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RESEARCH ARTICLE

Molecular Mechanisms of Resistance to First- and Second-Generation ALK Inhibitors in *ALK*-Rearranged Lung Cancer

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ABSTRACT Advanced, anaplastic lymphoma kinase (ALK)–positive lung cancer is currently treated with the first-generation ALK inhibitor crizotinib followed by more potent, second-generation ALK inhibitors (e.g., ceritinib and alectinib) upon progression. Second-generation inhibitors are generally effective even in the absence of crizotinib-resistant *ALK* mutations, likely reflecting incomplete inhibition of ALK by crizotinib in many cases. Herein, we analyzed 103 repeat biopsies from ALK-positive patients progressing on various ALK inhibitors. We find that each ALK inhibitor is associated with a distinct spectrum of *ALK* resistance mutations and that the frequency of one mutation, ALK^{G1202R}, increases significantly after treatment with second-generation agents. To investigate strategies to overcome resistance to second-generation ALK inhibitors, we examine the activity of the third-generation ALK inhibitor lorlatinib in a series of ceritinib-resistant, patient-derived cell lines, and observe that the presence of *ALK* resistance mutations is highly predictive for sensitivity to lorlatinib, whereas those cell lines without *ALK* mutations are resistant.

SIGNIFICANCE: Secondary *ALK* mutations are a common resistance mechanism to second-generation ALK inhibitors and predict for sensitivity to the third-generation ALK inhibitor lorlatinib. These findings highlight the importance of repeat biopsies and genotyping following disease progression on targeted therapies, particularly second-generation ALK inhibitors. *Cancer Discov; 6(10); 1118–33. ©2016 AACR.*

See related commentary by Qiao and Lovly, p. 1084.

INTRODUCTION

Anaplastic lymphoma kinase (*ALK*) rearrangements define a distinct molecular subtype of non–small cell lung cancer (NSCLC; refs. 1, 2). Recently, the therapeutic landscape for advanced ALK-positive NSCLC has been transformed by the development of increasingly potent and selective ALK inhibitors. Crizotinib was the first ALK inhibitor to enter clinical development (3). In randomized phase III trials, crizotinib produced significant improvements in objective response rates (ORR) and progression-free survival (PFS) compared to cytotoxic chemotherapy, establishing crizotinib as a standard treatment for advanced ALK-positive NSCLC (4, 5).

Although most patients respond to crizotinib, patients ultimately relapse on therapy, generally within 1 to 2 years (4, 5). Analysis of postprogression biopsy specimens has proven extremely valuable, facilitating a greater understanding of molecular mechanisms of crizotinib resistance (6, 7). Broadly speaking, such mechanisms have been classified as involving either on-target genetic alterations (e.g., *ALK* resistance mutations, *ALK* gene amplification) or off-target mechanisms of resistance (e.g., upregulation of bypass signaling pathways, such as EGFR, KIT, IGF1R, SRC, MEK/ERK, and others; refs. 6, 8–11). In published series to date, on-target resistance mechanisms have been found in approximately one third of patients progressing on crizotinib (6, 7).

Recently, several second-generation ALK inhibitors have demonstrated impressive activity in ALK-positive NSCLC (12–16). Two of these agents, ceritinib and alectinib, recently received approval by the FDA for the treatment of crizotinib-refractory, ALK-rearranged NSCLC. A third agent, brigatinib, has received breakthrough-therapy designation from the FDA. In preclinical models, second-generation ALK inhibitors overcome several crizotinib-resistant *ALK* mutations (17, 18). Furthermore, in phase I–II studies, these agents have demonstrated high ORRs (48%–71%) in crizotinib-resistant patients (12–16). Importantly, second-generation ALK inhibitors have also been active in patients without *ALK* resistance mutations or fusion gene amplification (12), suggesting that many cancers become resistant to crizotinib due to inadequate suppression of ALK. However, despite the efficacy of second-generation ALK inhibitors, patients almost invariably relapse. Thus far, descriptions of molecular mechanisms of resistance to second-generation ALK inhibitors have been limited to *in vitro* studies, case reports, and small clinical series, making it difficult to determine the scope of such alterations (17, 19–23).

Herein, we present the largest series of repeat biopsies from patients with ALK-positive NSCLC with resistance to ALK inhibitors, a majority of whom had acquired resistance. Using

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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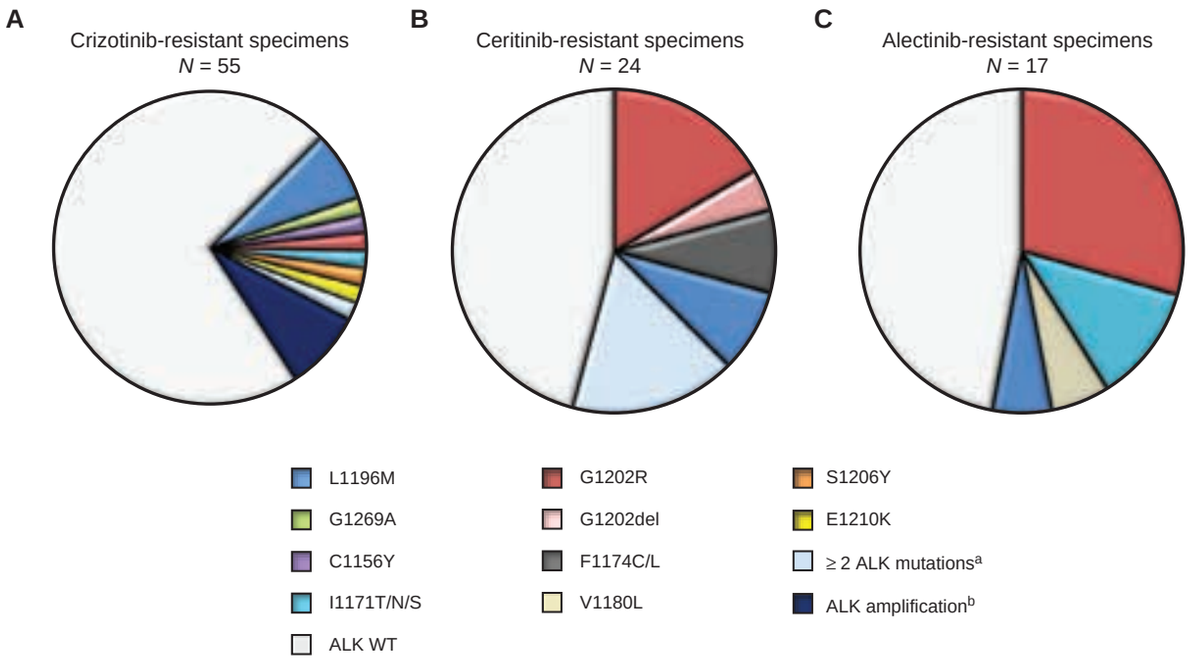

Figure 1. Overview of on-target mechanisms of resistance among ALK-positive specimens obtained from patients progressing on **A,** crizotinib; **B,** ceritinib; and **C,** alectinib. Pie charts depict the frequency and distribution of *ALK* resistance mutations and *ALK* fusion gene amplification in each cohort. Four patients underwent two separate biopsies while on crizotinib; one patient underwent two separate biopsies while on ceritinib. Note that if a specimen is listed as having ≥ 2 *ALK* resistance mutations, the individual mutations are not listed separately. [a]One post-crizotinib specimen harbored *ALK* G1269A and 1151Tins mutations. Four post-ceritinib samples contained ≥ 2 ALK resistance mutations. These included: I1171N+C1156Y, D1203N+F1174C, F1174L+G1202R, and C1156Y+G1202del+V1180L mutations. [b]ALK FISH to assess for fusion gene amplification was performed in only crizotinib-resistant specimens ($N = 36$), of which 8% had amplification. Ceritinib- and alectinib-resistant specimens were not assessed for *ALK* amplification by FISH. WT, wild-type.

a combination of genetic sequencing, histologic analyses, and functional drug screens, we find that the frequency and spectrum of *ALK* resistance mutations evolve as patients relapse on different ALK inhibitors. Moreover, in a series of ceritinib-resistant, patient-derived cell lines, we demonstrate that the presence of *ALK* resistance mutations is associated with sensitivity to the third-generation ALK inhibitor lorlatinib. In contrast, cell lines without *ALK* resistance mutations are resistant to lorlatinib. Together, these findings suggest a role for tailoring ALK inhibitor therapy based upon the underlying mechanisms of resistance.

RESULTS

Baseline Clinical Characteristics

Between January 2009 and June 2016, 83 ALK-positive patients underwent repeat biopsies following disease progression on first- or second-generation ALK inhibitors (Supplementary Table S1). All biopsies were procured from progressing lesions. Baseline clinical characteristics of these patients are summarized in Supplementary Table S2. A total of 103 biopsies were performed. Six (7%) patients underwent two separate biopsies while on the same ALK inhibitor (crizotinib, $N = 4$; ceritinib, $N = 1$; brigatinib, $N = 1$). Fourteen patients (18%) had paired repeat biopsies after disease progression on crizotinib and a second-generation ALK inhibitor (ceritinib, $N = 9$; alectinib, $N = 3$; brigatinib, $N = 2$; Supplementary Table S3).

