HHS Public Access

Author manuscript

Clin Cancer Res. Author manuscript; available in PMC 2021 November 15.

Published in final edited form as:

Clin Cancer Res. 2021 May 15; 27(10): 2899–2909. doi:10.1158/1078-0432.CCR-21-0032.

Spectrum of Mechanisms of Resistance to Crizotinib and Lorlatinib in *ROS1* Fusion-Positive Lung Cancer

Jessica J. Lin[1,2,*], **Noura J. Choudhury**[3,*], **Satoshi Yoda**[1,2,*], **Viola W. Zhu**[4], **Ted W. Johnson**[5], **Ramin Sakhtemani**[1,2], **Ibiayi Dagogo-Jack**[1,2], **Subba R. Digumarthy**[6], **Charlotte Lee**[1,2], **Andrew Do**[1,2], **Jennifer Peterson**[1,2], **Kylie Prutisto-Chang**[1,2], **Wafa Malik**[1,2], **Harper G. Hubbeling**[3], **Adam Langenbucher**[1,2], **Adam J. Schoenfeld**[3], **Christina J. Falcon**[3], **Jennifer S. Temel**[1,2], **Lecia V. Sequist**[1,2], **Beow Y. Yeap**[1,2], **Jochen K. Lennerz**[1,2], **Alice T. Shaw**[1,2,7], **Michael S. Lawrence**[1,2], **Sai-Hong Ignatius Ou**[4], **Aaron N. Hata**[1,2,#], **Alexander Drilon**[3,#], **Justin F. Gainor**[1,2,#]

[1]Department of Medicine, Massachusetts General Hospital Cancer Center, Boston, MA

[2]Harvard Medical School, Boston, MA

[3]Department of Medicine, Memorial Sloan Kettering Cancer Center and Weill Cornell Medical College, NY

[4]Department of Medicine, University of California Irvine, Orange, CA

[5]Pfizer Worldwide Research and Development, La Jolla, CA

[6]Department of Radiology, Massachusetts General Hospital, Boston, MA

[7]currently at Novartis Institutes for Biomedical Research, Cambridge, MA.

Abstract

Background: Current standard initial therapy for advanced, *ROS1* fusion-positive (ROS1+) non-small cell lung cancer (NSCLC) is crizotinib or entrectinib. Lorlatinib, a next-generation ALK/ROS1 inhibitor, recently demonstrated efficacy in ROS1+ NSCLC including in crizotinib-pretreated patients. However, mechanisms of lorlatinib resistance in ROS1+ disease remain poorly understood. Here, we assessed mechanisms of resistance to crizotinib and lorlatinib.

Patients and Methods: Biopsies from ROS1+ NSCLC patients progressing on crizotinib or lorlatinib were profiled by genetic sequencing.

Results: From 55 patients, 47 post-crizotinib and 32 post-lorlatinib biopsies were assessed. Among 42 post-crizotinib and 28 post-lorlatinib biopsies analyzed at distinct timepoints, *ROS1* mutations were identified in 38% and 46%, respectively. *ROS1* G2032R was the most common occurring in approximately a third of cases. Additional *ROS1* mutations included: D2033N (2.4%)

Corresponding Author: Dr. Justin F. Gainor, Department of Thoracic Oncology, Massachusetts General Hospital Cancer Center, 32 Fruit Street, Boston, MA 02114, USA. Telephone: 617-724-4000. jgainor@mgh.harvard.edu.
*These authors contributed equally.
#These authors contributed equally.

Conflicts of Interest:
All remaining authors have declared no potential conflicts of interest.

and S1986F (2.4%) post-crizotinib; L2086F (3.6%), G2032R/L2086F (3.6%), G2032R/S1986F/L2086F (3.6%), and S1986F/L2000V (3.6%) post-lorlatinib. Structural modeling predicted ROS1^{L2086F} causes steric interference to lorlatinib, crizotinib, and entrectinib, while it may accommodate cabozantinib. In Ba/F3 models, ROS1^{L2086F}, ROS1$^{G2032R/L2086F}$, and ROS1$^{S1986F/G2032R/L2086F}$ were refractory to lorlatinib but sensitive to cabozantinib. A patient with disease progression on crizotinib and lorlatinib and *ROS1* L2086F received cabozantinib for nearly 11 months with disease control. Among lorlatinib-resistant biopsies, we also identified *MET* amplification (4%), *KRAS* G12C (4%), *KRAS* amplification (4%), *NRAS* mutation (4%), and *MAP2K1* mutation (4%).

