshot tracings was abnormally high (Fig. 4A). To determine whether the higher *KIT* peak might reflect gene amplification, we performed *KIT* FISH on the resistant sample as well as the corresponding pre-crizotinib specimen. At the histological level, the resistant sample consisted of a lung adenocarcinoma with two different components: a bronchioloalveolar carcinoma (BAC) component and a solid growth component. As shown in Fig. 4B, the solid component, but not the BAC component or the pre-crizotinib specimen, demonstrated marked *KIT* gene amplification by FISH (more than five copies per cell). Consistent with the FISH results, we detected increased levels of KIT protein in the solid component of the resistance specimen, but not the pretreatment sample (solid adenocarcinoma) or the BAC component of the resistance specimen (Fig. 4C). We also observed increased expression of the KIT ligand, stem cell factor (SCF) in the stromal cells within the solid component (Fig. 4C), but not the

pre-treatment sample or the BAC component of the resistance specimen. Although the BAC component did not demonstrate evidence of *KIT* amplification or expression, we detected increased phospho-EGFR in this part of the tumor compared to the pre-crizotinib sample (Fig. 4C). Based on Ki67 staining, the proliferative index was higher in the solid, *KIT*-amplified component than in the BAC or the pretreatment sample (Fig. 4C). These findings suggest that multiple bypass tracks may be activated in the same patient, contributing to crizotinib resistance. Furthermore, we identified one case (MGH018) with both focal *KIT* amplification and a secondary *ALK* mutation (Table 1), supporting the notion of multiple and diverse mechanisms of TKI resistance within each individual patient.

To determine whether aberrant KIT activation was sufficient to confer resistance to crizotinib, we engineered crizotinib-sensitive H3122 cells to overexpress wild-type KIT via lentiviral infection. In the absence of exogenously added SCF, KIT-overexpressing H3122 cells remained highly sensitive to crizotinib (Fig. 4, D and E). However, in the presence of SCF, KIT-overexpressing H3122 cells were highly resistant to crizotinib and maintained downstream ERK and AKT signaling. Treatment with imatinib, a small-molecule inhibitor of KIT as well as ABL and PDGFR (platelet-derived growth factor receptor), completely reversed the resistant phenotype (Fig. 4, D and E). In contrast, the sensitivity of control H3122 cells was not affected by SCF or imatinib treatment. These results suggest that crizotinib resistance mediated by KIT requires stroma-derived SCF (as was observed in the patient sample, Fig. 4C) and that this form of resistance may be overcome by treating with combined ALK and KIT inhibitors.

DISCUSSION

Cancers harboring specific genetic abnormalities, such as *ALK*-positive cancers, *EGFR* mutant NSCLCs, and *BRAF* mutant melanomas, are highly sensitive to small-molecule kinase inhibitors, often leading to partial remissions. Although these provide substantial benefit, the remissions are relatively short-lived because the cancers become resistant to the kinase inhibitors. In the case of *ALK*-positive NSCLCs treated with crizotinib, the median duration of clinical benefit is 10 months (14).

Interrogation of patient samples and laboratory models in other oncogene addiction paradigms has identified some conceptual frameworks that underlie resistance to kinase inhibitors. To date, most mechanisms reside within one of two categories: genetic alteration of the drug target (that is, point mutations and/or gene amplification) or activation of bypass signaling [reviewed in (38, 39)]. Resistance mutations in the drug target markedly diminish the potency of the inhibitor against the kinase. Examples include *EGFR* T790M and *BCR-ABL* T315I. One approach to overcome this type of resistance is to identify more potent inhibitors capable of inhibiting the mutated target (40). The second type of resistance is caused by the activation of a parallel or downstream signaling pathway, obviating the need for the original drug target. One example is activation of MET in *EGFR* mutant NSCLCs that develop resistance to EGFR inhibitors (19, 29). In these resistant cancers, MET continues to activate critical intracellular signaling pathways, mainly PI3K-AKT and MEK-ERK, despite continued EGFR inhibition. A combination of EGFR and MET inhibitors effectively overcomes this resistance (18). In addition, there have been other findings in cancers with acquired resistance to EGFR inhibitors, such as conversion into small cell lung cancer and epithelial to mesenchymal transition (EMT), whose biological underpinnings remain obscure (17). Other hypotheses for potential causes of resistance, such as drug efflux and deficiencies in cell cycle arrest and apoptosis, have yet to be fully validated.

Here, we report results from a series of crizotinib-resistant lung cancer patients with *ALK*-positive NSCLC. In addition to the previously reported point mutations L1196M, C1156Y,

