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Sequential ALK Inhibitors Can Select for Lorlatinib-Resistant Compound *ALK* Mutations in ALK-Positive Lung Cancer

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Abstract

The cornerstone of treatment for advanced ALK-positive lung cancer is sequential therapy with increasingly potent and selective ALK inhibitors. The third-generation ALK inhibitor lorlatinib has demonstrated clinical activity in patients who failed previous ALK inhibitors. To define the spectrum of *ALK* mutations that confer lorlatinib resistance, we performed accelerated mutagenesis screening of Ba/F3 cells expressing EML4–ALK. Under comparable conditions, ENU mutagenesis generated numerous crizotinib-resistant but no lorlatinib-resistant clones

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harboring single *ALK* mutations. In similar screens with EML4-ALK containing single *ALK* resistance mutations, numerous lorlatinib-resistant clones emerged harboring compound *ALK* mutations. To determine the clinical relevance of these mutations, we analyzed repeat biopsies from lorlatinib-resistant patients. Seven of 20 samples (35%) harbored compound *ALK* mutations, including two identified in the ENU screen. Whole exome sequencing in three cases confirmed the stepwise accumulation of *ALK* mutations during sequential treatment. These results suggest that sequential ALK inhibitors can foster the emergence of compound *ALK* mutations, identification of which is critical to informing drug design and developing effective therapeutic strategies.

Keywords

Anaplastic lymphoma kinase; ALK; acquired resistance; crizotinib; ceritinib; alectinib; brigatinib; lorlatinib; and NSCLC

INTRODUCTION

Chromosomal rearrangements of the anaplastic lymphoma kinase (*ALK*) gene define a distinct molecular subset of non-small cell lung cancer (NSCLC) (1,2). *ALK* rearrangements lead to expression of constitutively activated ALK fusion proteins that function as potent oncogenic drivers. Since the discovery of *ALK* rearrangements in NSCLC one decade ago, numerous ALK inhibitors have been developed for the treatment of patients with advanced *ALK*-rearranged (i.e., ALK-positive) NSCLC (3). Until recently, the first-generation ALK inhibitor crizotinib was the standard therapy for newly diagnosed ALK-positive NSCLC, inducing responses in over 70% of patients with a median duration of response of approximately 11 months (4). For patients relapsing on crizotinib, more potent second-generation ALK inhibitors such as ceritinib, alectinib and brigatinib, have become standard treatments, re-inducing responses in the majority of crizotinib-resistant patients (5–8). Several recent randomized trials have demonstrated that second-generation ALK inhibitors may be more effective than crizotinib as first-line therapy (9–11). However, regardless of when patients receive second-generation ALK inhibitors, resistance almost always develops, leading to clinical relapse.

Multiple different molecular mechanisms can cause resistance to second-generation ALK inhibitors (12–15). In the largest study to date of clinical resistant specimens, one-half of cases resistant to a second-generation ALK inhibitor harbored *ALK* resistance mutations (i.e., on-target resistance) (12). The most common *ALK* mutation emerging on all second-generation inhibitors was the solvent front *ALK* G1202R substitution, accounting for approximately one-half of on-target resistance. Other less common *ALK* resistance mutations that were identified included *ALK* I1171 mutations with alectinib and *ALK* F1174 mutations with ceritinib. Importantly, based on analysis of patient-derived cell lines, those cancers harboring *ALK* resistance mutations remained ALK-dependent and sensitive to the pan-inhibitory, third-generation ALK inhibitor lorlatinib. In contrast, those cancers without identifiable *ALK* resistance mutations were ALK-independent and lorlatinib-insensitive, with resistance likely mediated by off-target mechanisms such as bypass signaling or lineage changes (12).

Consistent with these and other preclinical studies (16,17), lorlatinib has demonstrated clinical activity in resistant ALK-positive patients previously treated with two or more ALK inhibitors, including a second-generation inhibitor (18). In phase 1 testing, lorlatinib demonstrated a confirmed response rate of 42% and a median progression-free survival of 9.2 months in the subset of ALK-positive patients who had failed two or more ALK inhibitors. Based on repeat biopsies taken prior to lorlatinib, the presence of an *ALK* resistance mutation such as *ALK* G1202R predicted for clinical response to lorlatinib (18). The promising antitumor activity seen with lorlatinib has led to FDA breakthrough therapy designation, and has solidified the sequential treatment approach using first- and/or second-generation ALK inhibitors followed by lorlatinib.

As with other ALK inhibitors, acquired resistance to lorlatinib develops in essentially all patients. We previously reported a case of a patient with advanced ALK-positive lung cancer who had been treated with sequential crizotinib, ceritinib and lorlatinib (19). Molecular analysis of this patient's lorlatinib-resistant specimen revealed a novel compound resistance mutation, *ALK* C1156Y/L1198F, with both mutations residing on the same allele of the *ALK* fusion gene. *ALK* C1156Y/L1198F conferred resistance to lorlatinib but paradoxically re-sensitized the cancer to crizotinib. Indeed, the patient experienced a dramatic re-response when rechallenged with crizotinib (19). Outside of this single case report, no other mechanisms of resistance to lorlatinib have been described, hindering our ability to develop effective therapeutic strategies for patients who relapse on lorlatinib.

Here we report the results of preclinical studies aimed at discovering clinically relevant mechanisms of resistance to lorlatinib. Utilizing *in vitro* cell-based accelerated mutagenesis screens, we have identified numerous compound (but not single) *ALK* mutations that confer high-level resistance to lorlatinib. By analyzing a series of repeat biopsies taken from lorlatinib-resistant patients, we demonstrate that these compound *ALK* mutations develop in a stepwise fashion in patients treated with sequential ALK inhibitors. Our results identify novel compound ALK mutants and highlight the importance of therapeutic strategies aimed at preventing the emergence of highly refractory compound mutations.

RESULTS

ENU mutagenesis screening to identify lorlatinib-resistant *ALK* mutations

To predict *ALK* mutations conferring resistance to lorlatinib, we employed accelerated *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screening of Ba/F3 models of ALK-positive cancer. We utilized established Ba/F3 cell lines expressing either non-mutant EML4-ALK to model ALK inhibitor-naïve disease, or mutant EML4-ALK harboring a single *ALK* resistance mutation to model resistant disease post first- or second-generation ALK inhibitor. As previously reported (12), these Ba/F3 models exhibit different sensitivities to different ALK inhibitors, with lorlatinib retaining significant potency against all models, including EML4-ALK G1202R (Figure 1A).

To determine whether any single *ALK* mutations are capable of conferring resistance to lorlatinib, Ba/F3 cells expressing non-mutant EML4-ALK (either variant 1 (E13; A20) or variant 3a (E6a; A20), which represent the two most common EML4-ALK variants in

NSCLC) were chemically mutagenized with ENU, a potent inducer of point mutations (20). After treatment with ENU, mutagenized cells were cultured in the presence of a range of crizotinib or lorlatinib concentrations (100 nM–1000 nM) that simulate clinical drug exposures (Figure 1B). Emerging resistant clones were isolated and DNA was sequenced to identify *ALK* kinase domain mutations. As 100 nM of crizotinib was insufficient to prevent growth of non-mutagenized cells, this dose of crizotinib was excluded from our analysis; long-term cell proliferation assays confirmed that all other concentrations of crizotinib and lorlatinib prevented the outgrowth of non-mutagenized cells (Supplementary Figure S1) and results are summarized below.

As shown in Figure 1C, we observed numerous resistant clones emerging after treatment with 300–600 nM of crizotinib. These crizotinib-resistant clones harbored a variety of single *ALK* kinase domain point mutations, including the majority of crizotinib resistance mutations identified in clinical specimens (12,21–23). In contrast, no resistant clones emerged after treatment with 300–600 nM of lorlatinib (Figure 1C). This range of drug concentrations is comparable to the plasma exposures achieved *in vivo*, as most patients treated at standard dose lorlatinib have unbound drug levels exceeding 369 nM (18). Of note, a small number of clones did emerge after treatment with 100 nM of lorlatinib and were found to harbor predominantly *ALK* I1171N or, less commonly, *ALK* L1196M mutations. Of note, Ba/F3 cells expressing these two particular ALK mutants were slightly less sensitive to lorlatinib compared to other ALK mutants, as shown by the relatively higher IC_{50}'s in cellular assays (Figure 1A). However, these mutants were fully suppressed at higher (and clinically achievable) lorlatinib concentrations (Figure 1C). These results are consistent with previous studies that failed to identify a single *ALK* mutation sufficient to confer high level resistance to lorlatinib, and suggest that lorlatinib treatment may be able to suppress the emergence of all single *ALK* resistance mutations.