Conclusions: *ROS1* mutations mediate resistance to crizotinib and lorlatinib in over one-third of cases, underscoring the importance of developing next-generation ROS1 inhibitors with potency against these mutations including G2032R and L2086F. Continued efforts are needed to elucidate ROS1-independent resistance mechanisms.

Keywords

ROS1; non-small cell lung cancer; resistance; crizotinib; lorlatinib (provide 5)

INTRODUCTION

ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*) gene fusions define a unique molecular subset of non-small cell lung cancer (NSCLC) highly sensitive to treatment with ROS1 tyrosine kinase inhibitors (TKIs) (1,2). Crizotinib, a multitargeted anaplastic lymphoma kinase (ALK)/ROS1/MET proto-oncogene, receptor tyrosine kinase (MET) inhibitor, was the first TKI to receive US Food and Drug Administration (FDA) and European Medicines Agency (EMA) approval for the treatment of advanced *ROS1* fusion-positive lung cancer (3,4). In a phase 1 expansion cohort of *ROS1* fusion-positive lung cancer patients, crizotinib resulted in a significant objective response rate (ORR; 72%) and progression-free survival (PFS; median 19.2 months) (3,4), and additional single-arm studies of crizotinib have supported its efficacy (5–7). More recently, a ROS1/TRK/ALK inhibitor entrectinib has also attained approval on the basis of efficacy demonstrated in an integrated analysis of three phase I-II trials (ORR 77%; median PFS 19.0 months) (8).

Despite these advances and the significant initial benefit seen in most patients with *ROS1* fusion-positive lung cancer treated with ROS1 inhibitors, TKI resistance remains a major hurdle limiting duration of benefit to these therapies. Given the significant clinical and biological similarities between ROS1 and ALK, efforts to combat ROS1 inhibitor resistance have commonly drawn from the experiences in *ALK* fusion-positive NSCLC. Lorlatinib, a highly brain-penetrant, next-generation ALK and ROS1 inhibitor with FDA approval in *ALK* fusion-positive lung cancer, has recently demonstrated efficacy in *ROS1* fusion-positive disease with an ORR of 62% among treatment-naïve and 35% among crizotinib pre-treated patients (duration of response 25.3 and 13.8 months, and CNS ORR 64% and 50%, respectively) in a phase I-II study (9). The National Comprehensive Cancer Network (NCCN) Guidelines list lorlatinib as a subsequent option after disease progression on crizotinib or entrectinib in *ROS1* fusion-positive lung cancer (10). Nonetheless, resistance inevitably develops on lorlatinib as well.

Thus far, efforts to understand mechanisms of resistance to ROS1 inhibitors have been centered on ROS1-dependent resistance to the oldest TKI crizotinib. A number of crizotinib-resistant *ROS1* resistance mutations have been identified in patient samples, including the most commonly occurring *ROS1* G2032R (11–13) in addition to *ROS1* D2033N (14), L2026M (15), L1951R (16), and S1986F/Y (12,16). However, the frequency of *ROS1* mutations reported in these small case series has ranged widely from 8% to over 50% (12,13,15). Furthermore, outside of an isolated case report of *ROS1* G2032K mutation conferring resistance to lorlatinib (17), mechanisms of resistance to lorlatinib in *ROS1* fusion-positive lung cancer have not yet been elucidated.

Here, we present the largest multi-institutional series to date of repeat biopsies from patients with *ROS1* fusion-positive NSCLC progressing on crizotinib or lorlatinib, analyzed by gene sequencing in order to characterize the frequency and spectrum of resistance mechanisms.