and L1152R (15, 16), we observed multiple additional genetic changes in the *ALK* gene in resistant cancers. These include marked *ALK* gene amplification and new mutations, 1151Tins, and two point mutations (G1202R and S1206Y) in the solvent front of the kinase domain. Crizotinib preferentially binds to the inactive conformation of ALK (16, 41). On the basis of computational modeling (fig. S9A), the solvent front mutations may lead to resistance in a manner distinct from that of the 1151Tins mutation. The T1151 insertion is predicted to disrupt a critical hydrogen bond between T1151 and the carbonyl backbone of E1129. The location of E1129 on the P loop, adjacent to catalytic Lys^{1150}, has led molecular modelers to speculate that 1151Tins may lead to changes in the affinity of ALK for ATP (42) (fig. S9B). In contrast, the G1202R mutation leads to a large basic residue that would be predicted to cause steric interference with inhibitor binding (fig. S9C). Meanwhile, the S1206Y mutation may destabilize the interaction of the side-chain hydroxyl of Ser^{1206} with the carboxylate of D^{1203}. Additionally, the larger tyrosine side chain of S1206Y may lead to a number of conformational changes around the solvent; specifically, the bulkier tyrosine may clash with the ligand and may also result in destabilization of the complementary electrostatic interaction between the basic morpholine chemical moiety of the drug and the acidic E^{1210} (fig. S9, A and C). In total, these results indicate that the ALK kinase domain can develop multiple distinct mutations that can abrogate the capacity of crizotinib to inhibit ALK.

In contrast to acquired resistance to EGFR inhibitors or imatinib, resistance due to genetic alterations in the drug target was observed in only a minority of cases (28%), suggesting that alterations in the *ALK* gene may not be the predominant mechanism of crizotinib resistance in the clinic. Furthermore, several different ALK resistance mutations were observed. This is distinct from *EGFR* mutant NSCLCs with resistance to EGFR TKIs, in which *EGFR* T790M is essentially the sole resistance mutation observed in the clinic. The heterogeneity of *ALK* resistance mutations is more reminiscent of the wide array of secondary *BCR-ABL* mutations that confer resistance to imatinib.

From a therapeutic standpoint, this finding adds complexity to efforts to identify new ALK inhibitors to overcome crizotinib resistance. In our examination of new ALK inhibitors under active clinical development, we observed that they have differential potencies against the different resistance mutations. This raises the inconvenient possibility that distinct inhibitors may be needed to overcome specific subsets of resistance mutations. It is notable that all of the resistance mutations conferred some degree of relative resistance to each of the inhibitors examined. Thus, the ultimate success of these agents may depend on the concentrations of drug that are achievable in patients. Some mutations, such as G1202R and 1151Tins, caused profound resistance to all of the ALK inhibitors. At this point, the use of hsp90 inhibitors may be the most attractive option for these highly resistant mutations. Our data suggest that there will likely be a need to identify additional ALK inhibitors that can overcome these highly resistant *ALK* mutations.

In addition, we also observed activation of bypass signaling, namely, the KIT and EGFR signaling pathways, as potential resistance mechanisms. On the basis of laboratory studies, we anticipate that treating these resistant cancers will require combining an ALK inhibitor with the corresponding RTK inhibitor. However, such combinations may not necessarily be as active against the resistant cancer as crizotinib was in the TKI-naïve cancers. We observed that H3122 cells that developed EGFR activation (H3122 CR3) were sensitized to crizotinib by EGFR inhibition, but the combination therapy was still unable to induce robust apoptosis, possibly because of the suppression of BIM expression (Fig. 3, D and E). Thus, activation of EGFR is not the only difference between these resistant cells and the TKI-naïve H3122 cells. Recently, Jänne and colleagues reported activation of EGFR in a cell line with acquired resistance to crizotinib, and they also found that combined EGFR and ALK

inhibition failed to induce apoptosis in the resistant cells (16). Consistent with a role for EGFR in causing resistance to crizotinib in patients, we observed increased levels of phospho-EGFR in resistant tumor biopsies. Although these observations are certainly consistent with EGFR conferring resistance, they do not prove it. The most convincing proof would be clinical activity of combined EGFR and ALK inhibition in resistant cancers.

In contrast, the *KIT*-mediated resistance via gene amplification more closely parallels resistance to EGFR inhibitors caused by *MET* amplification because it is accompanied by genetic activation of the RTK. This resistance mechanism appeared to involve support by the cancer stroma because SCF was produced specifically in the stroma of the resistant cancer with *KIT* amplification. Accordingly, our laboratory studies suggest that ligand is necessary for amplified *KIT* to confer full resistance (Fig. 4, D and E). Our preclinical data suggest that a combination of imatinib and crizotinib may overcome this particular mechanism of resistance.

In some of the cases, multiple mechanisms of resistance were observed in the same patient. For example, the resistant specimen from MGH0NZ demonstrated two distinct histologies (Table 1 and Fig. 4). One was a solid tumor pattern with a high proliferative index and *KIT* amplification; the other was BAC with lower proliferation and EGFR activation. Additionally, in another case with an *ALK* resistance mutation (MGH021), there was clear evidence of increased EGFR activation, and in two other cases with point mutations (MGH011 and MGH018), there was high phospho-EGFR staining in the resistant specimen (Table 1). The potential for multiple, simultaneous resistance mechanisms supports the use of combination therapies to overcome resistance, namely, newer ALK inhibitors and hsp90 inhibitors in combination with other RTK inhibitors. If newer single-agent ALK inhibitors or hsp90 inhibitors fail to demonstrate clinical activity in resistant cancers harboring resistance mutations, there will be the possibility that the drugs effectively suppress the resistant ALK, but the presence of additional resistance mechanisms abrogates tumor responsiveness. Our laboratory models of resistance replicated the potential for heterogeneity of resistance mechanisms within a single cancer. The same *EML4-ALK* cell line, H3122, developed three different mechanisms of resistance: L1196M, 1151Tins, and EGFR activation. If a single cell line is capable of yielding multiple mechanisms of resistance, it is not surprising that a cancer that develops in a patient has a similar capacity. It is also noteworthy that the resistance mechanisms observed in the cell line models recapitulated those observed in the clinic, further validating such laboratory studies to identify clinically relevant mechanisms of resistance to targeted therapies.