Many patients will have disease progression on a second-generation ALK tyrosine kinase inhibitor (TKI) prior to lorlatinib, and about half of these cancers are likely to have on-target *ALK* resistance mutations (12). To recapitulate this clinical setting, we performed ENU mutagenesis screening of EML4-ALK-expressing Ba/F3 cells each harboring one of the most common *ALK* resistance mutations observed after failure of first- and second-generation ALK inhibitors (C1156Y, F1174C, L1196M, G1202R, and G1269A). Since each single EML4-ALK mutant exhibits a different sensitivity to lorlatinib (Figure 1A), we first determined the range of lorlatinib concentrations sufficient to prevent clonal outgrowth using long-term cell proliferation assays (Figure 2A, Supplementary Figure S2). After ENU mutagenesis, Ba/F3 cells were cultured in the presence of lorlatinib at concentrations that prevented the outgrowth of each single mutant. The minimum concentrations of lorlatinib used in the screen were 50 nM for C1156Y and F1174C, 100 nM for G1269A, and 300 nM for L1196M and G1202R.

For each single ALK mutant model, 12–49 lorlatinib-resistant clones were identified, each harboring a compound *ALK* mutation, *i.e.*, two mutations on the same allele (Figure 2B, Supplementary Figure S3). The exact compound mutations and the lorlatinib concentrations at which they emerged are shown in Figure 2B. Mutagenesis of C1156Y or L1196M Ba/F3 cells yielded lorlatinib-resistant clones harboring a multitude of different compound *ALK*

mutations. Among the nine different compound *ALK* C1156Y mutations, one was ALK C1156Y/L1198F, which we previously identified in a lorlatinib-resistant patient (19). Similarly, the L1196M model yielded eight different compound *ALK* L1196M mutations, two of which included L1198 mutations. The remaining three ALK mutant models – F1174C, G1269A and G1202R – yielded a smaller spectrum of compound *ALK* mutations (Figure 2B). For example, mutagenesis of G1202R Ba/F3 cells yielded lorlatinib-resistant clones harboring primarily the *ALK* G1202R/L1196M compound mutation. This compound mutation was the only one to emerge at the highest concentration of lorlatinib (1000 nM), suggesting it may represent a highly recalcitrant lorlatinib-resistant mutation. This mutation was also subsequently identified in a lorlatinib-resistant patient (see below). Overall, these results reveal a broad spectrum of compound *ALK* mutations that can mediate on-target resistance to lorlatinib.

Modelling lorlatinib resistance *in vitro* and *in vivo*

In a parallel approach to identifying on-target mechanisms of resistance to lorlatinib, we treated sensitive H3122 cells with increasing concentrations of lorlatinib for over 4 months until resistance emerged (Supplementary Figure S4A). Three independent resistant cell lines (H3122 LR-A, LR-B, and LR-C) were ultimately derived and maintained in 1 μM lorlatinib. All three cell lines were resistant to lorlatinib in cell viability assays (Supplementary Figure S4B), and none harbored a secondary mutation in the ALK tyrosine kinase domain. Of note, in similar studies of crizotinib resistance, we previously identified the *ALK* L1196M gatekeeper mutation in H3122 cells made resistant to crizotinib *in vitro* (24).

To model resistance to lorlatinib *in vivo*, we generated subcutaneous tumors from the sensitive EML4-ALK v1 cell line MGH006. Tumor-bearing mice were treated with lorlatinib by oral gavage, leading to tumor regression for more than 50 days, as previously reported (17). With continued lorlatinib treatment, three of six tumors showed regrowth consistent with the emergence of resistance (Supplementary Figure S4C). Three cell lines (MGH006 LR-B1, G3, and J2) were derived from the resistant tumors. As with the *in vitro* generated models, all three cell lines were resistant to lorlatinib in cell culture (Supplementary Figure S4D), and none harbored an *ALK* resistance mutation. Taken together, these results support the notion that no single *ALK* mutations confer resistance to lorlatinib.

Functional validation of lorlatinib-resistant compound *ALK* mutations *in vitro*

To confirm that the compound *ALK* mutations identified by ENU mutagenesis screening confer resistance to lorlatinib, we independently generated Ba/F3 cell lines expressing three of the identified compound mutations - *ALK* G1202R/L1196M, *ALK* G1202R/L1198F, and *ALK* L1196M/L1198F. Cells were treated with crizotinib, ceritinib, alectinib, brigatinib, or lorlatinib, and cell viability determined after 48 hours. Compared with *ALK* G1202R or L1196M single mutants, the compound *ALK* G1202R/L1196M mutant conferred high-level resistance to lorlatinib (IC_{50} 1,116 nM vs 37 or 18 nM with G1202R or L1196M, respectively; Figure 3A). The *ALK* G1202R/L1196M double mutant was also resistant to all first- and second-generation ALK inhibitors tested (Figure 3B). In contrast, *ALK* L1198F-containing compound mutants were resistant to lorlatinib and second-generation ALK

inhibitors, but exhibited sensitivity to crizotinib, as shown previously (19) (Supplementary Figure S5A,B). For all of these studies, similar results were obtained using either variant 1 or variant 3 of EML4-ALK (Supplementary Figure S5A,B).

We next assessed biochemical inhibition of compound *ALK* mutants by examining ALK phosphorylation across the different Ba/F3 models treated with lorlatinib. Consistent with the results of cell viability assays, lorlatinib treatment suppressed ALK phosphorylation of non-mutant ALK and single ALK mutants, but failed to suppress ALK phosphorylation of the ALK G1202R/L1196M, ALK G1202R/L1198F and ALK L1196M/L1198F compound mutants (Figure 3C, Supplementary Figure S6). Thus, the compound ALK mutants identified by ENU mutagenesis screening maintain ALK activation in the presence of lorlatinib.

To validate the findings made in Ba/F3 cells, we engineered the sensitive EML4-ALK v1 lung cancer cell line H3122 to overexpress EML4-ALK G1202R/L1196M. Consistent with the Ba/F3 data, H3122 cells overexpressing ALK G1202R/L1196M were highly resistant to lorlatinib compared to H3122 cells overexpressing ALK G1202R (IC_{50} 2,253 nM vs 46 nM, respectively; Supplementary Figure S7A). Additionally, lorlatinib treatment failed to suppress ALK phosphorylation of the compound ALK mutant, but was able to suppress ALK phosphorylation of both the non-mutant and single ALK G1202R mutant H3122 cells (Supplementary Figure S7B).

Molecular analysis of lorlatinib-resistant biopsies from patients

To identify clinical mechanisms of resistance to lorlatinib, we performed repeat biopsies of resistant tumors in 20 patients with ALK-positive lung cancer relapsing on lorlatinib. As shown in Table 1, these patients may have had primary (or intrinsic) resistance to lorlatinib, or have developed resistance after an initial response to lorlatinib (i.e., acquired resistance). Nineteen of the 20 patients had received two or more ALK inhibitors, including crizotinib and at least one second-generation ALK inhibitor; the remaining patient had received the second-generation ALK inhibitor brigatinib. Among the 20 cases, 11 had paired pre- and post-lorlatinib specimens (Supplementary Table S1). Pre-lorlatinib specimens were obtained on the ALK inhibitor just prior to lorlatinib, while post-lorlatinib specimens were obtained at the time of relapse on lorlatinib. Clinical history is summarized in Supplementary Table S2.