ALK Resistance Mutations in Crizotinib-Resistant Specimens

We first investigated the frequency of *ALK* resistance mutations in 51 ALK-positive patients progressing on crizotinib. Twenty-one patients received crizotinib as part of a clinical trial, with 18 (86%) experiencing an objective response by Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 (24). Among the remaining 30 patients, the median duration of crizotinib treatment was 7.6 months (range, 1.5–21.4 months). Two of these patients (6.7%) experienced disease progression on the first repeat tumor assessment, indicative of potential intrinsic resistance to therapy. Most biopsies (85%) were performed while patients were still receiving crizotinib or within one month of discontinuation. Biopsy sites included pleural fluid (31%), liver (22%), and nodal tissue (18%; Supplementary Table S2).

ALK resistance mutations were identified in 11 (20%) specimens (Fig. 1A) among 10 (20%) patients. Consistent with prior reports (6, 7), the most common *ALK* resistance mutations were L1196M and G1269A, but these were present in only 7% and 4% of all of the crizotinib-resistant specimens, respectively. The remaining *ALK* resistance mutations included: C1156Y (2%), G1202R (2%), I1171T (2%), S1206Y (2%), and E1210K (2%). Four patients underwent two separate biopsies on crizotinib. In three patients, both samples were negative for *ALK* mutations; 1 patient harbored ALK^{L1196M} in two separate pleural fluid specimens obtained

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approximately 1 month apart. No *ALK* resistance mutations were found among the 2 patients with intrinsic resistance.

Thirty-six crizotinib-resistant specimens underwent repeat ALK FISH. All were positive for *ALK* rearrangements. Three specimens (8.3%) demonstrated *ALK* gene amplification (defined as ALK/centromere 2 ratio > 2.0). No *ALK*-amplified tumor harbored a concomitant *ALK* resistance mutation. Thus, among 36 crizotinib-resistant specimens tested for both *ALK* resistance mutations and *ALK* gene amplification, 31% harbored on-target genetic alterations contributing to resistance. As ORRs to second-generation ALK inhibitors are reported to be 48% to 71% following progression on crizotinib (12–16), this low rate of on-target resistance mechanisms is consistent with previous studies demonstrating that crizotinib-resistant tumors without *ALK* point mutations also respond to second-generation ALK inhibitors (12).

ALK Resistance Mutations Are More Common after Treatment with Second-Generation ALK Inhibitors

To evaluate whether the scope of *ALK* resistance mutations changes following treatment with second-generation ALK inhibitors, we assembled cohorts of ALK-positive patients who underwent post-progression biopsies on ceritinib ($N = 23$), alectinib ($N = 17$), or brigatinib ($N = 6$). Baseline clinical characteristics for each cohort are summarized in Supplementary Table S2. Repeat biopsies were performed either on drug or within 1 month of discontinuation in 86%, 94%, and 100% of patients receiving ceritinib, alectinib, and brigatinib, respectively.

Among 23 patients undergoing ceritinib-resistant biopsies, 21 (91%) had received prior crizotinib. Details regarding prior crizotinib exposure and intervening therapies are provided in Supplementary Table S4. In nine cases, pre-ceritinib/post-crizotinib biopsies were also available. Notably, only two (22%) of these crizotinib-resistant specimens harbored on-target mechanisms of resistance. MGH011 was found to have an ALK^{S1206Y} mutation, whereas MGH034 harbored *ALK* fusion gene amplification. Three (13%) patients had previously received both crizotinb and alectinib, but none underwent biopsies following progression on either of those agents. Among the entire cohort of patients with ceritinib-resistant biopsies ($N = 23$), 13 were treated with ceritinib as part of a clinical trial, and 8 (62%) of these patients experienced an objective response (RECIST v1.0). The median duration of ceritinib treatment among the remaining 10 patients was 8 months (range, 1.1–9.2 months), suggesting that the majority of patients had acquired resistance to therapy. Two patients progressed on ceritinib on the first tumor assessment, indicative of intrinsic resistance.

In total, 24 separate post-ceritinib biopsies were performed. Over one-half of ceritinib-resistant specimens (13/24; 54%) harbored *ALK* resistance mutations (Fig. 1B), and 17% contained ≥2 *ALK* resistance mutations. The most common *ALK* mutations were G1202R (21%) and F1174C/L (16.7%), both of which have been previously described in ALK-positive NSCLC (6, 17). G1202R maps to the solvent-exposed region of ALK, where the bulkier, charged side chain is thought to lead to steric hindrance of most ALK inhibitors (6, 17, 25). ALK^{F1174} mutations map adjacent to the C-terminus of the αC helix and may stabilize an active conformation that increases the ATP-binding affinity of ALK (22, 26). In addition to G1202R and F1174C/L, we observed ALK^{C1156Y} mutations in two (8%) specimens. In preclinical models, we previously demonstrated that ceritinib has less activity against ALK^{C1156Y} (17). It should be noted, however, that both C1156Y-containing specimens in this series also harbored additional *ALK* mutations (MGH084-1: C1156Y and I1171N; MGH932-1: C1156Y, V1180L, and G1202del) that may have contributed to resistance.

In this analysis, we also identified an $ALK^{G1202del}$ mutation in two (8%) specimens. To directly evaluate whether $ALK^{G1202del}$ confers resistance to ceritinib, we engineered Ba/F3 cells to express *EML4–ALK* (E13;A20) harboring G1202del and examined ALK phosphorylation after treatment with various ALK inhibitors. We observed that G1202del confers moderate levels of resistance to ceritinib, alectinib, and brigatinib (Supplementary Figs. S1A–S1D and S2A–S2C). By contrast, crizotinib potency was less affected by G1202del. Structural data on the G1202del mutation is not available. Based upon structural modeling studies, however, we speculate that deletion of the glycine at position 1202 could shift the aspartic acid at position 1203 into the 1202 position, leading to disruption of tyrosine kinase inhibitor (TKI) binding (T. Johnson, personal communication). Importantly, although our functional models suggest that $ALK^{G1202del}$ may be a novel resistance mutation, G1202del appears to be quite distinct from ALK^{G1202R}. Indeed, in Ba/F3 models, ALK^{G1202R} results in much higher degrees of resistance to all currently available first- and second-generation ALK inhibitors (Supplementary Fig. S1D).

We next evaluated a cohort of 17 ALK-positive patients who underwent repeat biopsies following progression on alectinib. All 17 patients (100%) had previously received crizotinib; 3 patients (18%) had received both crizotinib and ceritinib. Only 2 patients (MGH087 and MGH090) underwent pre-alectinib/post-crizotinib biopsies, both of which were negative for *ALK* resistance mutations. Details regarding prior ALK inhibitor exposure and intervening therapies are available in Supplementary Table S5 and Supplementary Fig. S3. Within this cohort, the ORR with alectinib was 40% (RECIST v1.1; ref. 27). Among 17 alectinib-resistant biopsies, *ALK* resistance mutations were found in 9 (53%) specimens (Fig. 1C). Moreover, the most common *ALK* resistance mutation was G1202R, which was found in 29% of cases. Interestingly, among 3 patients with RECIST-defined progressive disease on a first repeat tumor assessment (i.e., intrinsic resistance), 2 harbored G1202R. Other *ALK* resistance mutations identified within the complete alectinib-resistant cohort included I1171T/S (12%), V1180L (6%), and L1196M (6%). Of note, several different ALK^{I1171} mutations (e.g., I1171T/N/S) have been reported previously among ALK-positive patients progressing on alectinib (23, 28). ALK^{V1180L} has also been previously shown to confer resistance to alectinib *in vitro* (29), but this represents the first clinical observation of an ALK^{V1180L} mutation in an alectinib-resistant patient. Interestingly, alectinib has demonstrated significant activity against the *ALK* gatekeeper mutation L1196M in preclinical models (18), yet this was observed in one (6%) post-alectinib biopsy. Of note, this patient (MGH988) had previously received crizotinib for 12.1 months, but no post-crizotinib/pre-alectinib biopsy was available. Therefore, it is possible that the L1196M mutation existed prior to treatment with alectinib in this case.