MATERIALS AND METHODS

Patients

We identified patients with advanced or metastatic NSCLC harboring *ROS1* fusion detected by local molecular profiling [e.g., fluorescent in situ hybridization (FISH), DNA-based next-generation sequencing (NGS), or targeted RNA sequencing]. Patients must have received ROS1 inhibitor(s) with post-treatment tumor or plasma analyzed by genotyping. Patients were identified at three institutions: Massachusetts General Hospital (MGH; Boston, MA; n=37), Memorial Sloan Kettering Cancer Center (MSKCC; New York, NY; n=11), and University of California Irvine (UCI; Irvine, CA; n=7). All studies were performed under Institutional Review Board-approved protocols at respective institutions.

Data Collection

Medical records were retrospectively reviewed and data extracted on clinical, pathologic, and molecular features. Data were updated as of September 2020. Time to progression (TTP) was measured from the time of therapy initiation to clinical/radiographic disease progression. Patients without documented progression were censored at last follow-up. Duration of therapy was measured from the time of therapy initiation to therapy discontinuation. Patients continuing on therapy were censored at last follow-up.

Biopsy Genotyping

All patients underwent tumor or plasma biopsy after treatment with crizotinib and/or lorlatinib and genotyping after providing informed consent. Of the 56 tissue biopsies, 54 were analyzed using: the MGH SNaPshot DNA-based genotyping panel and a separate RNA-based NGS assay (Solid Fusion Assay) (n=27) (18), FoundationOne (n=11; Foundation Medicine, Inc.; Cambridge, MA), MSK IMPACT (n=9) (19), OncoPanel (n=4) (20), Ion Ampliseq Comprehensive Cancer Panel (n=1; Thermo Fisher Scientific; Waltham, MA), Moffitt STAR Solid Tumor Assay (n=1; Moffitt, Tampa, FL), and University of Vermont Medical Center Solid Tumor Gene Panel (n=1; University of Vermont, Burlington, VT). Two tumor biopsies were analyzed by whole exome sequencing as described (21). Sixteen of the 27 samples analyzed by MGH SNaPshot additionally underwent analysis by

Sanger sequencing of the *ROS1* kinase domain (Supplementary Methods). Twenty-three liquid biopsies were genotyped using the Guardant360 cell-free DNA (cfDNA) (n=17; Guardant Health, Inc.; Redwood City, CA), FoundationACT (n=3; Foundation Medicine, Inc.; Cambridge, MA), or Resolution Bioscience (n=3; Resolution Bioscience, Inc.; Kirkland, WA) assays. *MET* FISH was performed on two post-treatment tumor samples as described (Supplementary Methods) (22).

Drug Sensitivity Assays

Ba/F3 cells expressing nonmutant *CD74-ROS1* or various mutant *CD74-ROS1* were generated as in Supplementary Methods (reagents are also included therein) and as described previously (23), and were plated (2,000–10,000) in triplicate into 96-well plates. Two days after drug treatment, the cell viability was measured by using CellTiter-Glo (Promega; Madison, WI) and a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, LLC; San Jose, CA). The luminescence values were normalized to untreated wells. GraphPad Prism (GraphPad Software; San Diego, CA) was used to display data and determine IC_{50} values by the four-parameter logistic regression model. Western blotting was performed per Supplementary Methods.

Structural Modeling

Co-crystal structure of lorlatinib in ROS1 (PDB 4UXL) and a close-in analogue of cabozantinib, foretinib, in MET (PDB 3LQ8) were used as starting points for modeling. Details are provided in Supplementary Methods.

Statistical Analysis

TTP and duration of therapy medians were estimated using the Kaplan-Meier method (Stata version 14.2). Fisher's exact test was used to compare the frequency of *ROS1* mutations between lorlatinib-resistant and crizotinib-resistant specimens based on a two-sided hypothesis.