All lorlatinib-resistant specimens underwent standard histopathology and molecular profiling using either the MGH SNaPshot next-generation sequencing (NGS) assay (25) or the FoundationOne platform (Table 1 and Supplementary Table S3). All samples showed NSCLC histology with no evidence of small cell transformation. As shown in Table 1, seven of the eight (88%) patients with primary resistance to lorlatinib had no detectable mutations within the ALK tyrosine kinase domain. In the four patients who had paired pre-lorlatinib specimens, none harbored *ALK* resistance mutations, suggesting the presence of ALK-independent mechanisms of resistance. In contrast, among the 12 patients with acquired resistance to lorlatinib, six (50%) developed compound *ALK* resistance mutations, including four with a double *ALK* mutation and two with a triple *ALK* mutation. In four cases with paired pre- and post-lorlatinib specimens, the pre-lorlatinib specimen harbored a single or

double *ALK* resistance mutation present in the compound mutant post-lorlatinib specimen, suggesting a stepwise accumulation of *ALK* resistance mutations during lorlatinib therapy. Interestingly, in two patients with acquired resistance, their pre-lorlatinib tumors harbored lorlatinib-sensitive *ALK* resistance mutations I1171N and G1202R, which were subsequently "lost" or no longer detectable post-lorlatinib.

In addition to *ALK*, a variety of co-occurring mutations were detected in lorlatinib-resistant specimens, with the most common being *TP53* mutations in 10 of the 20 cases (Figure 4). Among the co-occurring mutations identified in samples lacking *ALK* mutations, several were potential drivers of ALK independent resistance, including a *MAP3K1* mutation in MGH098 and an activating *NRAS* G12D mutation in MGH9107. Of note, the *NRAS* G12D mutation was most likely acquired as it was not detected in the patient's paired pre-lorlatinib specimen (Supplementary Table S3).

In one patient (MGH065), we identified a single *ALK* resistance mutation – *ALK* G1269A – at the time of lorlatinib relapse. A different *ALK* mutation - *ALK* L1196M - was identified in this patient's pre-lorlatinib sample. *ALK* G1269A is predicted to be sensitive to lorlatinib in biochemical and cellular studies (12), raising the possibility that an off-target mechanism(s) of resistance driving ALK-independent growth developed in a G1269A mutant clone. To test this hypothesis, we established a cell line from the lorlatinib-resistant biopsy specimen (MGH065-3H). This cell line was resistant to crizotinib, ceritinib, and lorlatinib in cell growth assays (Supplementary Figure S8A,B). Based on immunoblotting experiments, ALK phosphorylation was potently suppressed by ceritinib and lorlatinib (Supplementary Figure S8C), consistent with an ALK-independent mechanism(s) of resistance.

Clonal evolution of compound *ALK* resistance mutations with sequential ALK TKI therapy

Based on *in vitro* mutagenesis screening, preclinical modelling of acquired resistance, and molecular analysis of resistant patient samples, compound but not single *ALK* resistance mutations can cause resistance to lorlatinib. To determine the evolutionary origin of compound *ALK* resistance mutations in patients treated with sequential ALK inhibitors, we performed whole exome sequencing (WES) on serial biopsy samples from three patients. The first patient - MGH953 - was selected for further analysis as her lorlatinib-resistant tumor harbored the compound *ALK* G1202R/L1196M mutation also identified by *in vitro* mutagenesis screening. This patient had received treatment with sequential first, second, and third generation ALK inhibitors. While she had derived clinical benefit from each inhibitor, she developed resistance to each drug within five to eight months (Figure 5A). She underwent biopsy and molecular profiling at the time of each relapse, with no *ALK* mutation detected at diagnosis or post-crizotinib, *ALK* G1202R identified in a post-alectinib cell line derived from a malignant pleural effusion, and both *ALK* G1202R and L1196M mutations detected in *cis* in a post-lorlatinib malignant pleural effusion (Table 1 and Figure 5A).

We next performed WES of this patient's tumor and normal samples. We detected the compound *ALK* G1202R/L1196M mutations in *cis* in the lorlatinib-resistant sample, and the single *ALK* G1202R mutation in the alectinib-resistant sample. The *ALK* L1196M mutation was not detectable in any samples except for the lorlatinib-resistant specimen. The *ALK*

G1202R mutation was detectable in both the alectinib- and lorlatinib-resistant samples, but not the crizotinib-resistant specimen. Clonal analysis revealed a dominant clone in the pre-treatment specimen characterized by a set of 40 truncal mutations (Figure 5B). This clone gave rise to a crizotinib-resistant subclone harboring an additional 25 mutations, and subsequently an alectinib-resistant subclone harboring 21 additional mutations including *ALK* G1202R (Figure 5B). With chronic exposure to lorlatinib, this *ALK* G1202R subclone eventually evolved to acquire an additional 127 mutations including *ALK* L1196M. This double *ALK* G1202R/L1196M mutant subclone most likely led to the patient's relapse on lorlatinib.

The second patient – MGH086 - had also been treated with sequential first, second, and third generation ALK inhibitors and underwent multiple repeat biopsies throughout his course of disease (Figure 5C). We previously reported this patient's clonal evolution of resistance to sequential crizotinib followed by brigatinib (12). Clonal analysis demonstrated that an *ALK* E1210K mutant clone emerged on crizotinib and that under the selective pressure of brigatinib, this clone subsequently gave rise to two brigatinib-resistant compound *ALK* E1210K mutant subclones, *ALK* E1210K/S1206C and *ALK* E1210K/D1203N. These subclones were identified in excisional biopsies of recurrent left axillary disease taken approximately 14 and 21 months after starting on brigatinib, respectively (12). The patient continued on brigatinib and underwent three more excisional biopsies of the same site, before switching to lorlatinib, initially in combination with a checkpoint inhibitor (Supplementary Table S2). He had a significant response to lorlatinib lasting over 15 months at which time he underwent repeat biopsy of a lorlatinib-resistant subcutaneous metastasis. We performed WES on the last three brigatinib-resistant lesions and the one lorlatinib-resistant lesion. Clonal analysis of all sequenced specimens demonstrated that the parental *ALK* E1210K clone which first developed on crizotinib gave rise to three distinct brigatinib-resistant *ALK* E1210K double mutants (Figure 5D). The dominant subclone *ALK* E1210K/D1203N, which was detected in three of five brigatinib-resistant samples, initially responded to lorlatinib; however, after chronic exposure to lorlatinib, this subclone acquired a third *ALK* mutation at residue G1269, likely leading to failure of lorlatinib (Figure 5D).

Finally, patient MGH987 was treated with four sequential ALK TKIs - crizotinib, alectinib, ceritinib and lorlatinib - and had relatively brief responses to each TKI (Figure 5E). Repeat biopsy taken when the patient was relapsing on alectinib demonstrated a known alectinib-resistant mutation *ALK* I1171N. Based on this finding, the patient was switched to ceritinib and achieved another clinical response lasting over seven months. When he relapsed on ceritinib, no biopsy was performed and the patient was transitioned to lorlatinib. He had clinical improvement on lorlatinib, but after three months restaging scans demonstrated worsening disease. Repeat biopsy of the same site and SNaPshot NGS revealed a compound *ALK* I1171N/L1198F mutation. WES and clonal analysis demonstrated that the *ALK* I1171N mutant clone gave rise to the compound *ALK* I1171N/L1198F mutant subclone (Figure 5F), which is known to confer resistance to lorlatinib (19). Thus, in this patient as well as the prior two patients, sequential ALK targeted therapies fostered the stepwise accumulation of *ALK* resistance mutations, leading to lorlatinib-resistant double and triple mutants. While *ALK* L1198F-containing double mutants may be sensitive to crizotinib,

other compound mutants, particularly those containing *ALK* G1202R, are likely to be resistant to all available ALK TKIs.