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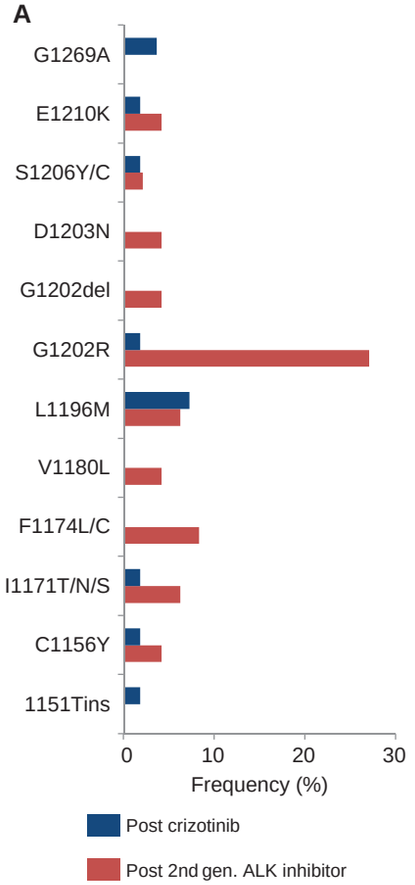
A



B

ALK resistance mutations[a]	Crizotinib (*N* = 55)	Ceritinib (*N* = 24)	Alectinib (*N* = 17)	Brigatinib (*N* = 7)
1151Tins	2%	0%	0%	0%
C1156Y	2%	8%	0%	0%
I1171T/N/S	2%	4%	12%	0%
F1174L/C	0%	17%	0%	0%
V1180L	0%	4%	6%	0%
L1196M	7%	8%	6%	0%
G1202R	2%	21%	29%	43%
G1202del	0%	8%	0%	0%
D1203N	0%	4%	0%	14%
S1206Y/C	2%	0%	0%	14%
E1210K	2%	0%	0%	29%
G1269A	4%	0%	0%	0%
ALK mutations[b]	**20%**	**54%**	**53%**	**71%**

Figure 2. *ALK* resistance mutations are more common after treatment with second-generation ALK inhibitors compared to crizotinib. **A,** comparison of the frequency and distribution of *ALK* resistance mutations in biopsy specimens obtained after disease progression on crizotinib (blue) or second-generation ALK inhibitors (red). Frequencies are expressed based upon the total numbers of biopsies in each cohort. **B,** breakdown of specific *ALK* resistance mutations in ALK-positive patients progressing on crizotinib, ceritinib, alectinib, or brigatinib. [a]For patients with ≥ 2 *ALK* resistance mutations in a biopsy, each individual mutation is incorporated into the frequencies above. [b]Each specimen with ≥ 2 *ALK* resistance mutations is considered only once in determining the total number of specimens with *ALK* resistance mutations.

We next evaluated a third cohort ($N = 6$) of ALK-positive patients who underwent biopsies following progression on brigatinib. Five of 6 patients had previously received crizotinib, and none had received another second-generation ALK inhibitor. Treatment histories for each patient are summarized in Supplementary Table S6. Median duration of treatment was 20.2 months (range, 12.1–44.4 months). No patients had intrinsic resistance to therapy. One patient (MGH086) underwent two separate brigatinib-resistant biopsies at the same anatomic site (see the next section). Overall, *ALK* resistance mutations were seen in 5 of 7 (71%) brigatinib-resistant specimens. Like patients progressing on ceritinib and alectinib, the most common *ALK* resistance mutation was G1202R, which was observed in three specimens.

Given the retrospective nature of this analysis, there are limited clinical data on the therapies patients received after disease progression on ceritinib, alectinib, and/or brigatinib. Immediate postprogression therapies are summarized in Supplementary Tables S4 to S6. Patients generally received systemic chemotherapy (25%) or were enrolled onto clinical trials (31%). Three patients (6%) received commercially available ALK inhibitors, whereas the remaining 38% of patients did not receive subsequent therapy or were lost to follow-up. Thus, we were unable to draw meaningful associations between our findings in postprogression biopsies and clinical outcomes to subsequent therapies.

Collectively, across all three biopsy cohorts ($N = 48$), *ALK* resistance mutations were present in 56% of ALK-positive patients progressing on second-generation ALK inhibitors (ceritinib, 54%; alectinib, 53%; and brigatinib, 71%). Thus, although *ALK* resistance mutations were observed in only 20% of ALK-positive patients progressing on crizotinib, treatment with more potent second-generation ALK inhibitors was associated with a significantly higher frequency of *ALK* resistance mutations ($P = 0.0002$) and a different spectrum of such mutations (Fig. 2A). Indeed, consistent with preclinical data, ALK^{G1202R} emerged as the most common *ALK* resistance mutation among patients receiving second-generation ALK inhibitors (Fig. 2A and B).

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Compound *ALK* Resistance Mutations Following Sequential ALK Inhibitor Therapy

Based upon experience from other targeted therapy settings [e.g., chronic myeloid leukemia (CML) and *EGFR*-mutant lung cancer; refs. 30–32], we hypothesized that sequential ALK inhibitor therapy may predispose patients to develop compound mutations. Indeed, we recently described the development of a compound *ALK* resistance mutation in a patient treated with crizotinib, ceritinib, and lorlatinib (33). To investigate the frequency of such dual alterations, we examined biopsies from patients progressing on second-generation ALK inhibitors, identifying 6 of 48 (12.5%) specimens harboring ≥2 *ALK* resistance mutations (Supplementary Table S7). In each case, patients had received crizotinib and a second-generation ALK inhibitor. In three specimens (MGH905-1, MGH086-0, and MGH086-1), *ALK* resistance mutations were in close enough proximity to confirm that they were present on the same allele or whole-exome sequencing (WES) demonstrated similar cancer cell fractions of each resistance mutation, suggesting that they were present on the same allele of the *ALK* fusion gene. The remaining cases did not have sufficient tissue available to make this determination.

In one of the patients above (MGH086), we were also able to investigate clonal evolution of compound *ALK* resistance mutations over time within the same anatomic site. The clinical course for MGH086 is summarized in Fig. 3A. Following identification of an *ALK* rearrangement, patient MGH086 was sequentially treated with crizotinib and brigatinib. Despite prolonged clinical benefit on each agent, he ultimately developed recurrent left axillary adenopathy requiring local excision/biopsy on three separate occasions (post-crizotinib $N = 1$, post-brigatinib $N = 2$; Fig. 3A and B). WES of the post-crizotinib specimen (MGH086-00) demonstrated an acquired ALK^{E1210K} mutation. E1210 maps to the ribose-binding pocket of ALK (26). ALK^{E1210K} confers resistance to crizotinib based

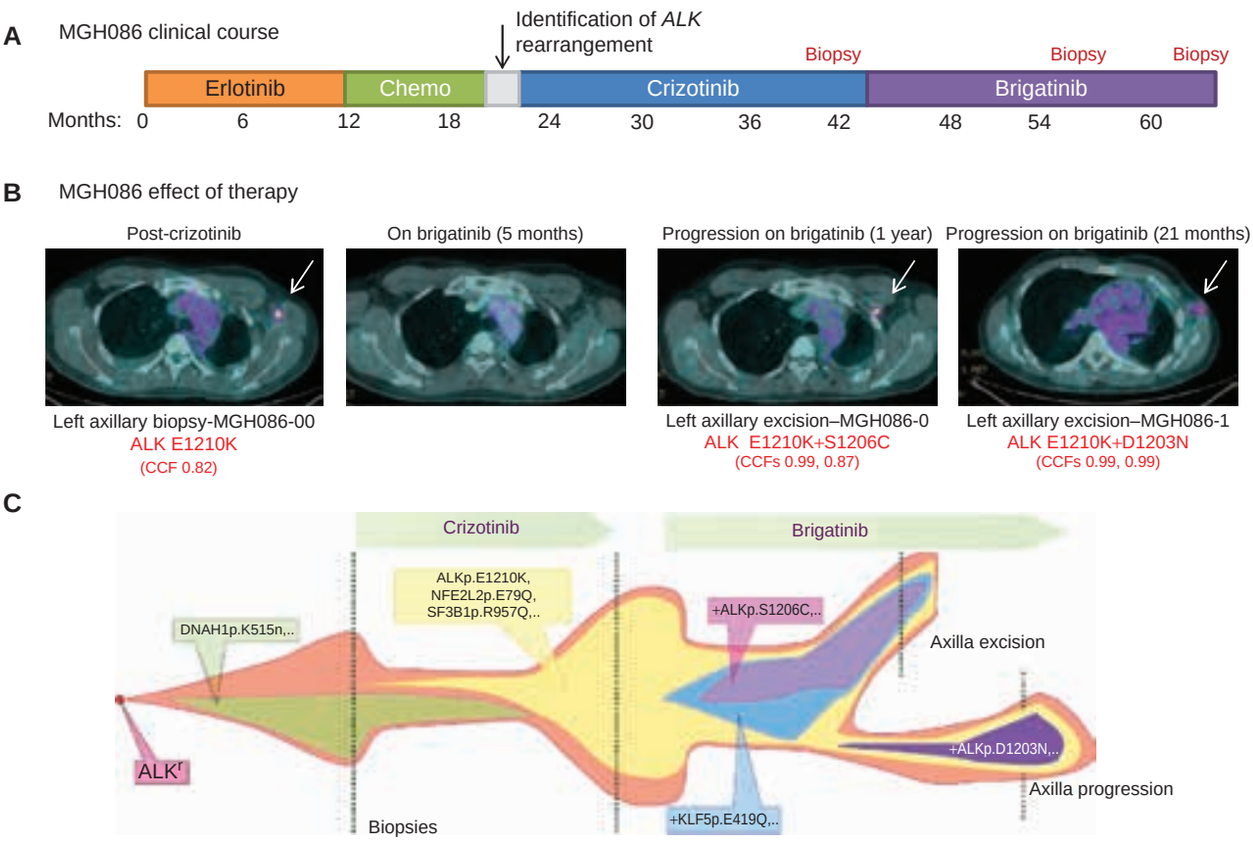

Figure 3. Clonal evolution of resistance to sequential ALK inhibitor therapy. **A,** the treatment course of patient MGH086. Of note, the patient received several lines of therapy, including the EGFR inhibitor erlotinib, before identification of an *ALK* rearrangement. The points at which the patient underwent biopsies are indicated in red. **B,** fused positron emission tomography (PET)–computed tomography (CT) images demonstrate a hypermetabolic, left axillary lymph node (white arrow) that developed at the time of disease progression on crizotinib. WES revealed an ALK^{E1210K} mutation [cancer cell fraction (CCF) 0.82]. This axillary lymph node initially responded to brigatinib but recurred after 12 months. WES of this brigatinib-resistant lesion (MGH086-0) demonstrated continued presence of the ALK^{E1210K} mutation and a new ALK^{S1206C} mutation. The patient remained on brigatinib. After an additional 9 months on brigatinib, he developed another recurrence in the left axilla (slightly more inferior than the prior lesion). Repeat WES (MGH086-1) revealed a new compound $ALK^{E1210K+D1203N}$ mutation. **C,** a model of clonal evolution of resistance to sequential ALK inhibitor therapy in patient MGH086. Using WES, we determined that a founder ALK^{E1210K} subclone was not present in a pre-crizotinib biopsy but later developed on crizotinib. When the patient was switched to brigatinib, the ALK^{E1210K} subclone expanded and ultimately acquired a new *ALK* mutation, S1206C. Surgical excision of this site of progression may have depleted the compound mutant ($ALK^{E1210K+S1206C}$), but microscopic parental E1210K clones may have persisted, ultimately acquiring ALK^{D1203N} in combination with ALK^{E1210K}.