RESULTS

Patient Characteristics and Outcomes on Crizotinib and Lorlatinib

Between 2014 and 2020, a total of 55 patients with advanced *ROS1* fusion-positive NSCLC who were treated with crizotinib and/or lorlatinib underwent post-treatment biopsies with sequencing analyses. Baseline characteristics are shown in Table 1. The median age at diagnosis was 50 (range, 22–81). All patients had adenocarcinoma and the majority (96%) were never or light smokers. The *ROS1* fusion partner was known for most patients (85%), *CD74* being the most common fusion partner detected in 44%.

All patients in the cohort received crizotinib. The median TTP on crizotinib was 10.1 months [95% confidence interval (CI), 6.9–12.4 months], and the median duration of therapy was 13.0 months (95% CI, 10.0–18.8 months). Fourteen of 55 patients did not undergo a post-crizotinib biopsy but were included as they had a subsequent post-lorlatinib biopsy. Among 41 patients who did have post-crizotinib biopsies, the median TTP on crizotinib was 10.5 months (95% CI, 6.9–18.8 months) with median duration of therapy of 14.7 months (95%

CI, 10.6–24.3 months). Twenty-five patients received lorlatinib and had post-lorlatinib biopsies. All 25 patients had received prior crizotinib; 3 had additionally received prior entrectinib (Supplementary Fig. S1). The median TTP on lorlatinib was 8.5 months (95% CI, 5.1–13.8 months), with median duration of therapy of 13.7 months (95% CI, 8.4–18.7 months).

From this cohort, 11 (20%) underwent both post-crizotinib and post-lorlatinib biopsies, 30 (55%) had at least one post-crizotinib biopsy (thus, a total of 41 patients had post-crizotinib biopsies), and 14 (25%) had at least one post-lorlatinib biopsy (thus, a total of 25 patients had post-lorlatinib biopsies) (Figure 1; Supplementary Fig. S1). A subset of patients had paired tissue and plasma analyses at the time of disease progression, or underwent serial repeat biopsies on the same TKI (Figure 1; Supplementary Fig. S1). In total, 47 post-crizotinib and 32 post-lorlatinib biopsies were analyzed.

ROS1 Resistance Mutations After Crizotinib

We first examined the distribution of ROS1-dependent resistance in patients progressing on crizotinib. The 47 post-crizotinib biopsies included 5 sets of paired tissue and plasma collected at the same timepoint, thus representing 42 timepoint-distinct biopsies. The gene alterations detected in the crizotinib-resistant biopsies are summarized in Supplementary Fig. S2 and Supplementary Table 1.

ROS1 resistance mutations were identified in 16 cases (38%). Consistent with the prior literature (12,13), the most common *ROS1* resistance mutation detected in approximately a third of cases was the solvent front mutation, *ROS1* G2032R. Other *ROS1* resistance mutations included *ROS1* D2033N (2%) and *ROS1* S1986F (2%) (Figure 2A), both of which have been described in *ROS1* fusion-positive NSCLC (12,14,16). Of note, one case (MGH0003.A) was found to harbor *ROS1* G2032R in the post-crizotinib plasma [variant allele fraction (VAF) 0.4%] but not in the paired crizotinib-resistant liver specimen. This patient subsequently continued on crizotinib with the addition of chemotherapy and had initial disease response. Nine months later, the patient again experienced disease progression, and repeat paired biopsies of the plasma and growing pleural effusion (MGH0003.B) identified *ROS1* G2032R in both specimens (VAF 5.7% in the plasma) [described in ref. (13)]. Therefore, the discordance in the detection of *ROS1* G2032R in the initial crizotinib-resistant liver versus plasma samples (MGH0003.A) likely represented either tumor heterogeneity or early emergence of *ROS1* G2032R not captured by tissue genotyping.

ROS1 Resistance Mutations After Lorlatinib

Next, we analyzed 32 lorlatinib-resistant tissue and plasma samples which included 4 sets of paired tissue/plasma, representing a total of 28 distinct timepoint lorlatinib-resistant cases. *ROS1* resistance mutations were detected in 13 post-lorlatinib cases (46%) (Figure 2, Supplementary Fig. S2). While the frequency of *ROS1* kinase domain mutations was numerically higher in lorlatinib-resistant as compared to crizotinib-resistant specimens (46% vs 38%, respectively), this difference was not statistically significant (p=0.621).