Structural and biochemical basis for *ALK* G1202R/L1196M-mediated resistance to lorlatinib

We selected the compound *ALK* G1202R/L1196M mutant for more detailed biochemical and structural studies since 1) *ALK* G1202R is the most common *ALK* resistance mutation emerging after failure of second-generation ALK TKIs, and 2) *ALK* G1202R/L1196M was discovered in our ENU mutagenesis screen and subsequently identified in a lorlatinib-resistant patient. The *ALK* L1196M gatekeeper mutation was one of the first crizotinib-resistant mutations identified and was proposed to mediate resistance through steric interference with crizotinib binding (26). Lorlatinib is active against *ALK* L1196M, but with some decrement in potency in cellular assays (Figure 1A) and loss of binding by an order of magnitude compared to non-mutant ALK, as measured by the inhibitory constant (K_i) (16). To determine how the co-occurrence of L1196M and G1202R leads to lorlatinib resistance, we first compared the co-crystal structures of non-mutant ALK and ALK L1196M bound to lorlatinib (Figure 6A). Aside from the ALK L1196M substitution and movement in the ATP-phosphate binding loop (P-loop) leading to a maximal separation of 3.1 Å at residue 1126, the structures of the non-mutant ALK and ALK L1196M kinase domains were highly similar. Binding of the ligand lorlatinib was also almost identical (Figure 6A). Thus, the decreased binding of lorlatinib to ALK L1196M may be due to subtle overall structural changes or motion of the protein-ligand complex, rather than a clash with the methionine at position 1196. Furthermore, in kinetic assays, the enzymatic activity of the L1196M mutant ($k_{cat}/K_{M,substrate}$, a measure of catalytic efficiency) was increased approximately 3- to 5-fold compared to non-mutant ALK (Supplementary Table S4). Taken together, these results suggest that the decreased potency of lorlatinib against ALK1196M may reflect both increased enzyme activity and decreased drug binding.

We next performed molecular dynamics (MD) simulations on the single ALK G1202R mutant and the ALK G1202R/L1196M double mutant with lorlatinib bound in the ATP pocket. MD simulations suggested that the orientation of the polar portion of the sidechain of ALK G1202R was directed into solvent due to interactions with residue E1210, at times forming 0, 1 or 2 hydrogen bonds. On average, the simulations revealed about 1 hydrogen bond between E1210 and G1202R. Initially we suspected that the increased bulk of the ALK G1202R side chain might perturb the ligand position and consequently reduce the quality of hinge interactions to the 2-aminopyridine of lorlatinib. However, there was no difference in the average hydrogen bonding partner distances and angles between lorlatinib and non-mutant ALK, ALK G1202R and ALK G1202R/L1196M in the simulations (Supplementary Figure S9A). Instead MD simulations suggested that introduction of the arginine at position 1202 increases steric bulk near the pyrazole of the ligand which destabilizes both the ligand and P-loop above. The net effect of the G1202R mutation is that the pyrazole of lorlatinib moves “up” approximately 0.5 Å above where it is normally seen in the non-mutant ALK or ALK L1196M structures (Figure 6B). With G1202R, the P-loop opens wider by a similar amount (0.5 Å) near the pyrazole, and in combination with L1196M, the P-loop opens 0.7 Å near the pyrazole and about 0.4 Å in the inner portion of the P-loop near V1130 (Figure 6B,

Supplementary Figure 9B). This increased distance between the protein and ligand would be expected to weaken favorable CH-π interactions and van der Waals contacts, and may incur additional ligand strain. The need to adopt this wider conformation may also increase strain in the P-loop or favor dissociation of the ligand. Of note, based on MD simulations, the *ROS1* resistance mutation G2032R, which is analogous to *ALK* G1202R, may confer resistance to ROS1 inhibitors through a similar mechanism involving P-loop conformational changes leading to shorter residence time (27).

Taken together, these results suggest that ALK G1202R destabilizes lorlatinib binding due to steric and conformational effects induced by the arginine substitution. While this leads to a decrease in potency of lorlatinib (Figure 1A), exposures in the clinic are still sufficient to adequately inhibit the single ALK G1202R mutant (18). However, when combined with L1196M, which both reduces binding affinity of lorlatinib and enhances the enzymatic activity of ALK, the additive effects of both mutations significantly impair lorlatinib's potency, fueling the emergence of resistance.

DISCUSSION

The current therapeutic paradigm for patients with advanced ALK-positive NSCLC is to treat with sequential ALK targeted therapies, often moving from first- to second- to third-generation ALK inhibitors (3). While this treatment approach has dramatically improved clinical outcomes, acquired resistance invariably develops and leads to clinical relapse. In this report, we have focused on resistance to the third-generation ALK inhibitor lorlatinib (16), which is widely anticipated to become a standard therapy for ALK-positive patients after failure of one or more prior ALK inhibitors. Using *in vitro* cell-based accelerated mutagenesis screening, we identified multiple different compound *ALK* mutations that confer resistance to lorlatinib, but no single *ALK* mutations capable of causing high-level lorlatinib resistance. Importantly, in lorlatinib-resistant tumor specimens from patients, on-target resistance mechanisms consisted only of compound and not single *ALK* mutations. Furthermore, two of the compound *ALK* mutations identified in our mutagenesis screen – *ALK* C1156Y/L1198F and *ALK* G1202R/L1196M – were also identified in patients, validating the utility of this screen in discovering clinically-relevant mechanisms of resistance. Consistent with previous work (19), we show that resistance to ALK inhibition is a dynamic and clonal process, with a founder single *ALK* mutant clone in the pre-lorlatinib tumor giving rise to a lorlatinib-resistant compound *ALK* mutant subclone.

The evolution of recurrent on-target resistance mutations in ALK-positive NSCLC is highly reminiscent of other oncogene-addicted cancers. For example, in *EGFR*-mutant, T790M-positive NSCLC, the third-generation EGFR inhibitor osimertinib is initially highly effective (28), but tumors eventually develop acquired resistance. In approximately 30% of cases, resistance is due to acquisition of the *EGFR* C797S mutation, usually in *cis* with T790M (29). Like *ALK* G1202R/L1196M, the EGFR mutant containing T790M/C797S is resistant to all available kinase inhibitors. However, in contrast to *EGFR*-mutant NSCLC in which T790M is essentially the only on-target resistance mutation observed after failure of first- and second-generation inhibitors, numerous (>10) different single *ALK* kinase domain mutations can emerge in tumors resistant to first- and second-generation ALK inhibitors

(12). This multitude of single *ALK* mutations serves as the substrate for the diverse array of compound *ALK* mutations driving resistance to lorlatinib. A similar stepwise accumulation of on-target resistance mutations leading to multiple different compound resistance mutations has been observed in chronic myelogenous leukemia (CML) treated with sequential ABL TKIs (30,31). Thus, resistance in ALK-positive lung cancer may more closely mirror resistance in BCR-ABL-driven CML rather than *EGFR*-mutant NSCLC.

Our studies on resistance to lorlatinib have several important implications for the field of ALK-positive lung cancer. First, with the increasing availability of second- and third-generation ALK inhibitors, the vast majority of ALK-positive patients will be treated with sequential ALK inhibitors, and approximately 35% will develop compound *ALK* resistance mutations on lorlatinib. Based on *in vitro* mutagenesis screening, we identified 24 different compound *ALK* mutations associated with resistance to lorlatinib, two of which were also discovered in patients. Comprehensive cataloging of compound *ALK* mutations that emerge in the clinic is needed to determine the most common pairings and identify the most promising targets for drug development. As the solvent front *ALK* G1202R mutation is the most common kinase domain mutation seen after second-generation ALK inhibitors (12), G1202R-containing compound mutations may become the most common on-target resistance mechanism in patients relapsing after sequential second- and third-generation inhibitors. The compound G1202R/L1196M mutant is refractory to all known ALK inhibitors, and based on structural modeling studies, we predict that other G1202R-containing compound mutations may be similarly recalcitrant. However, not all compound *ALK* mutations are necessarily refractory to currently available ALK inhibitors. We previously showed that the compound ALK C1156Y/L1198F mutant is resistant to next generation inhibitors but sensitive to crizotinib (19). In our mutagenesis screening, we identified this and other L1198-containing compound mutations, all of which are predicted to be sensitive to crizotinib. Thus, in patients treated with sequential ALK inhibitors, repeat biopsies at the time of resistance are critical to identify refractory versus resensitizing compound mutations and to select the most effective therapeutic strategies.