With crizotinib's recent FDA approval, many *ALK*-positive patients will benefit from this therapy over the next few years. Acquired resistance will be the major limitation preventing this therapy from having greater impact. Although this study has identified several resistance mechanisms, there are still many left to be discovered. In our study, there were many NSCLC patients for whom a resistance mechanism was not identified. Clinical trials are planned and under way to evaluate the efficacy of new ALK inhibitors, hsp90 inhibitors, and combination strategies to overcome resistance. It will be incumbent upon the medical and scientific communities to obtain biopsies of relapsing patients before enrolling them in these studies. Effective interpretation of the results will require detailed understanding of the specific resistance mechanisms that can develop. On the basis of our understanding of EGFR TKI resistance, certain resistance mechanisms may preexist in crizotinib-naïve patients (18, 43, 44). Therefore, the most effective therapeutic strategy for *ALK*-positive lung cancers may ultimately require first-line combinatorial strategies that target not only ALK and ALK resistance mutations but also emerging alternative pathways of resistance.

MATERIALS AND METHODS

Patients

The 18 *ALK*-positive NSCLC patients with acquired crizotinib resistance underwent biopsy of their resistant tumors between January 2009 and July 2011. Standard histopathology was performed to confirm the diagnosis of malignancy and the histological subtype. For all samples, total nucleic acid was isolated as described. In cases with sufficient tissue, we also performed FISH and IHC studies as described below. The electronic medical record was reviewed retrospectively to obtain clinical information under an Institutional Review Board–approved protocol.

Reagents and cell culture conditions

H3122, H3122-derived resistant cells (H3122 CRs), HCC827, PC9, and A549 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (R-10). MGH006 cells and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (D-10). Ba/F3, immortalized murine bone marrow–derived pro–B cells, were cultured in D-10 with or without IL-3 (0.5 ng/ml) (Invitrogen). Crizotinib and NVP-TAE684 were purchased from ChemieTek, and 17-AAG was from Selleck. CH5424802 and ASP-3026 were purchased from Active Biochem. Each compound was dissolved in dimethyl sulfoxide (DMSO) for cell culture experiments. HiPerFect reagent was from Qiagen. Human SCF was obtained from Cell Signaling Technology. The human phospho-RTK array kit was purchased from R&D Systems.

Generation of H3122 CR2 and CR3 cells

H3122 CR2 and CR3 cells were established in the same manner as H3122 CR1 (12). Briefly, H3122 cells were seeded at ~70% confluence in 15-cm dishes in R-10. Crizotinib was added at a starting concentration of 30 nM, and cells were maintained in fresh drug-containing medium changed every ~72 hours. Cells were passaged once they reached confluence. After every two passages at a given concentration of drug, the concentration of crizotinib was increased in half-log intervals until a final concentration of 1 μM was achieved. The resulting pool of resistant cells (designated H3122 CR2 or CR3) was maintained in R-10 with 1 μM crizotinib. From the H3122 CR2 and CR3 pool, we derived 10 clones of each from single cells by limiting dilution.

Survival assays

For 72-hour drug treatments, 3000 cells were plated in replicates of six into 96-well plates. After drug treatments, cells were incubated with CellTiter-Glo assay reagent (Promega) for 10 min, and luminescence was measured with a Centro LB 960 microplate luminometer (Berthold Technologies).

Fluorescence in situ hybridization

Two-color FISH for *ALK* rearrangement was done on 3:1 methanol/acetic acid–fixed cell lines with the LSI ALK Dual Color, Break Apart Rearrangement Probe (Abbott-Vysis) following the manufacturer's protocols. KIT gene copy number was detected with a Spectrum Orange–labeled BAC RP11-977G3 (Invitrogen/Life Technologies) and a control CEP4 Spectrum Green probe (Abbott Molecular). Images were captured with an Olympus BX61 fluorescence microscope equipped with a charge-coupled device camera, and analysis was done with the CytoVision software (Applied Imaging). High-level amplification is defined as a ratio of KIT to CEP4 signals that is greater than 5.

Immunoblotting

Cells were resuspended in lysis buffer (20 mM tris, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM EGTA, and protease and phosphatase inhibitors), incubated on ice for 10 min, and centrifuged for 5 min (15,000 rpm). Protein concentration determination and immunoblotting were performed as previously described (45). β-Actin, BIM, phospho-KIT (pY^{719}), KIT, phospho-ERBB3 (pY^{1289}), phospho-ERK (T^{202}/Y^{204}), ERK, phospho-AKT (S^{473} and T^{308}), phospho-ALK (Y^{1604}), and ALK antibodies were obtained from Cell Signaling Technology. Phospho-EGFR (pY^{1173}) was purchased from Epitomics. Phospho-EGFR (pY^{1068}) was purchased from Abcam. AKT1/2/3, EGFR, and ERBB3 antibodies were purchased from Santa Cruz.