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upon *in vitro* mutagenesis screens, but this alteration has not been described in clinical NSCLC samples previously (26). We next performed WES on the first brigatinib-resistant excision specimen (MGH086-0), which revealed an *ALK*^{E1210K} mutation and a new *ALK*^{S1206C} mutation (Fig. 3B). Notably, WES of the second brigatinib-resistant excision specimen (MGH086-1) also demonstrated the *ALK*^{E1210K} mutation, but *ALK*^{S1206C} was no longer observed. Instead, a new *ALK*^{D1203N} mutation was found. Clonal analysis suggested that the E1210K mutation emerged as an early resistant clone after treatment with crizotinib. Subsequent treatment with brigatinib enriched for this clone, which eventually acquired a compound *ALK*^{S1206C} mutation. It is possible that surgical excision of the patient's left axillary node may have physically removed the E1210K+S1206C clone, but parental E1210K clones may have still been present microscopically. Ultimately, these clones may have acquired an *ALK*^{D1203N} mutation with continued brigatinib exposure (Fig. 3C).

These data demonstrate that compound *ALK* resistance mutations can arise in ALK-positive patients treated with sequential ALK inhibitors. Moreover, as we discuss in greater detail below, such compound mutations can confer high levels of resistance to ALK inhibitors.

Broad Assessment of Genetic Mutations in Resistant Cancers

ALK resistance mutations appear to be the predominant mechanism of resistance to second-generation ALK inhibitors. Nonetheless, we observed that 44% of post–second-generation ALK TKI biopsies were negative for *ALK* mutations. To investigate the potential role of alternative mechanisms of resistance, such as upregulation of bypass signaling pathways, we first performed targeted next-generation sequencing (NGS) on post-ceritinib, post-alectinib, and post-brigatinib biopsy specimens using the Massachusetts General Hospital (MGH) Snapshot NGS platform or FoundationOne NGS (Supplementary Tables S8–S10; Fig. 4).

Twenty-seven specimens had sufficient tissue for analysis. Beyond *ALK* resistance mutations, 15 (56%) specimens showed genetic alterations in at least one other gene. *TP53* mutations were the most common, present in 9 (33%) specimens. Of note, we were unable to determine whether these mutations were present prior to treatment with second-generation ALK inhibitors due to a lack of baseline tissue for analysis. No *KRAS* or *EGFR* mutations were identified. Missense mutations in *DDR2* (L610F), *BRAF* (G15V), *FGFR2* (F645L), *MET* (T992I), *NRAS* (A155T), and *PIK3CA* (G106V) were each identified in one (3.7%) specimen, none of which were overlapping. Mutations in *DDR2, BRAF, NRAS,* and *FGFR2* did not occur in known hotspot residues, nor have they been observed in NSCLCs in the Catalogue of Somatic Mutations in Cancer database (COSMICv76); thus, the impact of these alterations on ALK inhibitor resistance is uncertain. *MET*^{T992I} was observed in one specimen in this series (MGH040-2). *MET*^{T992I} has been reported at low frequencies in various malignancies, but functional studies have demonstrated that this variant lacks transformative capacity and does not affect MET phosphorylation status (34). Thus, *MET*^{T992I} was unlikely to be a major driver of resistance in this patient—particularly because this specimen harbored a concomitant *ALK*^{G1202R} mutation. By contrast, the alectinib-resistant specimen MGH074-2 showed no *ALK* resistance mutations but harbored a *PIK3CA*^{G106V} mutation. Previous studies have shown that *PIK3CA*^{G106V} is a gain-of-function mutation that localizes to the p85/adaptor-binding domain of p110α and results in increased AKT phosphorylation (35). In addition, *PIK3CA* mutations have been associated with acquired resistance to EGFR inhibitors in EGFR-mutant NSCLC (36). Due to tissue availability, we were unable to assess this patient's pre-alectinib specimen for the presence of *PI3KCA*^{G106V}; thus, it remains unclear when this alteration arose. Among the remaining 26 cases that underwent NGS, no additional *PIK3CA* mutations were identified; however, we previously reported a *PIK3CA*^{H1047R} mutation in a ceritinib-resistant specimen (MGH034-2) that was identified by the Snapshot allele-specific assay (10, 37).

To identify other potential off-target mechanisms of resistance to second-generation ALK inhibitors, we established six ceritinib-resistant, patient-derived cell lines. All samples underwent targeted NGS of 1,000 known cancer genes (Supplementary Table S11; Fig. 4; ref. 10). Samples without *ALK* resistance mutations (MGH034-2A, MGH049-1A, and MGH075-2E) were also evaluated using a combination drug screen to identify potential novel mechanisms of resistance (10). As we previously reported (10), MGH034-2A harbored an acquired *MAP2K1*^{K57N} mutation. Furthermore, treatment with a MEK inhibitor (AZD6244) resensitized these cells to ceritinib, suggesting that reactivation of the MAPK pathway promoted resistance in this specimen. In addition, for MGH049-1A, we previously observed that agents targeting SRC, EGFR, and PI3K resensitized cells to ALK inhibition, implicating these signaling pathways as mediators of resistance in this model (10). Notably, no genetic alterations in these pathways were identified in our 1,000-gene NGS analysis. Finally, we performed the same pharmacologic screen on MGH075-2E, identifying dasatinib as the only hit (Supplementary Fig. S4A and S4B). In addition to ABL and EPH receptors, dasatinib potently inhibits SRC family kinases, which have been associated with resistance to ALK inhibitors previously (10). Of note, we could not identify a genetic basis for SRC activation using a 1,000-gene NGS panel. Prior biochemical experiments using cell line models sensitive to combined ALK and SRC inhibition have suggested potential cross-talk between ALK and SRC, with ALK inhibition leading to upregulation of SRC signaling (10).

Collectively, these observations reinforce our finding that *ALK* resistance mutations are the predominant mechanisms of resistance to second-generation ALK inhibitors. Although several potential bypass signaling tracts were identified in individual patients, no high-frequency, recurring genetic alterations beyond *TP53* mutations were observed.

Epithelial–Mesenchymal Transition Is Associated with ALK Inhibitor Resistance

In preclinical models, ceritinib, alectinib, and brigatinib have demonstrated activity against the *ALK*^{L1196M} gatekeeper mutation (17, 18, 38). However, we identified three ALK-positive patients with *ALK*^{L1196M} following treatment with second-generation ALK inhibitors (ceritinib, $N = 2$; alectinib, $N = 1$), suggesting that additional resistance mechanisms may be responsible. In one of these specimens (MGH067-1),