Second, as the sequential treatment approach fosters the stepwise accumulation of *ALK* resistance mutations, culminating in a potentially refractory compound mutation, a more effective therapeutic strategy may be to prevent the early development of single *ALK* mutations by using pan-inhibitory molecules such as lorlatinib upfront. Consistent with previous preclinical studies (17), mutagenesis screening using cell lines expressing non-mutant EML4-ALK failed to identify any single *ALK* kinase domain mutations capable of conferring high level resistance to lorlatinib. However, by using cell lines already harboring single *ALK* resistance mutations, we discovered numerous different compound *ALK* mutants conferring variable degrees of resistance to lorlatinib. Similarly, among the 20 clinical specimens obtained from lorlatinib-resistant patients, seven harbored compound resistance mutations, including double and triple *ALK* mutations. Of note, one lorlatinib-resistant case did harbor a single *ALK* resistance mutation G1269A. However, analysis of the cell line derived from this case showed that ALK was effectively inhibited by lorlatinib and suggests that alternative, or ALK-independent, signaling pathways were likely driving resistance in this case. Taken together, our results suggest that compound *ALK* mutations, but not single *ALK* mutations, may be necessary for cancers to become resistant to

lorlatinib. As compound mutants are less likely to emerge in a treatment-naïve, non-mutant ALK molecule, we speculate that upfront treatment with lorlatinib could completely suppress or at least significantly delay on-target resistance, leading to more durable clinical benefit than the current sequential treatment approach.

Third, regardless of the line in which it is used, a broadly potent ALK inhibitor such as lorlatinib may ultimately foster the development of ALK-independent resistance mechanisms. While roughly 50% patients relapsing on a second generation ALK inhibitor may harbor an *ALK* resistance mutation and remain ALK-dependent, the remaining 50% of patients do not harbor *ALK* mutations (12). These patients' cancers are likely driven by off-target, or ALK-independent, mechanisms of resistance, such as bypass signaling or lineage changes. To date, a variety of different bypass signaling pathways have been reported as mediating resistance to ALK inhibitors, including EGFR, MET, c-KIT, SRC, RAS/MAPK, and SHP2 among others (12,15,23,32,33). Lineage changes ranging from epithelial-to-mesenchymal transition (EMT) to small cell transformation have also been reported in resistant ALK-positive tumors (12,34,35). Among the 20 lorlatinib-resistant biopsies we performed, 12 (60%) had no detectable *ALK* mutations and likely harbored ALK-independent mechanisms of growth and survival. Of these 12 cases, two patients - MGH9092 and MGH040 - harbored *ALK* resistance mutations prior to lorlatinib, exhibited durable responses to lorlatinib, and then "lost" the *ALK* mutations when they became resistant to lorlatinib (Table 1), suggesting elimination of on-target resistance and outgrowth of an ALK-independent clone. As we optimize therapeutic approaches to overcome and even prevent on-target resistance, cancers will almost certainly develop a diverse array of off-target mechanisms of resistance. Improved understanding of these off-target resistance mechanisms will be critical to developing combination strategies that effectively suppress both on- and off-target resistance.

Over the last decade, numerous experimental systems have been explored to model acquired resistance to targeted therapies *in vitro*. In this study, we utilized cell-based accelerated mutagenesis screens to predict *ALK* mutations conferring resistance to lorlatinib. This methodology provides an efficient system for generating many different *ALK* point mutations, and has been used successfully to identify the most common on-target resistance mutation in *ROS1*-rearranged lung cancer, *ROS1* G2032R, analogous to *ALK* G1202R (36–38). Similar mutagenesis screens have also been used to define the resistance profiles of imatinib and other ABL inhibitors in CML (39). However, there are several limitations of ENU mutagenesis screening. ENU mutagenesis an artificial means of inducing point mutations, and ENU is known to lead to specific patterns of single-nucleotide substitutions. In this mammalian cell system, GC to AT, AT to GC, and AT to TA substitutions preferentially occur (40,41). This mutational bias could lead to over-representation of mutations that are unlikely to develop *in vivo*, or alternatively failure to induce all clinically relevant resistance mutations. Under the conditions of the screen, ENU mutagenesis would also not capture more complicated genetic alterations of *ALK* such as the triple compound *ALK* mutations identified in patients MGH086 and MGH087 (Table 1).

In summary, we have shown through *in vitro* cell based mutagenesis screening and molecular analysis of patient samples that treatment with sequential first-, second-, and

third-generation ALK inhibitors fosters the development of diverse compound *ALK* mutations, some of which are highly refractory to all available ALK inhibitors. It is tempting to speculate that upfront treatment with the third-generation inhibitor lorlatinib may be able to prevent the emergence of single and subsequently compound *ALK* mutations, potentially improving clinical outcomes. This hypothesis will be formally addressed in an ongoing randomized phase 3 trial comparing lorlatinib with crizotinib as first-line therapy in advanced ALK-positive lung cancer (NCT03052608). However, our data also suggest that highly potent target inhibition with lorlatinib could ultimately select for ALK independent resistance mechanisms which may be difficult to overcome once established. Thus, optimal first-line therapy may require development of lorlatinib-based combinations to prevent both ALK-dependent and ALK independent resistance.

METHODS

Cell lines and Reagents

Ba/F3 immortalized murine bone marrow-derived pro-B cells were obtained from the RIKEN BRC Cell Bank (RIKEN BioResource Center) in 2010 and cultured in DMEM with 10% FBS with (parental) or without (EML4–ALK) IL3 (0.5 ng/mL). cDNAs encoding EML4-ALK variant 1 (E13; A20) and variant 3a (E6a; A20) containing different point mutations were cloned into retroviral expression vectors, and Ba/F3 cells were infected with the virus as previously described (24). After retroviral infection, Ba/F3 cells were selected in puromycin (0.7 μg/mL) for 2 weeks. IL3 was withdrawn from the culture medium for more than 2 weeks before experiments. Patient-derived cell lines were established as previously described (23,32). MGH006 was developed in 2010 from a malignant pleural effusion from a TKI-naïve, ALK-positive patient, and MGH065-3H was developed in 2015 from a lorlatinib-resistant lymph node biopsy. H3122 was provided by the Center for Molecular Therapeutics at Massachusetts General Hospital (MGH) in 2010. H3122 and H3122-derived resistant lines were cultured in RPMI1640 supplemented with 10% FBS. MGH006, MGH006-resistant cell lines, and MGH065-3H were cultured in DMEM with 10% FBS. Cell lines were sequenced to confirm the presence of *ALK* rearrangement and *ALK* mutations. Additional authentication was performed by SNP fingerprinting in 2017. The expression vectors of *EML4-ALK* were transfected with Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Lorlatinib was purchased from Selleck Chemicals; for the H3122 and MGH006 experiments, lorlatinib was provided by Pfizer. Crizotinib and ceritinib were purchased from Selleck Chemicals. Alectinib and brigatinib were purchased from MedChem Express.

ENU Mutagenesis Screen

The ENU mutagenesis screen protocol was based on procedures published by Bradeen and colleagues and O'Hare and colleagues (39,42). The cells were exposed to a final concentration of 100 μg/mL N-ethyl-N-nitrosourea (ENU; Sigma) for 16 hours and were collected and washed with PBS. After a 24-hour incubation in normal media, the cells were incubated with various concentrations of lorlatinib or crizotinib, in 96-well plates (1×10^6–3×10^6 cells per well). Plates were visually inspected for media color change and for cell growth throughout the 4-week experiment. The contents of wells exhibiting cell growth were

digested with proteinase K, and *ALK* kinase domain was PCR-amplified from gDNA with Fast Start PCR Master (Roche). The PCR fragments were sequenced bidirectionally by Sanger sequencing. Each figure represents the combined results of two independent experiments.

Survival Assays

Ba/F3 cells (2,000) were plated in triplicate into 96-well plates. Forty-eight hours after drug treatment, cells were incubated with CellTiter-Glo (Promega) and luminescence was measured with a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices LLC). GraphPad Prism (GraphPad Software, San Diego, CA) was used to graphically display data and determine IC_{50} values by a nonlinear regression model utilizing a four-parameter analytic method.

Western Blot Analysis

A total of 2×10^6 Ba/F3 cells were treated in 6-well plates for 6 hours with lorlatinib. Cell protein lysates were prepared as previously described (24). Phospho-ALK (Y1282/1283), ALK, and β-Actin antibodies were obtained from Cell Signaling Technology.

Animals

All animal studies were conducted in accordance with the guidelines as published in the Guide for the Care and Use of Laboratory Animals. Experiments were approved by the Institutional Animal Care and Use Committee of MGH. Female Nu/Nu mice aged 6–8 weeks were obtained from Charles River laboratories. Mice were maintained in laminar flow units in sterile filter-top cages with Alpha-Dri bedding.