Apoptosis assay

Cells were collected and stained with annexin V and propidium iodide (PI) (5 μg/ml) for 10 min. Cells were then assayed with a FACSDiva (BD Biosciences) flow cytometer, and the data were analyzed with the FlowJo software (Tree Star).

Retroviral infection

cDNAs encoding EML4-ALK variant1, EML4-ALK variant1 L1196M, G1202R or S1206Y mutants, or KIT were cloned into 1520 retroviral expression vectors, and virus was produced as previously described (46). After retroviral infection, Ba/F3 or H3122 cells were selected in puromycin (1.0 μg/ml) for 2 weeks. For Ba/F3 cells infected by EML4-ALK variants, IL-3 was withdrawn from the culture medium for 2 weeks before experiments.

In vitro kinase assay

The IC_{50} values of compounds against ALK (V^{1058}-P^{1620}) wild type, ALK (V^{1058}-P^{1620}) L1196M (gatekeeper mutation), and two ALK (V^{1058}-P^{1620}) solvent front mutants G1202R and S1206Y were assessed in Caliper off-chip mobility shift assay format, which measures both phosphorylated and unphosphorylated fluorescence-labeled substrate and calculates a ratiometric value to determine percent inhibition. Before the assays, the K_m (Michaelis constant) value of ATP was determined for each ALK kinase involved: wild type (33 μM), L1196M (24 μM), G1202R (23 μM), and S1206Y (20 μM).

In an ALK wild-type K_m ATP IC_{50} test, the reactions in 1.2× Hepes assay buffer [60 mM Hepes (pH 7.3), 1.2 mM dithiothreitol (DTT), 0.012% Tween 20, bovine serum albumin (60 μg/ml)] were run with varying concentrations of inhibitory compounds in low-volume 384-well plates. After spotting of 2 μl of 6× inhibitor in 5% DMSO and addition of 5 μl of 2.4× enzyme mix [at reaction endpoint, 1 nM enzyme, 33 μM ATP, and 1.5 μM Srctide (5FAM-GEEPLYWSFPAKKK-NH_2) peptide substrate in 1.2× Hepes assay buffer], plates were preincubated for 10 min at room temperature. Five microliters of $MgCl_2$ (10 mM in final) in Hepes assay buffer was added to initiate the reactions, and plates were incubated for 90 min at room temperature. The reactions were quenched by the addition of 5 μl of reaction stop buffer [100 mM Hepes (pH 7.3), 120.7 mM EDTA, 0.77% Coating Reagent 3 (Caliper), 0.01% Tween 20] and read in a Caliper LC3000 instrument. Percent inhibition values were calculated for each inhibitory concentration in Caliper's HTS Well Analyzer software. IC_{50} values were obtained by fitting the percent inhibition data to a four-parameter sigmoidal IC_{50} equation with ActivityBase (IDBS). In parallel, an ALK wild-type high ATP IC_{50} test was also carried out with 0.5 nM protein and 5 mM ATP in the same assay system. In the assay plates, 2 μl of 5% DMSO spotted as max control, and 2 μl of 300 mM EDTA in 5% DMSO as min control subsequently.

For the ALK mutants, the protein concentrations were determined to generate levels of substrate phosphorylation similar to that of the wild type in their IC_{50} tests at a certain reaction time.

Production of recombinant ALK proteins

Recombinant baculoviruses were used to infect *Spodoptera frugiperda* (*Sf*21) insect cells at a multiplicity of infection (MOI) of 1. Infected *Sf* 21 cells were harvested via centrifugation 48 hours after infection. The cell pellets were lysed by French press in 25 mM tris (pH 7.5), 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.5 mM EDTA, phosphatase inhibitors (5 mM NaF, 1 mM sodium orthovanadate, 10 mM glycerol-2-phosphate), and Roche Complete EDTA-free protease inhibitors. The lysates were clarified by centrifugation at 40,000 rpm (Sorvall Discovery 90 T647.5 rotor) for 30 min. Clarified lysates were batch-captured on glutathione resin (GE 17-0756-01) for 1 to 2 hours at 4°C. Glutathione *S*-transferase–ALK proteins were eluted from washed resin with 100 mM tris (pH 8.0), 150 mM NaCl, 10% glycerol, 0.02% Triton X-100, 1 mM DTT, and 20 mM glutathione. Fractions containing ALK protein were pooled and passed over a Superdex 200 (16/60) column equilibrated with 25 mM tris (pH 7.5), 150 mM NaCl, 10% glycerol, 2 mM DTT, 0.02% Triton X-100, and 0.5 mM EDTA. Purified ALK fractions were pooled, concentrated, and snap-frozen in liquid nitrogen and stored at −80°C.

RNA preparation, quantitative real-time PCR, and sequencing

Quantitative RT-PCR was performed essentially as described (27). Total RNA from cell lines was extracted with an RNeasy Mini Kit (Qiagen). RNA (1 μg) was reverse-transcribed with Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. mRNA was quantified with SYBR Green with a PCR LightCycler 480 (Roche Diagnostics) and normalized by the amount of actin mRNA. For the sequencing of ALK or EGFR, the entire coding region or kinase domain of ALK or EGFR was PCR-amplified from cDNA and sequenced bidirectionally by Sanger dideoxynucleotide sequencing. PCR primers and conditions are available upon request.