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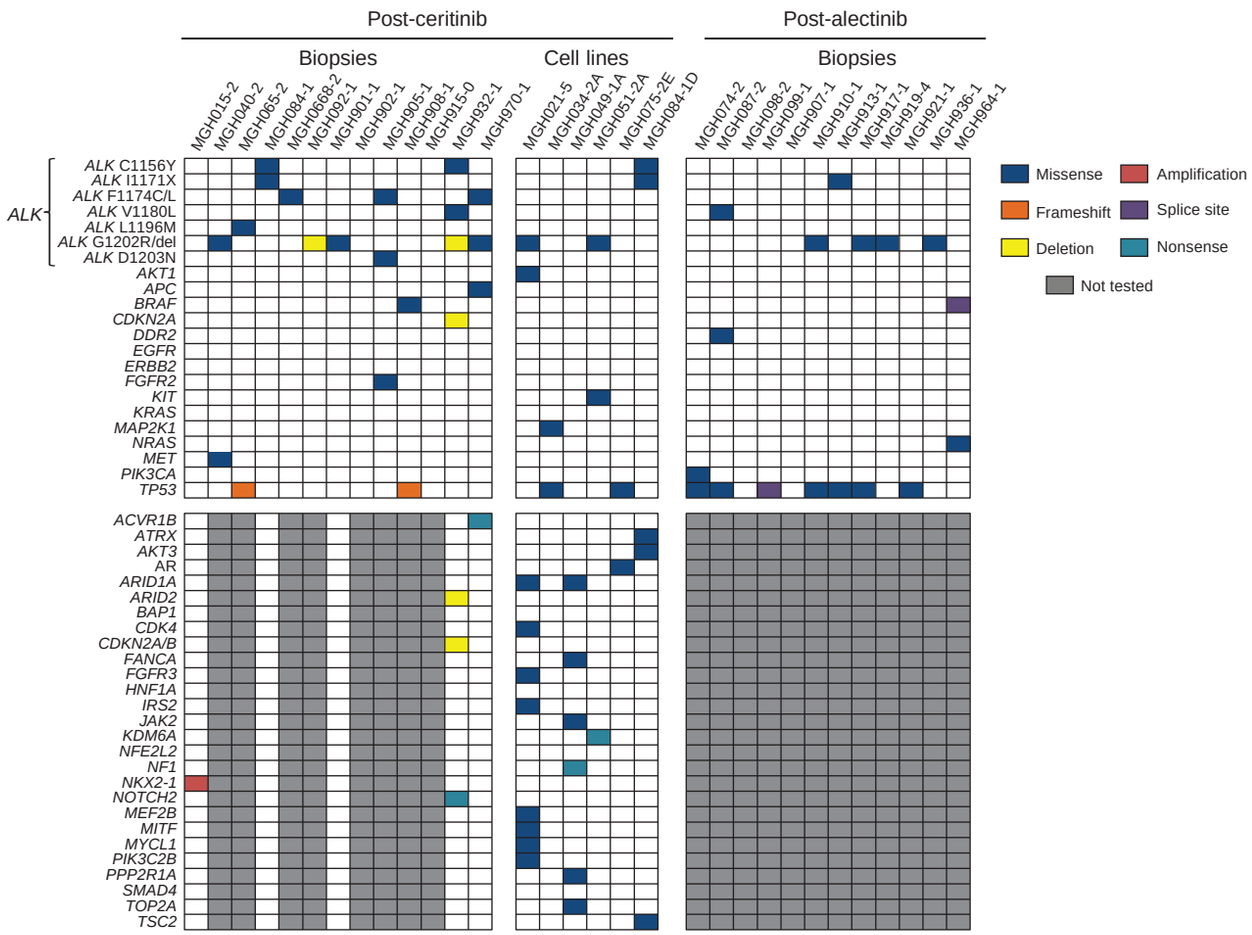

Figure 4. Summary of genetic alterations in resistant biopsies among patients progressing on ceritinib or alectinib. Specimens underwent targeted NGS using the MGH NGS assay or the FoundationOne platform. Only genes with at least one genetic alteration detected in resistant specimens are depicted. If a particular gene was not evaluated in a given specimen due to the type of sequencing platform used, it is represented in dark gray. Within this cohort, the most commonly mutated genes were *ALK* and *TP53*. Recurrent alterations in other genes were uncommon. In addition, six ceritinib-resistant, patient-derived cell lines underwent NGS using a 1,000-gene panel (see Genotype Assessments in Methods). Genes from this panel are shown if the following criteria are met: (i) a genetic alteration was present in at least one of the patient-derived cell lines, and (ii) the gene was also included in either the MGH NGS or FoundationOne panels. Please see Supplementary Table S11 for a comprehensive assessment of all genetic alterations identified within these cell lines.

obtained from an ALK-positive patient treated with crizotinib, alectinib, and ceritinib (Fig. 5A), the post-ceritinib biopsy revealed a malignant spindle-cell neoplasm with no morphologic or immunohistochemical markers of epithelial differentiation (Fig. 5B). In contrast to the patient's initial diagnostic specimen, the post-ceritinib biopsy showed diffuse vimentin expression and loss of E-cadherin staining, consistent with epithelial-to-mesenchymal transition (EMT). Of note, ALK FISH was positive for a rearrangement (Supplementary Fig. S5A–S5C), but immunohistochemical staining for ALK showed negative to weak staining (Supplementary Fig. S5D–S5F). Post-crizotinib and/or post-alectinib biopsies were not performed in this patient; thus, we could not determine when the *ALK*^{L1196M} mutation was acquired. However, we suspect that it was acquired before ceritinib. Indeed, it is possible that EMT, rather than the *ALK*^{L1196M} mutation, may have contributed more significantly to ceritinib resistance in this patient.

Based upon the above observation, we performed immunohistochemical staining on 11 other ceritinib-resistant biopsy specimens with sufficient tissue for analysis to investigate the frequency of EMT in ALK-positive patients progressing on second-generation ALK inhibitors. EMT is generally associated with loss of E-cadherin staining and gain of vimentin expression (36). In total, we observed transition to a more mesenchymal phenotype in 5 of 12 (42%) specimens (Fig. 5C). Interestingly, among these five specimens, three harbored *ALK* resistance mutations, including the two cases with *ALK*^{L1196M}. Notably, post-crizotinib/pre-ceritinib biopsies were not available in these cases but baseline diagnostic specimens were available for comparison in all five cases (Supplementary Table S12). Four of these were negative for EMT. In one patient (MGH065), the baseline biopsy showed strong vimentin expression, but preserved E-cadherin staining, which was lost in the ceritinib-resistant cancer. Of note, histologic transformation to small cell lung cancer (SCLC) was not observed in these

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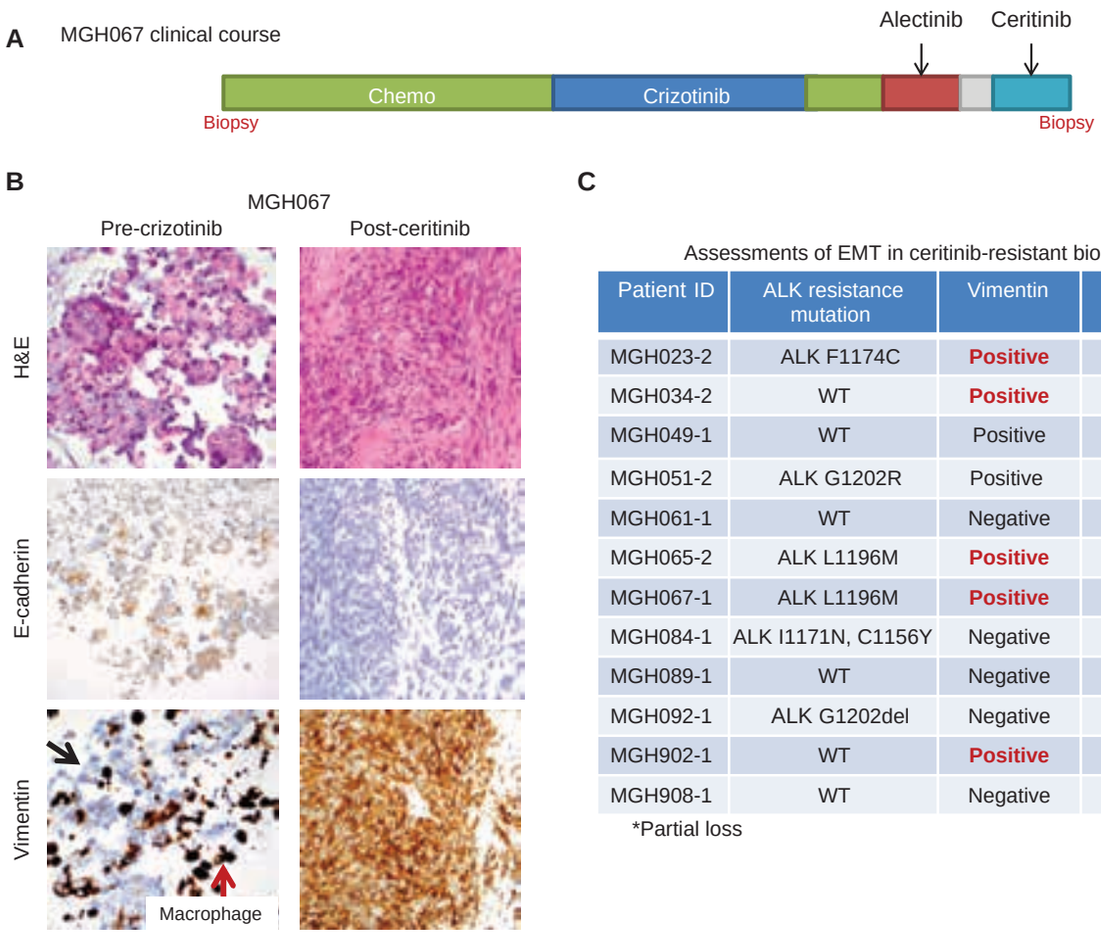

Assessments of EMT in ceritinib-resistant biopsies

Patient ID	ALK resistance mutation	Vimentin	E-cadherin
MGH023-2	ALK F1174C	**Positive**	**Negative**
MGH034-2	WT	**Positive**	**Negative**
MGH049-1	WT	Positive	Positive
MGH051-2	ALK G1202R	Positive	Positive
MGH061-1	WT	Negative	Positive
MGH065-2	ALK L1196M	**Positive**	**Negative**
MGH067-1	ALK L1196M	**Positive**	**Negative**
MGH084-1	ALK I1171N, C1156Y	Negative	Positive
MGH089-1	WT	Negative	Positive
MGH092-1	ALK G1202del	Negative	Positive
MGH902-1	WT	**Positive**	**Negative***
MGH908-1	WT	Negative	Positive

*Partial loss

Figure 5. EMT is associated with ceritinib resistance. **A,** clinical course of patient MGH067. **B,** pre-crizotinib and post-ceritinib biopsies (lung and subcutaneous lesions, respectively) from MGH067 underwent hematoxylin and eosin (H&E) staining, and immunostaining for E-cadherin and vimentin. The post-ceritinib biopsy shows a loss of E-cadherin staining and gain of vimentin expression, consistent with EMT. Black arrow indicates a lack of vimentin staining of tumor cells in the pre-crizotinib biopsy. Red arrow depicts vimentin staining of alveolar macrophages in the same specimen. **C,** 12 ceritinib-resistant biopsy specimens underwent E-cadherin and vimentin staining to assess for EMT. In total, five specimens demonstrated immunohistochemical features consistent with EMT. WT, wild-type.

cases. Together, these findings suggest that EMT was acquired during ALK inhibitor therapy and may therefore play a role in resistance to second-generation ALK inhibitors. However, EMT may not be the sole driver of resistance in these patients, because several specimens with EMT had concomitant alterations that may also contribute to resistance.