Patients and Treatment

Patients with ALK-positive NSCLC and disease progression on an ALK inhibitor underwent repeat biopsies of lorlatinib-resistant tumors between November 2014 and October 2017. Standard histopathology was performed to confirm the presence of malignancy. Electronic medical records were retrospectively reviewed to obtain clinical data and treatment histories. All patients provided signed informed consent under an Institutional Review Board (IRB)-approved protocol. This study was conducted in accordance with the Belmont Report and the U.S. Common Rule.

Genotype Assessments

All post-lorlatinib biopsies were analyzed for *ALK* resistance mutations using either the MGH SNaPshot NGS platform (25) or the commercially available FoundationOne platform. The MGH SNaPshot platform uses anchored multiplex polymerase chain reaction (PCR) to detect single-nucleotide variants (SNVs) and insertions/deletions within 39 cancer-related genes (version 1) or 91 cancer-related genes (version 2) including *ALK* (exons 22, 23, and 25 with version 1 or exon 21–23 and 25 with version 2), and to detect copy number variants in 92–94 cancer-related genes (version 2). This assay can detect SNV and indel variants at ≥5% allelic frequency in target regions with sufficient read coverage.

For WES, genomic DNA was extracted from formalin-fixed paraffin-embedded samples. Whole-exome capture libraries were constructed from 100ng of extracted tumor and normal DNA following shearing, end repair, phosphorylation and ligation to barcoded sequencing adapters. Ligated DNA was size-selected for lengths between 200–350bp and subjected to exonic hybrid capture using SureSelect v2 Exome bait (Agilent). Samples were multiplexed and sequenced on multiple Illumina HiSeq flowcells (paired end 76bp reads) to average depth of coverage of 41–250× and 90–107× for tumor and normals, respectively.

Massively parallel sequencing data were processed using two consecutive pipelines as previously described (19).

Enzyme Kinetic Assays and Computational Methods

These are described in detail in Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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STATEMENT OF SIGNIFICANCE

Treatment with sequential first-, second-, and third-generation ALK inhibitors can select for compound *ALK* mutations that confer high-level resistance to ALK targeted therapies. A more efficacious long-term strategy may be upfront treatment with a third-generation ALK inhibitor in order to prevent the emergence of on-target resistance.

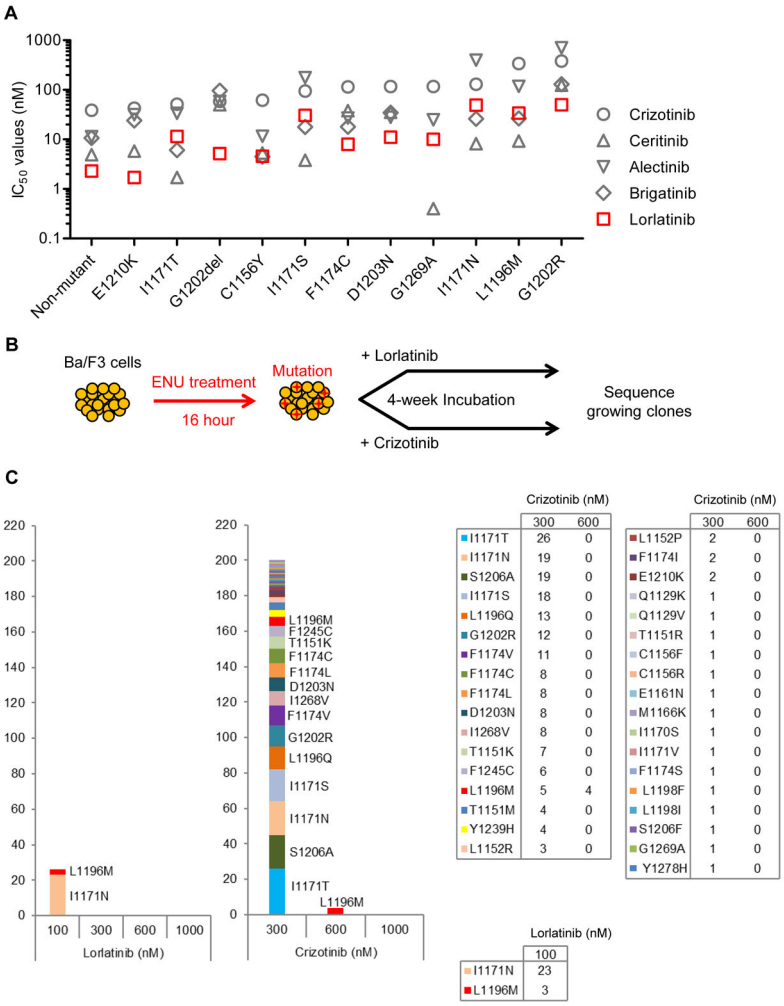

Figure 1. No single *ALK* mutations confer high level resistance to lorlatinib

A, for reference are shown previously reported IC_{50} values of first-, second-, and third-generation ALK inhibitors on cellular ALK phosphorylation in Ba/F3 cells expressing non-mutant or mutant EML4-ALK (adapted from ref (12)). **B,** scheme of ENU mutagenesis screen using Ba/F3 cells. **C,** summary of the type and number of *ALK* kinase domain mutations identified in the mutagenesis screen using Ba/F3 cells harboring non-mutant EML4-ALK (either variant 1 or variant 3). Numerous crizotinib-resistant clones were identified, as shown in the right panel. In contrast, no lorlatinib-resistant clones were identified at comparable and clinically achievable drug concentrations, as shown in the left panel. Shown are combined data from two independent experiments.

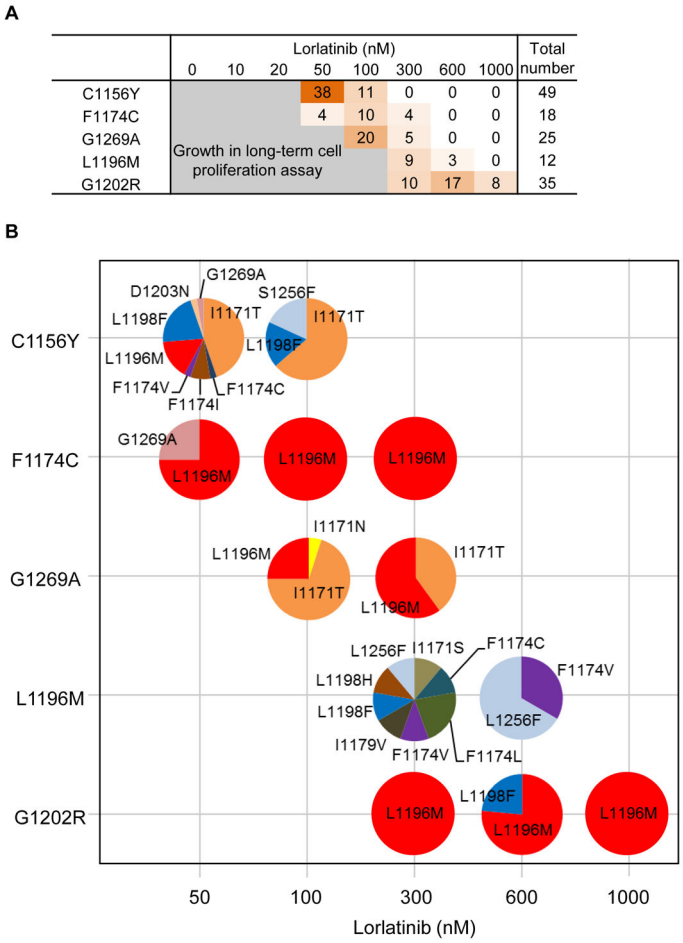

Figure 2. Multiple different compound *ALK* mutations can cause resistance to lorlatinib

A, shown is a summary of the lorlatinib concentrations used in ENU mutagenesis screening of each Ba/F3 EML4-ALK mutant model. Grey wells indicate lorlatinib concentrations that were insufficient to prevent clonal outgrowth in long-term cell proliferation assays; screening was performed at lorlatinib concentrations that could prevent clonal outgrowth (see Supplementary Figure S2). The numbers of growing clones at each drug concentration in each model are shown. **B,** summary of compound *ALK* mutations identified in growing, lorlatinib-resistant clones after ENU mutagenesis. Each mutant EML4-ALK model is listed on the left. The x-axis depicts increasing concentrations of lorlatinib (from 50 to 1000 nM). Each pie chart depicts the secondary *ALK* mutation(s) that were identified and that led to lorlatinib resistance, as well as the relative abundance of each compound mutation. Shown are combined data of two independent experiments using Ba/F3 cells expressing mutant EML4-ALK (variant 1).