Isolation of gDNA preparation for *ALK* exon PCR

gDNA was isolated from cell pellets with a DNeasy kit (Qiagen) according to the manufacturer's protocol. Exons 20 to 28 corresponding to the *ALK* TK domain were PCR-amplified from gDNA with FastStart PCR Master (Roche). PCR primers and conditions are available upon request.

Immunohistochemistry

IHC staining was performed on representative tissue sections from formalin-fixed and paraffin-embedded tissue blocks. IHC was performed with an anti–phospho-EGFR–specific antibody (Cell Signaling Technology, dilution 1:800 in SignalStain Antibody Diluent) or an anti-SCF (C19H6) antibody (Cell Signaling Technology, dilution 1:50 in SignalStain Antibody Diluent) according to the manufacturer's protocol.

Statistical analysis

All data are shown as means ± SD. Statistical analysis was performed with two-tailed Student's *t* test. Significance was established for *P* values of <0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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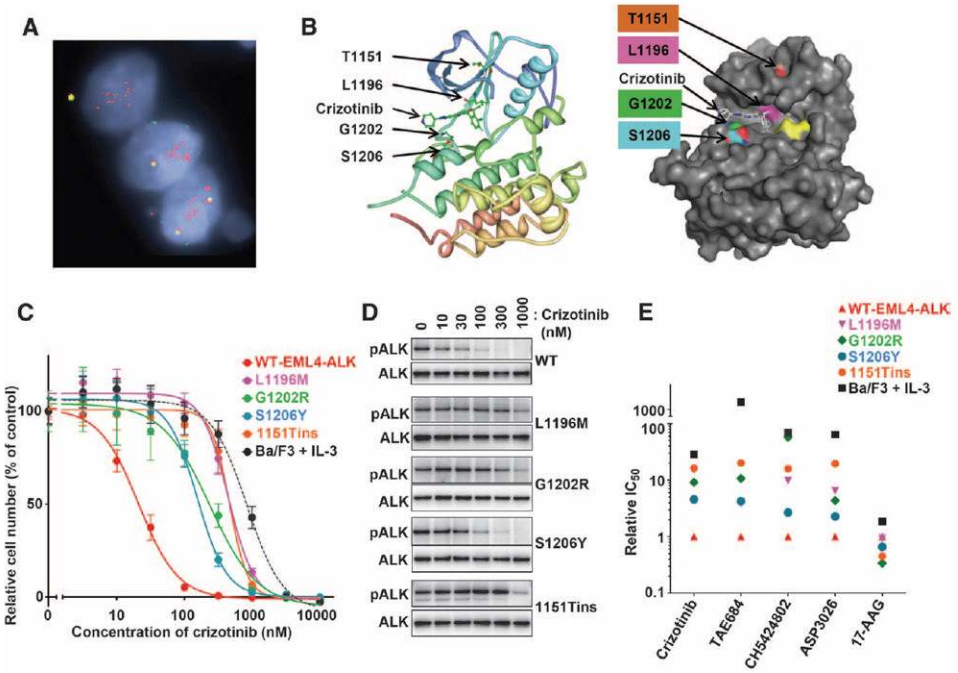

Fig. 1. *ALK* gene amplification and multiple *ALK* resistance mutations in cancers with acquired crizotinib resistance

(**A**) FISH analysis of *ALK* demonstrates high-level *ALK* gene amplification in one resistant tumor. Amplified, rearranged *ALK* appears as a cluster of isolated red signals in this resistant specimen. (**B**) Three-dimensional mapping of each identified ALK mutation based on the crystal structure of ALK. Each of the four ALK mutations is mapped on a ribbon (left) or surface (right) diagram. In the surface structure model, each mutated residue is shown in a different color, and yellow depicts the DFG motif. Figures were drawn using the PyMOL software with the crystal structure information of Protein Data Bank ID 2XP2. (**C**) Ba/F3 cells were transformed by expression of either wild-type (WT) *EML4-ALK* or *EML4-ALK* harboring one of the four identified resistance mutations (L1196M, G1202R, S1206Y, or 1151Tins). Parental Ba/F3 cells (cultured with IL-3) or EML4-ALK–expressing Ba/F3 cells (cultured without IL-3) were treated with the indicated doses of crizotinib for 48 hours. Cell survival was measured using CellTiter-Glo. Each concentration was measured in sextuplicate, and the average and SD are shown. (**D**) Ba/F3 cells transformed by WT EML4-ALK or EML4-ALK harboring the indicated resistance mutation were treated with the indicated concentrations of crizotinib for 1 hour. Cell lysates were probed with phospho-ALK (pALK) and ALK-specific antibodies. (**E**) Differential sensitivity conferred by ALK TK mutations to next-generation ALK inhibitors and the hsp90 inhibitor 17-AAG. The relative IC_{50} of each drug across six different Ba/F3 cell lines, including parental, IL-3–dependent Ba/F3 cells as well as transformed Ba/F3 cells expressing the indicated EML4-ALK constructs, is shown. For each drug, the IC_{50} values for the various cell lines have been normalized to that of crizotinib-sensitive Ba/F3 cells expressing WT EML4-ALK. The values are the average from three independent experiments. The raw data from a representative experiment are shown in fig. S2 and tabulated in fig. S2F.