The most common *ROS1* mutation identified in 9 post-lorlatinib samples (32%) was again *ROS1* G2032R. Four of these 9 cases with *ROS1* G2032R had matched post-crizotinib/pre-lorlatinib specimens analyzed (Supplementary Tables 1–2). In 3 of these 4 cases, *ROS1* G2032R was pre-existing in the post-crizotinib samples, and TTP on lorlatinib for these patients ranged from 3.2 to 6.1 months. In the remaining case, MGH0024, the post-crizotinib/pre-lorlatinib liver specimen (MGH0024.A) was found to harbor a *ROS1* S1986F mutation, while whole exome sequencing of the subsequent lorlatinib-resistant liver specimen (TTP on lorlatinib of 5.1 months) revealed *ROS1* G2032R and L2086F mutations in addition to the previously seen *ROS1* S1986F. We were unable to determine whether the mutations were present *in cis* or *in trans* due to the distance between the nucleotides. Of note, sequencing of the post-lorlatinib plasma (MGH0024.B) from this patient also detected *ROS1* G2032R (VAF 12.3%), which had been absent in the post-crizotinib/pre-lorlatinib plasma (MGH0024.A). This liquid NGS platform did not assess the exons covering *ROS1* S1986 or L2086 mutations. Therefore, in the case of MGH0024, *ROS1* G2032R was most likely acquired on lorlatinib.

Outside of *ROS1* G2032R, we identified a *ROS1* L2086F mutation—alone or together with other *ROS1* mutation(s)—in 3 lorlatinib-resistant biopsies (11%): the aforementioned MGH0024, MGH0026, and MGH0035 (Supplementary Fig. S2). $ROS1^{L2086F}$ is analogous to ALK^{L1256F}, which confers resistance to crizotinib and lorlatinib in *ALK* fusion-positive lung cancer in preclinical models (24,25). In addition, a *ROS1* double mutation, S1986F and L2000V *in cis*, was observed in the lorlatinib-resistant left-sided pleural fluid sample of MGH0032.B (Figure 2A). This patient's crizotinib-resistant/pre-lorlatinib right pleural fluid had detected no *ROS1* mutations. In total, three post-lorlatinib biopsies (11%) were found to harbor ≥2 *ROS1* resistance mutations (Figure 2A), of which two included G2032R. All 3 cases had received prior crizotinib.

Overall, 11 of the 28 lorlatinib-resistant cases had matched post-crizotinib/pre-lorlatinib biopsies analyzed by NGS (Supplementary Table 2). Six of these 11 cases (55%) had no *ROS1* mutation detected in either post-crizotinib or post-lorlatinib specimen. Three cases (27%) acquired new *ROS1* mutations on lorlatinib. Specifically, in two cases (18%), no *ROS1* mutations were detected post-crizotinib whereas either *ROS1* G2032R (MGH0039.B) or S1986F/L2000V (MGH0032.B) mutations were identified post-lorlatinib; and in one case (9%), *ROS1* S1986F was detected pre-lorlatinib with the identification of two additional *ROS1* mutations G2032R and L2086F post-lorlatinib (MGH0024.B). The remaining three cases had *ROS1* G2032R in both post-crizotinib and post-lorlatinib biopsies (MGH0036, MGH0039, UCI0002).

Preclinical Activity of ROS1 TKIs Against Mutant ROS1 Kinases

To assess the functional role and potential clinical implications of the spectrum of *ROS1* mutations detected in our cohort, we generated Ba/F3 cell lines expressing nonmutant *CD74-ROS1* or mutant *CD74-ROS1* harboring G2032R, L2000V, L2086F, S1986F/L2000V, S1986F/L2086F, S1986F/G2032R, G2032R/L2086F, or S1986F/G2032R/L2086F. Cells were treated with the FDA-approved ROS1 inhibitors crizotinib and entrectinib, or other TKIs with activity against ROS1 (lorlatinib, repotrectinib, cabozantinib, ceritinib, brigatinib,

and taletrectinib), as well as alectinib (an ALK TKI without any ROS1 activity), and cell viability was measured after two days to determine the potency of these TKIs against the various mutant ROS1 kinases.