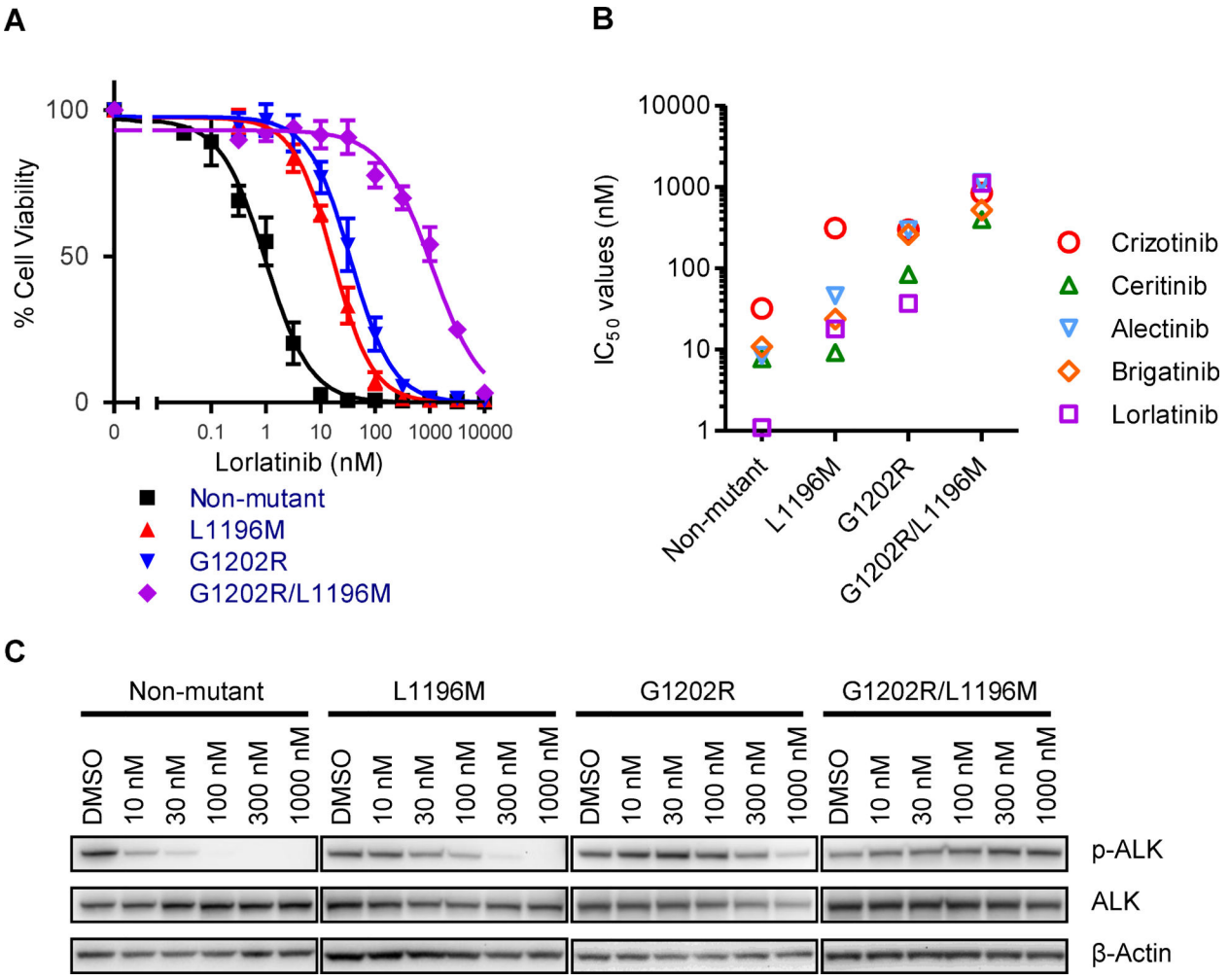

Figure 3. Functional validation of the lorlatinib-resistant *ALK* G1202R/L1196M compound mutant identified by ENU mutagenesis

A, cell viability assay of Ba/F3 cells expressing EML4-ALK variant 1, either non-mutant, single mutant (L1196M or G1202R), or compound mutant (G1202R/L1196M). Data are mean ± s.e.m. of three replicates. **B,** comparison of lorlatinib's activity with that of other ALK inhibitors in the same Ba/F3 models. Shown are absolute IC_{50} values. The compound mutant confers resistance to all generations of ALK inhibitors. Data are mean of three replicates. **C,** ALK phosphorylation in the same Ba/F3 models treated with lorlatinib, as assessed by immunoblotting of cell lysates. Lorlatinib potently suppresses ALK activation in non-mutant and single mutant EML4-ALK models, but fails to inhibit ALK in Ba/F3 cells expressing the compound mutant.

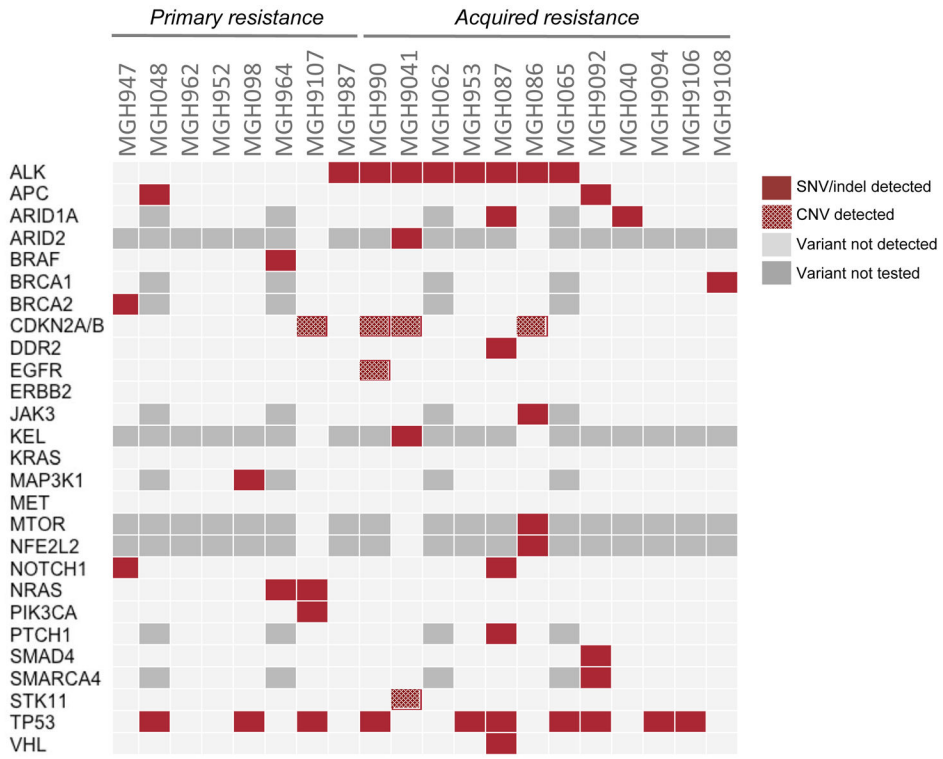

Figure 4. Summary of the genetic landscape of lorlatinib-resistant cancers

All clinical specimens underwent targeted NGS testing using either the MGH SNaPshot assay or the FoundationOne platform (see Supplementary Table S3). Shown here are known oncogenes (*ALK*, *BRAF*, *EGFR*, *ERBB2*, *KRAS*, and *MET*) and genes for which an alteration was detected in at least one sample. SNV, single nucleotide variant; indel, insertion or deletion; CNV, copy number variant.