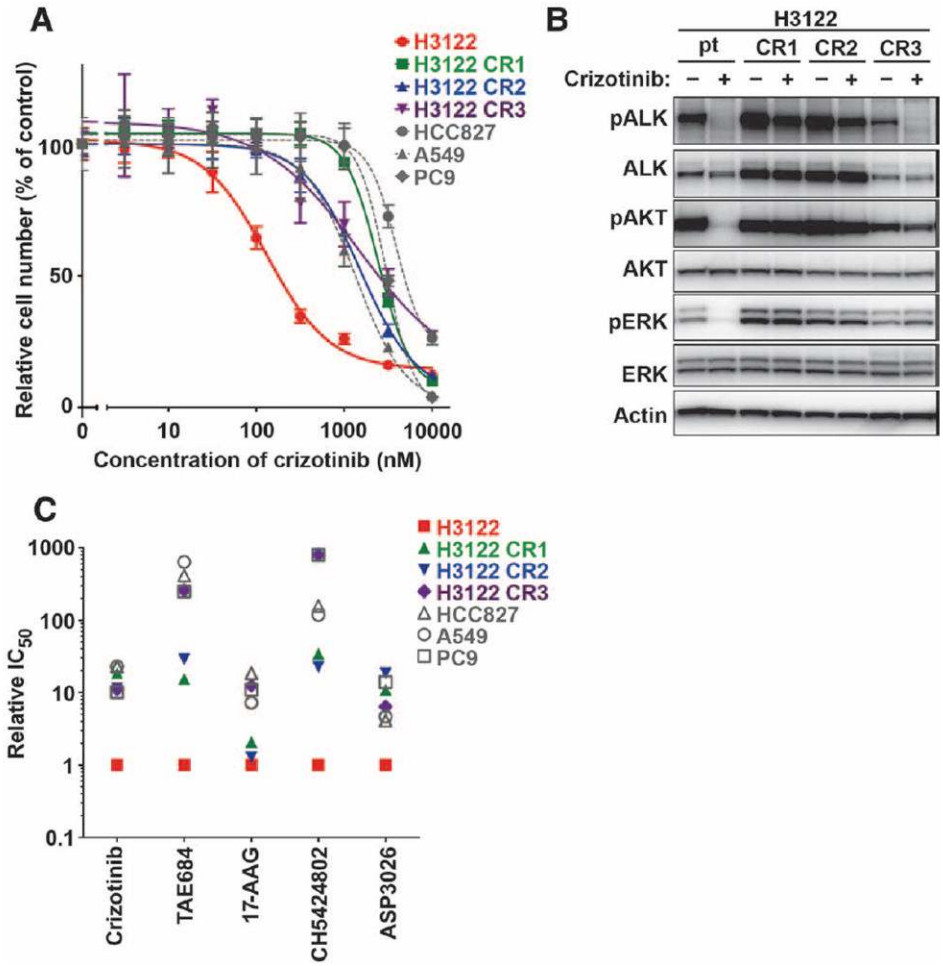

Fig. 2. Heterogeneity of resistance mechanisms from cell lines with acquired resistance to crizotinib

(**A**) Three independently derived, crizotinib-resistant cell lines (H3122 CR1, CR2, and CR3) were treated with the indicated doses of crizotinib for 72 hours. As controls, parental H3122 cells and three ALK WT cell lines (HCC827, PC9, and A549) were also treated in parallel. Cell survival was measured using a CellTiter-Glo viability assay. Each concentration was measured in sextuplicate, and the average and SD are shown. (**B**) ALK signaling in crizotinib-resistant H3122 cell lines. Parental (pt) H3122 cells and the three different crizotinib-resistant H3122 cell lines (CR1, CR2, and CR3) were treated with 1 μM crizotinib for 6 hours. Cell extracts were immunoblotted with antibodies directed against the indicated proteins. (**C**) Differential sensitivity of H3122 CR1, CR2, and CR3 cells to ALK and hsp90 inhibitors. Shown is the relative IC_{50} for each drug across seven different cell lines, including the three crizotinib-resistant H3122 cell lines as well as three WT ALK controls (A549, HCC827, and PC9). For each drug, the IC_{50} values for the various cell lines have been normalized to that of parental H3122 cells. The values are the average from three independent experiments. The raw data from a representative experiment are shown in fig. S5.