We and others have previously reported *ROS1* G2032R as a solvent front mutation conferring resistance to crizotinib (11,12,26). In the Ba/F3 models, $ROS1^{G2032R}$ was indeed refractory to crizotinib, entrectinib, ceritinib, and brigatinib. Lorlatinib was not potent against this mutation, with an IC_{50} of 196.6 nM (vs 0.7 nM with $ROS1^{wild\text{-}type}$) (Figure 3A–B). The next-generation ROS1/TRK inhibitor repotrectinib and a type II TKI cabozantinib maintained potency against $ROS1^{G2032R}$ (IC_{50} 23.1 nM and 17.5 nM, respectively), while taletrectinib had moderate potency against $ROS1^{G2032R}$ (IC50 53.3 nM, vs 2.6 nM with $ROS1^{wild\text{-}type}$).

$ROS1^{L2086F}$ conferred resistance to lorlatinib in this Ba/F3 model (Figure 3A–B, Supplementary Fig. S3), in concordance with the clinical detection of *ROS1* L2086F in the lorlatinib-resistant biopsies. $ROS1^{L2086F}$ was notably refractory to the majority of other currently available ROS1 inhibitors. Brigatinib had a relatively lower IC_{50} for $ROS1^{L2086F}$ although remained 17-fold less potent against this mutant compared to the nonmutant kinase (IC_{50} 159.3 nM with $ROS1^{L2086F}$ vs 9.4 nM with $ROS1^{wild\text{-}type}$). The L2086F-harboring compound mutant kinases $ROS1^{S1986F/L2086F}$, $ROS1^{G2032R/L2086F}$ and $ROS1^{S1986F/G2032R/L2086F}$ were also highly refractory to lorlatinib, crizotinib and entrectinib, as well as ceritinib, taletrectinib, and repotrectinib. Only cabozantinib, a type II multitargeted TKI, maintained strong potency across these *ROS1* mutations. Consistent with the observed effects on cell viability, the phosphorylation of $ROS1^{L2086F}$ was sustained in the presence of most ROS1 inhibitors even at the concentrations of 300 nM (Figure 3C). By contrast, cabozantinib suppressed $ROS1^{L2086F}$ (and all L2086F-harboring compound mutant ROS1 kinases) at 30 nM (Supplementary Fig. S4).

As *ROS1* L2000V mutation has not previously been described, we also assessed the functional consequence of the *ROS1* S1986F/L2000V mutation detected in a lorlatinib-resistant case. Both $ROS1^{L2000V}$ and $ROS1^{S1986F/L2000V}$ resulted in relatively decreased sensitivity to crizotinib (IC_{50} 37.1 nM or 159.4 nM, respectively, vs 5.4 nM with $ROS1^{wild\text{-}type}$) (Figure 3A, Supplementary Fig. S3, Supplementary Fig. S5). On the other hand, neither mutant kinase caused resistance to lorlatinib in the Ba/F3 model (IC_{50} 2.5 nM or 2.4, respectively, vs 0.7 nM with $ROS1^{wild\text{-}type}$), highlighting the importance of functionally validating on-target genetic alterations.

Structural Modeling of $ROS1^{L2086F}$ and Clinical Benefit from Cabozantinib

Next, we performed structural modeling of the refractory *ROS1* L2086F mutation. $ROS1^{L2086F}$ was predicted to cause severe steric clash against the binding of lorlatinib, in line with the Ba/F3 drug sensitivity results. In particular, the fluorophenyl group of lorlatinib that fills the pocket formed by G2101, N2084, and R2083 clashes with $ROS1^{L2086F}$ (Figure 4A), with the rigid, cyclic nature of lorlatinib not permitting independent bond rotation for better accommodation of the larger phenyl side chain at L2086 (Supplementary Fig. S6A). On the other hand, the binding of cabozantinib could be less affected, as modeled using a close analogue foretinib (27) and MET in DFG-out state (there are no reported structural

data for cabozantinib bound to ROS1, or for ROS1 DFG-out conformation) (Supplementary Fig. S6B–C).