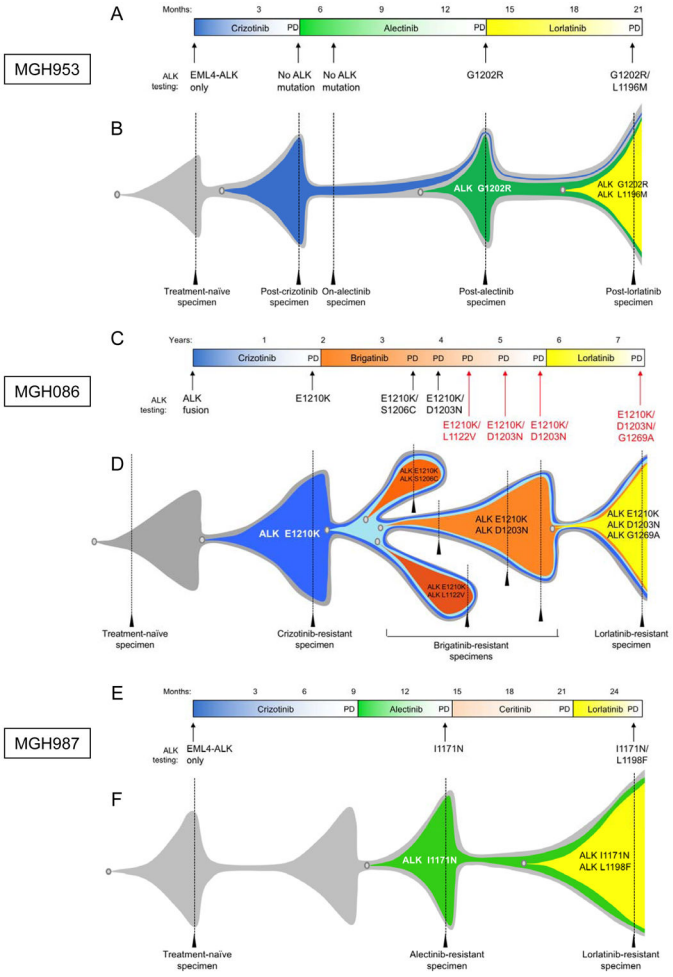

Figure 5. Clonal evolution of resistance to sequential ALK targeted therapies

A, treatment course of patient MGH953. This patient received sequential first-, second-, and third-generation ALK inhibitors, with initial response and then relapse on each drug. At each progression event, the patient's recurrent malignant pleural effusion was drained and a cytology block was prepared. The resistant cancers underwent SNaPshot NGS profiling, with the *ALK* sequencing results shown below the timeline. **B**, clonal analysis based on whole exome sequencing of MGH953 samples. The alectinib-resistant clone harboring *ALK* G1202R acquired an additional *ALK* L1196M mutation on the same allele, leading to clinical relapse on lorlatinib. **C**, treatment course of patient MGH086. This patient was also treated with sequential first-, second-, and third-generation ALK inhibitors, with initial response and then relapse on each drug. All five brigatinib-resistant specimens were excisions of a recurring left axillary nodal mass, while the post-lorlatinib specimen was an excisional biopsy of a growing subcutaneous metastasis. **D**, clonal analysis based on whole exome sequencing of MGH086 samples. Of note, we previously reported the results of clonal analysis up to the second brigatinib-resistant specimen (ref. 12). Here we have extended the clonal analysis with the addition of three brigatinib-resistant and one lorlatinib-resistant specimens (indicated in red). The dominant brigatinib-resistant clone harboring *ALK* E1210K/D1203N acquired an additional *ALK* G1269A mutation, leading to clinical

relapse on lorlatinib. **E,** treatment course of patient MGH987. This patient received four sequential ALK inhibitors, including lorlatinib. Repeat biopsies were performed at the time of resistance to alectinib and lorlatinib; both resistant specimens were derived from a progressive liver metastasis. **F**, clonal analysis based on whole exome sequencing of MGH987 samples. The alectinib-resistant clone harboring *ALK* I1171N acquired an additional *ALK* L1198F mutation on the same allele.

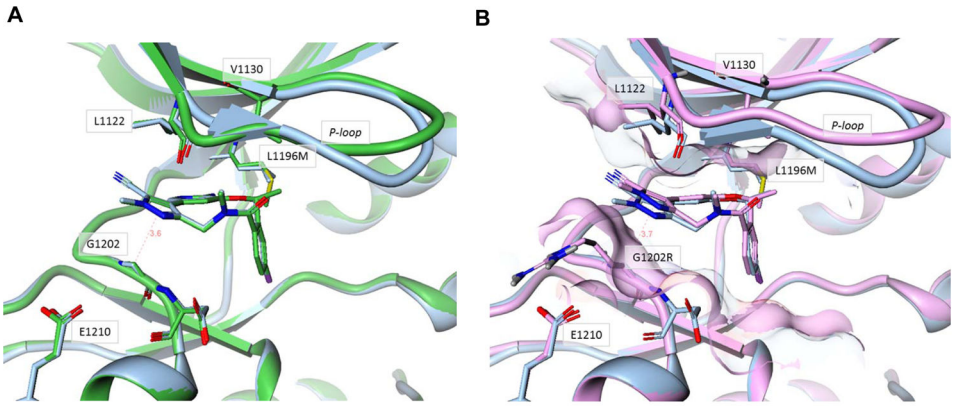

Figure 6. Structural basis for lorlatinib resistance mediated by the compound *ALK* G1202R/L1196M mutant

A, co-crystal structures of non-mutant (blue) and ALK L1196M (green) tyrosine kinase domains bound to lorlatinib. **B**, aligned co-crystal structure of non-mutant ALK (blue) and model of the compound ALK G1202R/L1196M mutant (pink) based on MD simulations. The model comes from modifying the crystal structure of ALK L1196M with lorlatinib to replicate differences that were seen between the non-mutant and mutant kinase domains in MD simulations.

Table 1

ALK mutations in pre- and post-lorlatinib biopsies

Patient ID	Resistance*	Pre-lorlatinib†	Post-lorlatinib
MGH947	Primary	--	No ALK mutation
MGH048	Primary	--	No ALK mutation
MGH962	Primary	--	No ALK mutation
MGH952	Primary	No ALK mutation	No ALK mutation
MGH098	Primary	No ALK mutation	No ALK mutation
MGH964	Primary	No ALK mutation	No ALK mutation
MGH9107	Primary	No ALK mutation	No ALK mutation
MGH987	Primary	--	ALK I1171N + L1198F§
MGH990	Acquired	--	ALK I1171N + D1203N
MGH9041	Acquired	--	ALK G1202R + G1269A
MGH062	Acquired	ALK C1156Y	ALK C1156Y + L1198F‡
MGH953	Acquired	ALK G1202R	ALK G1202R + L1196M**
MGH087	Acquired	ALK G1202R	ALK G1202R + L1204V + G1269A**
MGH086	Acquired	ALK E1210K + D1203N	ALK E1210K + D1203N + G1269A**
MGH065	Acquired	ALK L1196M	ALK G1269A
MGH9092	Acquired	ALK I1171N	No ALK mutation
MGH040	Acquired	ALK G1202R	No ALK mutation
MGH9094	Acquired	--	No ALK mutation
MGH9106	Acquired	--	No ALK mutation
MGH9108	Acquired	--	No ALK mutation

*Resistance is classified as primary (i.e., best response is worsening disease) or acquired (i.e., initial response to lorlatinib or stable disease ≥ 6 months, followed by worsening disease).

†Pre-lorlatinib biopsies were obtained on the ALK TKI used prior to lorlatinib.

§These mutations were shown to be in *cis* by amplifying *EML4-ALK* from frozen tumor-derived cDNA, subcloning the PCR products into pCR4-TOPO and sequencing individual bacterial colonies, as described in ref. 19.

‡These mutations were previously reported to be in *cis* (ref. 19).

**These mutations were shown to be in *cis* by SNaPshot NGS testing. In the case of the triple *ALK* mutant in MGH087, *ALK* G1202R and L1204V were confirmed to be in *cis* by SNaPshot NGS; in the triple *ALK* mutant in MGH086, *ALK* E1210K and D1203N were confirmed to be in *cis* by FoundationOne.