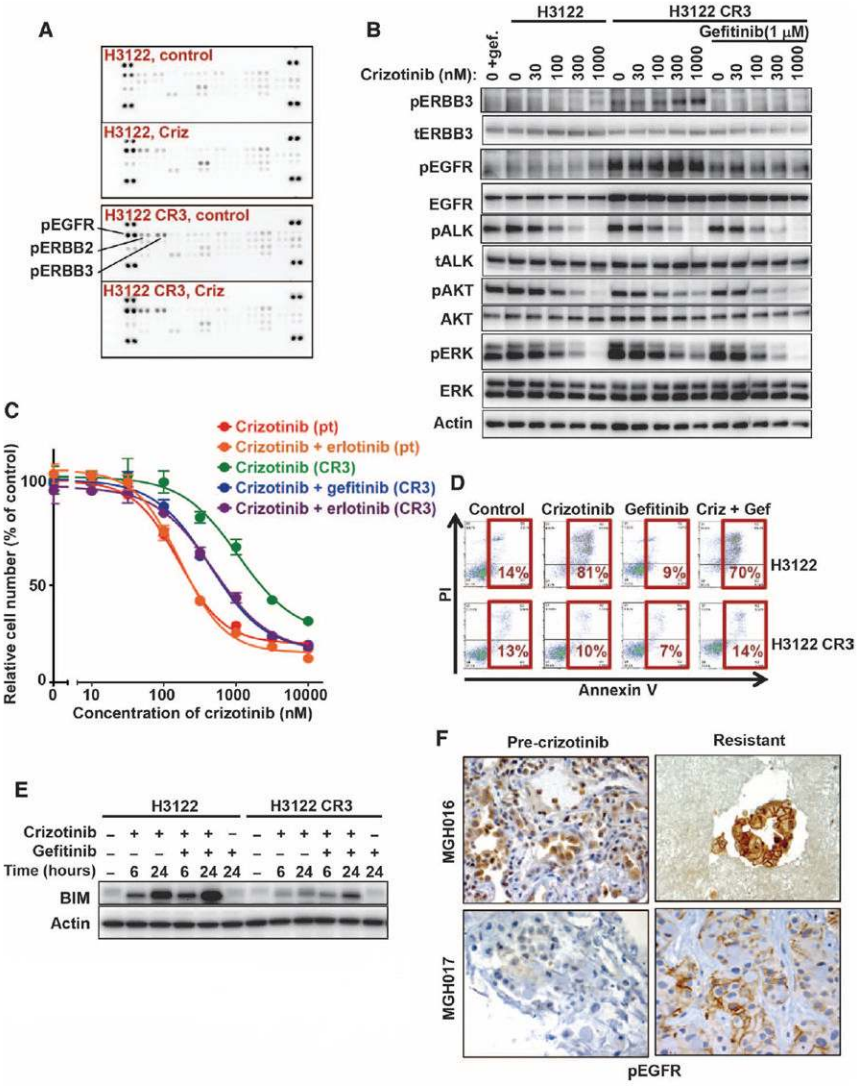

Fig. 3. Activation of EGFR in cell lines and patients with acquired resistance to crizotinib

(**A**) Parental H3122 and H3122 CR3 cells were incubated in the absence (control) or presence (Criz) of 1 μM crizotinib for 6 hours, and lysates were incubated with phospho-RTK arrays (R&D Systems). The positions of phospho-EGFR, phospho-ERBB2, and phospho-ERBB3 are indicated. (**B**) H3122 CR3 cells were treated for 6 hours with the indicated concentrations of crizotinib in the presence or absence of gefitinib. Cell lysates were probed with the indicated antibodies. (**C**) Sensitization of resistant H3122 CR3 cells by treatment with both crizotinib and an EGFR TKI (gefitinib or erlotinib). Parental H3122 and H3122 CR3 cells were treated with the indicated doses of crizotinib in the presence or absence of 2 μM gefitinib or 1 μM erlotinib for 72 hours. Cell survival was determined using the CellTiter-Glo viability assay. Each concentration was measured in sextuplicate, and the average and SD are shown. (**D**) Lack of apoptosis induction in H3122 CR3 cells treated with crizotinib (Criz) and gefitinib (Gef). Parental H3122 and H3122 CR3 cells were treated with 1 μM crizotinib, 2 μM gefitinib, or the combination. After 72 hours, cells were stained with Alexa Fluor 633–labeled annexin V and PI and analyzed by flow cytometry. The percentage of cells undergoing apoptosis is shown. (**E**) Defective up-regulation of BIM in H3122 CR3 cells treated with crizotinib and gefitinib. H3122 and H3122 CR3 cells were

treated with 1 μM crizotinib, 2 μM gefitinib, or both drugs for 6 or 24 hours. Lysates were probed with BIM and actin-specific antibodies. (**F**) Increased EGFR activation in crizotinib-resistant tumors. Pre-crizotinib and crizotinib-resistant tumors were stained using a phospho-EGFR–specific antibody. Shown are two cases, MGH016 and MGH017, both of which demonstrate stronger plasma membrane staining of phospho-EGFR in the resistant cancer than in the corresponding pre-crizotinib sample.