Lorlatinib Is Active against *ALK* Resistance Mutations That Develop on Second-Generation ALK Inhibitors

To investigate whether cancers that develop resistance to second-generation ALK inhibitors remain susceptible to continued ALK inhibition, we examined the activity of the third-generation ALK inhibitor lorlatinib (PF-06463922). Lorlatinib is a potent, highly selective ALK/ROS1 inhibitor that is currently being evaluated in an ongoing phase II clinical trial (NCT01970865). We first engineered Ba/F3 cells to express wild-type *EML4–ALK* (E13;A20) or *EML4–ALK* harboring various *ALK* mutations (Fig. 6). Ba/F3 cells were treated with crizotinib, ceritinib, alectinib, brigatinib, and lorlatinib. Notably, lorlatinib was the only ALK inhibitor to potently inhibit ALK phosphorylation across all single *ALK* secondary mutations, including ALK^{G1202R} (IC_{50} 49.9 nmol/L). Next, based upon our observations of compound *ALK* resistance mutations in a subset of patients, we also evaluated the activity of various ALK inhibitors against compound *ALK* resistance mutations. We found that crizotinib, ceritinib, alectinib, and brigatinib were all inactive against D1203N+E1210K and F1174C+D1203N. Conversely, lorlatinib retained significant potency against the *ALK* double-mutant D1203N+E1210K and intermediate potency against D1203N+F1174C.

To further examine the activity of lorlatinib in ALK-resistant models, we next evaluated a series of ceritinib-resistant, patient-derived cell lines ($N = 6$). Three cell lines harbored *ALK* resistance mutations (MGH021-5A, MGH051-2C, and MGH084-1D), whereas three cell lines (MGH034-2A,

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Cellular ALK phosphorylation mean IC_{50} (nmol/L)

Mutation status	Crizotinib	Ceritinib	Alectinib	Brigatinib	Lorlatinib
Parental Ba/F3	763.9	885.7	890.1	2774.0	11293.8
EML4–ALK V1	38.6	4.9	11.4	10.7	2.3
EML4–ALK C1156Y	61.9	5.3	11.6	4.5	4.6
EML4–ALK I1171N	130.1	8.2	397.7	26.1	49.0
EML4–ALK I1171S	94.1	3.8	177.0	17.8	30.4
EML4–ALK I1171T	51.4	1.7	33.6[a]	6.1	11.5
EML4–ALK F1174C	115.0	38.0[a]	27.0	18.0	8.0
EML4–ALK L1196M	339.0	9.3	117.6	26.5	34.0
EML4–ALK L1198F	0.4	196.2	42.3	13.9	14.8
EML4–ALK G1202R	381.6	124.4	706.6	129.5	49.9
EML4–ALK G1202del	58.4	50.1	58.8	95.8	5.2
EML4–ALK D1203N	116.3	35.3	27.9	34.6	11.1
EML4–ALK E1210K	42.8	5.8	31.6	24.0	1.7
EML4–ALK G1269A	117.0	0.4	25.0	ND	10.0
EML4–ALK D1203N+F1174C	338.8	237.8	75.1	123.4	69.8
EML4–ALK D1203N+E1210K	153.0	97.8	82.8	136.0	26.6

$IC_{50} \leq 50$ nmol/L

$IC_{50} > 50 < 200$ nmol/L

$IC_{50} \geq 200$ nmol/L

Figure 6. Lorlatinib potently inhibits *ALK* resistance mutations, including ALK^{G1202R}. Absolute IC_{50} values of crizotinib, ceritinib, alectinib, brigatinib, and lorlatinib on cellular ALK phosphorylation in Ba/F3 cells harboring wild-type *EML4–ALK* variant 1 or various *EML4–ALK* resistance mutants are depicted. [a]In Ba/F3 cells, ALK^{F1174C} and ALK^{I1171T} appear sensitive to ceritinib and alectinib, respectively; however, these mutations may not be susceptible to these agents *in vivo* based upon prior clinical reports. ND, not done.

MGH049-1A, and MGH075-2E) were wild-type for *ALK* secondary mutations. Notably, alectinib and ceritinib had minimal effects on cell growth across all patient-derived cell lines (GI_{50} values 131 nmol/L to >10,000 nmol/L and 115 nmol/L to >10,000 nmol/L, respectively; Fig. 7; Supplementary Fig. S6A and S6B). By contrast, lorlatinib markedly inhibited cell growth in 3 of 6 patient-derived cell lines (Fig. 7A and B; Supplementary Fig. S6A). Interestingly, this activity was restricted to cell lines with *ALK* resistance mutations (MGH021-5A, MGH051-2C, MGH084-1D; Supplementary Fig. S7A–S7D). The remaining three cell lines that lacked *ALK* resistance mutations (MGH034-2A, MGH049-1A, and MGH075-2E) were insensitive to lorlatinib (GI_{50} values >10,000 nmol/L; Fig. 7C and D; Supplementary Figs. S8A–S8D and S9A–S9C). These data suggest that in the setting of acquired resistance to second-generation ALK inhibitors, the presence of *ALK* resistance mutations indicates continued dependency on ALK signaling and susceptibility to the pan-inhibitory ALK inhibitor lorlatinib (Fig. 7E and F). In contrast, the absence of *ALK* resistance mutations after failure of second-generation ALK inhibitors may indicate loss of ALK dependency and resistance to lorlatinib.

DISCUSSION

In this study, we present the largest systematic analysis of ALK inhibitor resistance to date and the first study

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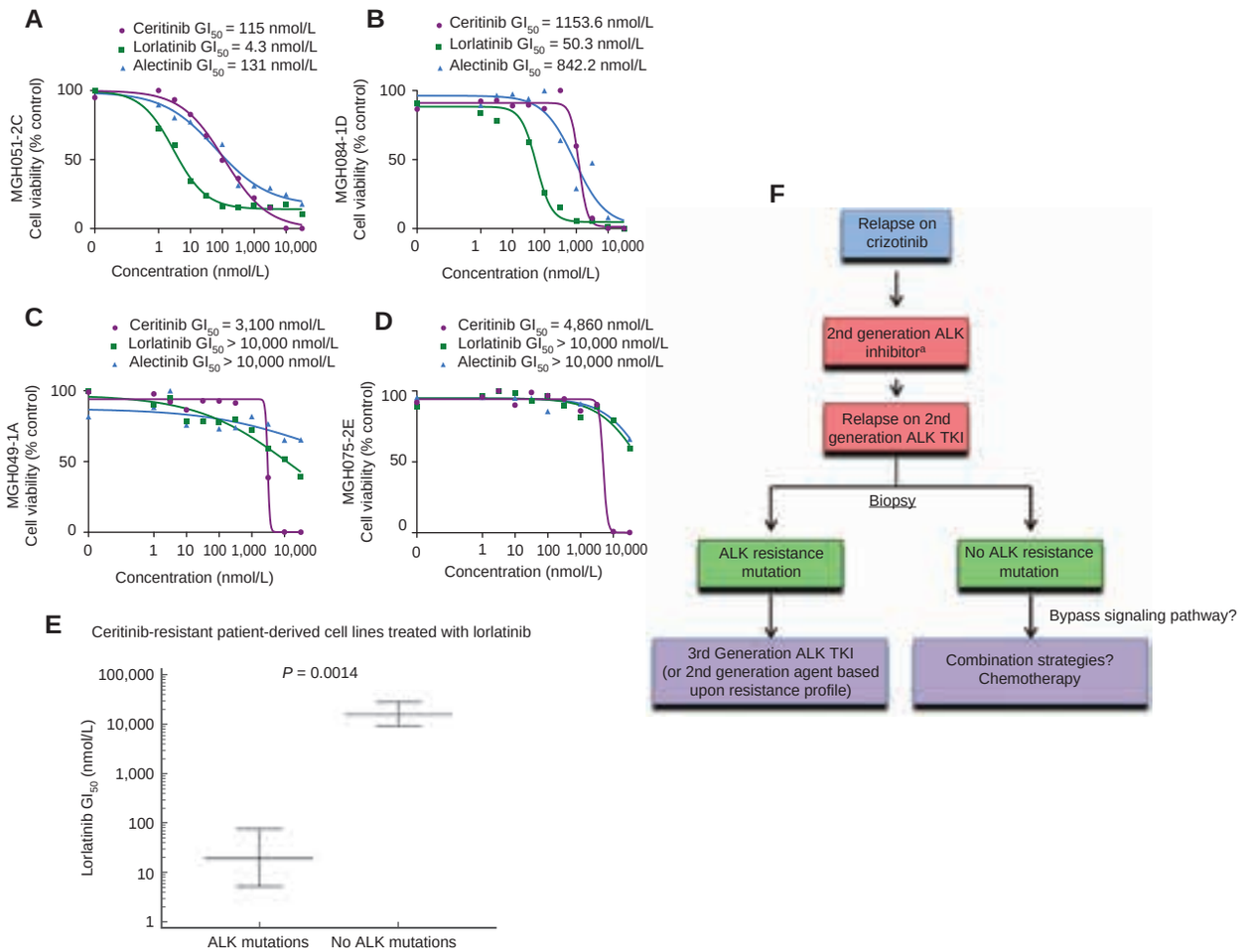

Figure 7. *ALK* resistance mutations predict for sensitivity to lorlatinib in patient-derived cell line models of acquired resistance to ceritinib. **A** and **B,** cell viability assays of two representative ceritinib-resistant, patient-derived cell lines harboring ALK resistance mutations (MGH051-2C [*EML4-ALK*^{G1202R}] and MGH084-1D [*EML4-ALK*^{I1171N,C1156Y}]) treated with ceritinib, alectinib, and lorlatinib. The number of cells seeded and the duration of treatment were adjusted for each cell line in order to have a consistent proliferation index (3.5–5) at the end of treatment. Values are presented as means ($N = 3$). **C** and **D,** cell viability assays of two representative, ceritinib-resistant, patient-derived cell lines without ALK resistance mutations (MGH049-1A [*EML4–ALK*^{WT}] and MGH075-2E [*EML4–ALK*^{WT}]) treated with ceritinib, alectinib, and lorlatinib. The number of cells seeded and the duration of treatment were adjusted for each cell line in order to have a consistent proliferation index (3.5–5) at the end of treatment. Values are presented as means ($N = 3$). **E,** comparison of cell viabilities of ceritinib-resistant, patient-derived cell lines treated with lorlatinib based upon *ALK* resistance mutation status. **F,** proposed schema for the clinical approach to ALK-positive patients with acquired resistance. This paradigm incorporates repeat biopsies and decision-making based upon *ALK* resistance mutation status following disease progression on second-generation ALK inhibitors. [a]Choice of second-generation ALK inhibitors may be affected by identification of specific *ALK* resistance mutations, such as G1202R and I1171N/S/T, which can be rarely seen after crizotinib.

to evaluate mechanisms of resistance across a spectrum of first- and second-generation ALK inhibitors. Consistent with earlier reports (6, 7), we found that only a minority of ALK-positive patients (~20%) developed *ALK* resistance mutations on crizotinib. By contrast, *ALK* resistance mutations were present in over one-half of patients progressing on second-generation ALK inhibitors, likely reflecting the greater potency and selectivity of these agents compared to crizotinib. In parallel, we observed that the spectrum of *ALK* resistance mutations was different following progression on second-generation ALK inhibitors compared to crizotinib. Most notably, *ALK*^{G1202R}, which was present in only 2% of crizotinib-resistant biopsies, emerged as the most common *ALK* resistance mutation after treatment with second-generation ALK inhibitors. These findings are consistent with data from Ba/F3 models, which demonstrate that *ALK*^{G1202R} confers high levels of resistance to all currently available second-generation ALK inhibitors.

Although *ALK*^{G1202R} was a common shared resistance mutation in each second-generation ALK inhibitor cohort, it is noteworthy that the spectrum of other *ALK* resistance mutations appeared to differ across agents. For example, *ALK*^{F1174} mutations were observed in several post-ceritinib biopsy specimens (4/24; 16.7%), but were otherwise absent from post-alectinib and post-brigatinib biopsies. Although this comparison is limited due to the relatively small sample

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size, such observations may reflect the structural differences between ALK inhibitors. Indeed, prior reports suggest that *ALK*^{F1174} mutations confer resistance to ceritinib but remain sensitive to alectinib (17, 23). Conversely, several investigators have described *ALK*^{I1171} mutations that mediate resistance to alectinib, while conferring sensitivity to ceritinib (23, 29, 39). Consistent with these reports, we observed *ALK*^{I1171} mutations to be the second-most-common *ALK* resistance mutations in post-alectinib specimens (N = 2; 12%). Of note, one (4.1%) post-ceritinib sample also contained an *ALK*^{I1171N} mutation (in combination with *ALK*^{C1156Y}), but this patient had previously received crizotinib, alectinib, and ceritinib. Additional biopsies between therapies were unavailable; thus, it is unclear whether the *ALK*^{I1171N} mutation was acquired as a result of ceritinib or alectinib. Nonetheless, our findings that different *ALK* resistance mutations impart differential sensitivities to second-generation ALK inhibitors may have important clinical implications. Specifically, this provides support for a new paradigm in which particular *ALK* resistance mutations inform the choice of subsequent ALK-targeted therapies, especially after failure of two ALK inhibitors. Tailoring of ALK therapy after failure of crizotinib may also be important in the small proportion of cases with uncommon and refractory mutations like *ALK*^{G1202R}.

From a therapeutic standpoint, overcoming resistance to ALK inhibitors may be further complicated by the emergence of compound *ALK* resistance mutations. In this study, we identified six specimens with ≥2 *ALK* resistance mutations. In all cases, patients had received multiple ALK inhibitors, suggesting that sequential use of ALK inhibitors may facilitate the development of compound *ALK* mutations. Furthermore, in Ba/F3 models, we demonstrated that compound *ALK* mutations conferred increased drug resistance. However, one important exception to this observation has emerged. We recently reported the case of an ALK-positive patient treated with crizotinib, ceritinib, and lorlatinib, who ultimately acquired a dual *ALK*$^{L1198F+C1156Y}$ mutation at the time of disease progression (33). Interestingly, *ALK*^{L1198F} paradoxically resensitized cells to crizotinib, again underscoring the importance of serial biopsies in ALK-positive NSCLC. Of note, the emergence of compound resistance mutations in ALK-positive NSCLC is analogous to the experience with other targeted therapies. For example, in CML, sequential use of different ABL inhibitors has been shown to select for more drug-resistant, compound *BCR–ABL* mutations (30, 40). Similarly, in EGFR-mutant NSCLC, compound drug-resistant T790M/C797S mutations have been described following sequential treatment with first- and third-generation EGFR inhibitors (31, 33, 41). Ultimately, new therapeutic strategies, such as up-front TKI combinations, may be needed to suppress the emergence of on-target resistance mechanisms, particularly compound resistance mutations.

We also investigated potential off-target resistance mechanisms using a combination of NGS, histologic analyses, and functional drug screens. We previously identified upregulation of bypass signaling tracts involving SRC and the MAPK pathway (10). We also observed *PIK3CA* mutations in two patients. *PIK3CA* mutations have been implicated in resistance to other targeted therapies, such as EGFR inhibitors (36). Nonetheless, outside of *TP53* mutations, recurring genetic alterations were uncommon in this series. We did however observe evidence of EMT in some patients. EMT is believed to enhance cell motility and invasiveness (42). Preclinical studies have also suggested that EMT is associated with resistance to crizotinib and ceritinib (43–45), but clinical specimens were not examined in these reports. Of note, other phenotypic changes, such as transformation to SCLC, have been shown to mediate resistance in EGFR-mutant lung cancer and rarely in ALK-positive NSCLC (36, 46–48). However, we did not observe transformation to SCLC in any specimens in this series.

Beyond characterizing mechanisms of resistance to second-generation ALK inhibitors, this study also aimed to investigate the therapeutic impact of these findings. In particular, we evaluated the preclinical activity of the third-generation ALK inhibitor lorlatinib. Using a combination of Ba/F3 models and patient-derived cell lines, we demonstrated that lorlatinib was active against all single *ALK* resistance mutations. Moreover, lorlatinib was the only ALK inhibitor that retained significant activity against *ALK*^{G1202R}. Such findings are consistent with preliminary phase I data in which lorlatinib has demonstrated an ORR of 44% among ALK-positive patients treated with two or more ALK inhibitors, with responses noted in ALK-positive patients harboring *ALK*^{G1202R} mutations (49).

More broadly, our data suggest that therapeutic approaches to crizotinib resistance and resistance to second-generation ALK inhibitors will differ. In particular, we anticipate that repeat biopsies to identify *ALK* resistance mutations will play a larger role in guiding therapy decisions after progression on second-generation ALK TKIs compared to crizotinib. With respect to the latter, the absence of *ALK* resistance mutations has not been shown to affect ORRs or PFS to a second-generation ALK inhibitor among crizotinib-resistant patients (12, 50). Thus, such patients generally remain ALK-dependent, even in the absence of *ALK* resistance mutations—likely reflecting the relatively low potency of crizotinib against ALK. By contrast, data from our patient-derived cell line models suggest that *ALK* resistance mutation status after disease progression on second-generation ALK inhibitors is likely to be critically important in predicting sensitivity to the third-generation ALK inhibitor lorlatinib. Indeed, lorlatinib was active only in ceritinib-resistant, patient-derived cell lines harboring *ALK* resistance mutations in this study. Altogether, our findings support a new therapeutic paradigm in which clinicians tailor ALK inhibitor therapy based upon resistance mechanisms following disease progression on second-generation ALK inhibitors (Fig. 7F).