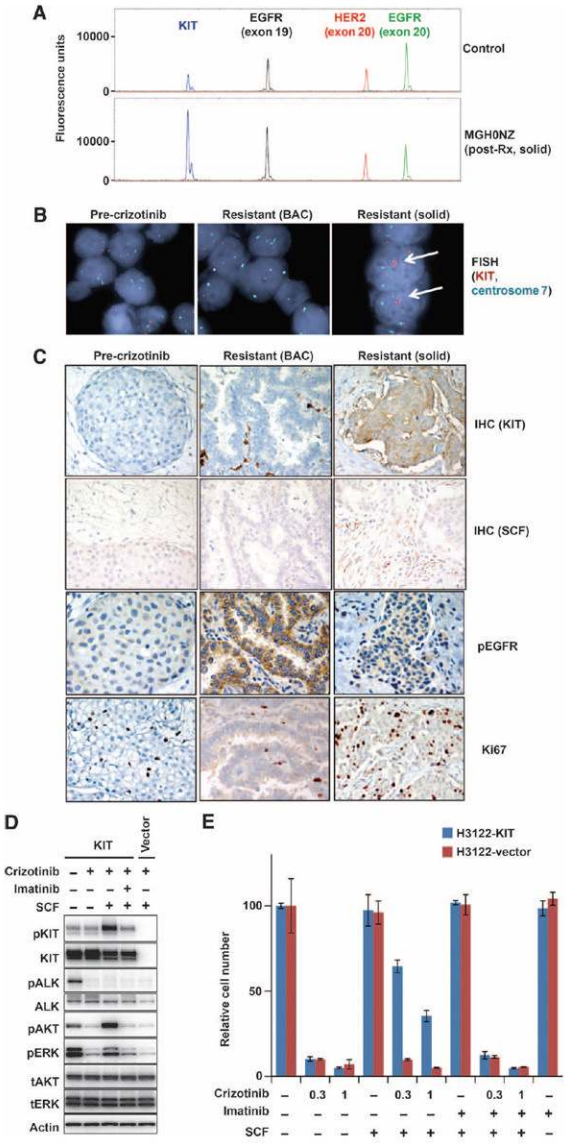

Fig. 4. Aberrant activation of KIT/SCF mediates acquired resistance to crizotinib

(**A**) Snapshot panel showing relatively increased amplitude of the WT *KIT* peak (arrow) in patient MGH0NZ compared to normal control, suggesting *KIT* gene amplification. This snapshot panel tests for mutations within *KIT* (blue), *EGFR* exon 19 (black), *HER2* (red), and *EGFR* exon 20 (green). (**B**) Confirmation of *KIT* amplification by FISH analysis. *KIT*/ centromere 7 FISH was performed on both pretreatment and resistant specimens from MGH0NZ. Amplified *KIT* appears as a cluster of red signals (arrows) and was detected in the solid but not the BAC component of the resistant specimen. Aqua signals indicate centromere 7. (**C**) IHC staining for KIT, SCF, phospho-EGFR (pEGFR), and Ki67 expression. Within the resistant specimen, the solid, but not the bronchioloalveolar, component showed strong KIT expression in the tumor cells and SCF expression in the stromal cells. Conversely, the bronchioloalveolar, but not the solid, component showed strong phospho-EGFR expression. Note that the KIT-positive cells in the BAC represent CD117-positive mast cells. (**D**). H3122 cells were infected with retrovirus expressing KIT or empty vector control. After 2 weeks of selection in puromycin, cells were treated for 6 hours with crizotinib (1 μM), human SCF (100 ng/ml), imatinib (1 μM), or a combination, as

indicated. Cell extracts were immunoblotted to detect the indicated proteins. (**E**) Control and KIT-overexpressing H3122 cells were seeded in 12-well plates and treated with crizotinib (1 μM), human SCF (100 ng/ml), imatinib (1 μM), or their combination as indicated for 7 days. Cell viability was measured using a crystal violet assay. Experiments were performed in triplicate, and the average and SD are shown.

Table 1

ALK-positive NSCLC patients with acquired resistance to crizotinib. Adeno, adenocarcinoma; NA, tissue not available; BAC, bronchioloalveolar component.

Patient	Duration (months)*	Timing (months)†	Histology	ALK fusion	ALK-amplified	ALK mutation	Phospho-EGFR (pre-crizotinib)	Phospho-EGFR (post-crizotinib)	CKIT FISH
MGH0NZ	20	0	Adeno	Positive	No	No	1+	1+ (solid) 2+ (BAC)	Amplified (solid)
MGH001	4	3.5	Adeno	Positive	No	No	NA	1+	No
MGH010	8	0	Adeno	Positive	No	No	2+	1+	No
MGH011	34	0	Adeno	Positive	No	S1206Y	2+	2+	No
MGH013	9	0	Adeno	Positive	No	No	NA	2+	No
MGH016	6	6	Adeno	Positive	No	No	1+	2+	No
MGH017	23+	0	Adeno	Positive	No	No	0	2+	No
MGH018	10	0.5	Adeno	Positive	No	G1202R	2+	2+	Focal amplification
MGH019	8	<0.5	Adeno	Positive	No	No	NA	NA	NA
MGH020	13	0	Adeno	Positive	No	L1196M	NA	NA	NA
MGH021	12	3	Adeno	Positive	No	1151Tins	1+	2+	No
MGH022	6	0	Adeno	Positive	No	No	NA	2+	No
MGH023	12	0	Adeno	Positive	No	No	2+	1+	No
MGH024	15	0	Adeno	Positive	No	No	2+	2+	No
MGH025	11	0	Adeno	NA	NA	No	NA	2+	No
MGH027	4	1	Adeno	NA	NA	No	NA	NA	NA
MGH028	14	1	Adeno	NA	NA	No	1+‡	NA	NA
MGH029	8	0	Adeno	Positive	Yes	No	NA	0	NA

*Approximate duration of crizotinib therapy. "+" indicates that the patient remains on crizotinib.

†Approximate time between the last crizotinib dose and the repeat biopsy. "0" indicates that the patient was still taking crizotinib at the time of the biopsy.

‡This sample had focal areas with 2+ staining.