This study has several important limitations. First, resistance to targeted therapies may be heterogeneous (10, 51), and a single biopsy may not adequately capture the full scope of resistance in a given patient. However, in general, it is not feasible to obtain biopsies of multiple sites in patients with NSCLC. In the future, circulating tumor DNA (ctDNA) platforms may enable us to evaluate tumor heterogeneity, while also allowing clinicians to readily monitor the evolution of resistance over time. A second

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limitation is that the sample sizes of several resistant-biopsy cohorts were relatively small, and patients did not always have adequate tissue for comprehensive molecular analysis or generation of patient-derived cell lines. As a result, we prioritized tissue for assessments of on-target mechanisms of resistance. Nonetheless, it is possible that our targeted *ALK* sequencing panels did not adequately capture low-frequency variants or previously undescribed *ALK* resistance mutations. Another limitation of this analysis is that pre-crizotinib and/or pre–second-generation ALK inhibitor biopsies were generally not available; thus, we were unable to determine whether certain genetic alterations existed prior to the development of resistance to second-generation ALK inhibitors.

In summary, we demonstrate that the frequency and spectrum of *ALK* resistance mutations differs depending on the ALK inhibitor. Moreover, resistance profiles may evolve over time and in response to sequential ALK inhibitors. Moving forward, it will be important to incorporate repeat biopsies into clinical trials of next-generation ALK inhibitors both before treatment and at progression. As biopsies are not always feasible, and resistance may be heterogeneous, noninvasive techniques, such as ctDNA, will be crucial to develop and validate in parallel. Together, such efforts may facilitate discovery of novel mechanisms of resistance and new insights into the impact of heterogeneity on treatment response. On a practical level, this work will also allow clinicians to personalize ALK-targeted strategies based upon the presence or absence of specific *ALK* resistance mutations, which may ultimately translate into improved patient outcomes.

METHODS

Patients and Treatment

Patients with ALK-positive NSCLC underwent repeat biopsies of resistant tumors between January 2009 and June 2016. Tumor histology was classified according to World Health Organization criteria. Electronic medical records were retrospectively reviewed to obtain clinical data and treatment histories. All patients provided signed informed consent under an Institutional Review Board–approved protocol. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Genotype Assessments

All postprogression biopsies were analyzed for *ALK* resistance mutations. Testing methodologies included the MGH NGS platform, the FoundationOne NGS platform, and Sanger dideoxynucleotide sequencing of complementary DNA (cDNA) and genomic DNA (gDNA; Supplementary Table S2). The MGH NGS platform (v1.1.4) uses anchored multiplex polymerase chain reaction (PCR) to detect single-nucleotide variants (SNV) and insertions/deletions within 39 cancer-related genes, including ALK (exons 22, 23, and 25; ref. 52). The FoundationOne platform (Foundation Medicine) uses NGS to evaluate the entire coding sequence of 315 cancer-related genes as well as select introns from 28 genes commonly altered in solid tumors (53). A subset of specimens underwent Sanger sequencing of the entire *ALK* kinase domain as previously described (6).

For WES, genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) samples. Whole-exome capture libraries were constructed from 100 ng of extracted tumor and normal DNA. Ligated DNA was size-selected for lengths between 200 and 350 bp and subjected to exonic hybrid capture using SureSelect v2 Exome bait (Agilent). Samples were multiplexed and sequenced on Illumina HiSeq flowcells (paired-end 76-bp reads) to an average on-target coverage depth ranging from 134 to 210× for all tumors and normal DNA, respectively. Massively parallel sequencing data were processed using two consecutive pipelines as previously described (33).

Patient-derived cell lines (MGH021-5A, MGH034-2A, MGH049-1A, MGH051-2, and MGH075-2E) were analyzed by NGS. RNA bait-based hybridization capture was performed to capture over 1,000 known cancer genes (RightOn Cancer Sequencing Kit, developed in collaboration with Elim BioPharma), as previously described (10).

FISH

ALK FISH was performed on FFPE tissue using dual-color, break-apart rearrangement probes (Abbott-Vysis; ref. 6). Multicolor FISH to assess for gene amplification was performed using a mix of custom FISH probes: Kreatech MET (7q31) blue, EGFR (7p11) green, and HER2/ERBB2 (17q12) red (Leica Biosystems).

Immunohistochemistry

ALK immunohistochemistry was performed using an anti-ALK monoclonal antibody (clone 5A4, Novocastra) at 1:50 dilution with Leica automation (Leica BOND-III, Leica Microsystems). Immunohistochemical staining for vimentin and E-cadherin were performed as previously described (36).

Cell Lines and Reagents

Patient-derived cell lines were established as previously described (10). MGH075-2E and MGH049-1A were developed from malignant, ceritinib-resistant pleural effusions (March 2014 and July 2012, respectively). MGH084-1D, MGH034-2A, and MGH051-2C were established from ceritinib-resistant liver biopsies (April 2014, September 2012, and February 2013, respectively). MGH021-5A was derived from a malignant pleural effusion (June 2011). Cell lines were sequenced to confirm the presence of *ALK* rearrangements identified by clinical testing of biopsy specimens from the same patients. Additional authentication was not performed. Cells were grown either in RPMI-1640 or in DMEM (Invitrogen) supplemented with 10% FBS and 1X Antibiotic–Antimycotic.

Ba/F3 immortalized murine bone marrow–derived pro-B cells were obtained from the RIKEN BRC Cell Bank (RIKEN BioResource Center) and cultured in DMEM supplemented with 10% FBS and IL3 (0.5 ng/mL). Cells were infected with lentiviral vectors (pLenti) expressing either wild-type *EML4–ALK* variant 1 (E13;A20) or *EML4–ALK* harboring different *ALK* resistance mutations. Infected cells were selected in puromycin (0.8 μg/mL) for 2 weeks. After selection, IL3 was withdrawn from the culture medium for at least 2 weeks before experiments.

Ceritinib, brigatinib, and lorlatinib were purchased from Selleckchem. Alectinib was purchased from MedChem Express. Each compound was dissolved in DMSO for cell culture experiments.

Drug Screens

Patient-derived cell lines lacking known *ALK* resistance mutations underwent a combination drug screen consisting of 77 agents, as previously described (10). Cells were treated with vehicle or varying concentrations of drugs to be screened in the absence or presence of 0.3 μmol/L ceritinib for 72 hours. Cell viability was determined as detailed below.

Antibodies and Immunoblotting

Cells (5×10^5) were seeded in 6-well plates and treated with indicated agents for 6 hours. Lysates were prepared as previously described, and

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equal volumes of total cell lysate were processed for immunoblotting (6). Antibodies against phospho-ALK (Y1282/1283), ALK, phospho-AKT (S473), AKT, phospho-ERK (T202/Y204), ERK, phospho-S6, and S6 were obtained from Cell Signaling Technology. GAPDH was purchased from Millipore.

Survival Assays

Because the patient-derived cell lines used in this study have different growth kinetics, the number of cells seeded and the duration of treatment were adjusted for each cell line in order to have a consistent proliferation index (3.5 to 5) at the end of treatment (Supplementary Table S13). Cells were seeded into 96-well plates and treated with serial dilutions of different ALK inhibitors over time. Each condition was performed in triplicate. For Ba/F3 cells, 2,000 cells were plated into 96-well plates and treated for 48 hours.

At the end of treatment, cells were incubated with a CellTiter-Glo assay reagent (Promega) for 20 minutes, and luminescence was measured with a Centro LB 960 Microplate Luminometer (Berthold Technologies). GraphPad Prism version 5.0 were used to graphically display data. IC_{50} values were determined by a nonlinear regression model utilizing a four-parameter analytic method.

Growth Assays

The indicated number of cells per cell line (Supplementary Table S14) were seeded in 6 wells per condition in 96-well plates and treated with vehicle or indicated drugs at a concentration of 300 nmol/L. Cell growth kinetics was measured over time using Real-Time Glo reagent (Promega) according to the manufacturer's protocol.

Disclosure of Potential Conflicts of Interest

J.F. Gainor is consultant/advisory board member for Bristol-Myers Squibb, Genentech, Ariad, Novartis, Merck, Clovis, Boehringher Ingelheim, Jounce Therapeutics, and Kyowa Hakko Kirin. S. Gadgeel has received speakers bureau honoraria from Roche/Genentech and is a consultant/advisory board member for Roche/Genentech, Novartis, and Pfizer. L.P. Le has ownership interest (including patents) in ArcherDx and is a consultant/advisory board member for the same. R.S. Heist is a consultant/advisory board member for Boehringer Ingelheim and Ariad. L.V. Sequist is a consultant/advisory board member for AstraZeneca, Clovis Oncology, Novartis, Merrimack, Genentech, Ariad, and Taiho. A.J. Iafrate has ownership interest (including patents) in ArcherDx and is a consultant/advisory board for Roche. J.A. Engelman is an employee at Novartis, reports receiving a commercial research grant from Novartis, and is a consultant/advisory board member for Novartis, Chugai, and Roche. A.T. Shaw is a consultant/advisory board member for Pfizer, Novartis, Blueprint Medicines, Roche, Genentech, Ariad, Daiichi-Sankyo, Ignyta, Taiho, EMD Serono, and Loxo. No potential conflicts of interests disclosed by the other authors.

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CANCER DISCOVERY

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