MGH0026 was one of the lorlatinib-resistant cases in this cohort found to have an acquired *ROS1* L2086F mutation. This patient with advanced *ROS1* fusion-positive NSCLC had received prior chemotherapy, immunotherapy, followed by crizotinib with disease progression in the brain (Figure 4B). Approximately 17 months after subsequent therapy with lorlatinib, biopsy of a progressing lorlatinib-resistant lymph node revealed the *CD74-ROS1* fusion and the *ROS1* L2086F mutation (other alterations shown in Supplementary Fig. S2). On the basis of the above preclinical data, this patient was then treated with cabozantinib. The patient had disease stabilization on cabozantinib, with duration of therapy lasting nearly 11 months (Figure 4C).

Landscape of ROS1-Independent Alterations in Resistant Tumors

ROS1 resistance mutations were not identified in approximately 50–60% of this cohort, suggesting a role for ROS1-independent or bypass mechanism (Figure 2B). Therefore, we investigated potential ROS1-independent mechanisms of resistance in these specimens using the NGS results.

MET amplification is a known mechanism of bypass signaling across multiple subsets of NSCLC including EGFR-, ALK-, and RET-driven NSCLC (22,28–32). *MET* amplification was identified in two (2.9%) biopsies of our ROS1 cohort. MGH0034 with advanced *CD74-ROS1* fusion-positive NSCLC had no evidence of *MET* amplification in the treatment-naïve pleural fluid (*MET* FISH negative with *MET*:*CEP7* ratio of 1.0), received crizotinib for 4.7 months followed by lorlatinib. The lorlatinib-resistant brain specimen demonstrated the known *CD74-ROS1* fusion and acquired *MET* gain confirmed by FISH (*MET*:*CEP7* ratio of 6.3) (Supplementary Fig. S2). In addition, acquired *MET* amplification was detected in the crizotinib-resistant adrenal biopsy of patient MGH0014 by NGS and *MET* FISH (*MET*:*CEP7* ratio >25:1) (Supplementary Fig. S2). In this patient, paired crizotinib-resistant plasma analysis revealed *MET* gain as well as a *MET* L1195V mutation (VAF 3.6%; VAF of *ROS1-SLC34A2* 0.6%) (Figure 2B), which is known to cause resistance to MET inhibitors including crizotinib (33). While we identified additional *MET* alterations in three (4.3%) biopsies, these consisted of *MET* V1271M (UCI0003) and *MET* R469Q (MGH0037.B) variants of unclear significance, and a *WAC* intron 3 to *MET* intron 17 fusion of unclear significance in a lorlatinib-resistant case with *ROS1* G2032R (MGH0022) (Supplementary Fig. S2; Supplementary Table 1).

Although activating *EGFR* mutations E709K and L858R were detected in the crizotinib-resistant lung nodule of UCI0004.A (sample insufficient for *ROS1* fusion assessment), this case was from a patient who initially presented with multifocal bilateral lung nodules. The biopsied oligoprogressing lung nodule was treated with stereotactic body radiation therapy, and the patient continued on crizotinib for >4 years thereafter before switching to lorlatinib. Plasma biopsy at the time of progression on lorlatinib detected neither *ROS1* fusion nor *EGFR* mutations, but rather identified a *KRAS* Q61H mutation, raising the possibility of multifocal lung cancer. Three additional cases had *EGFR* variants of unlikely functional consequence (MGH0039.A with *EGFR* R521K polymorphism; and UCI0003 and UCI0007

with *EGFR* S1120R and R377S mutations, respectively, both outside the protein kinase domain).

Alterations in genes affecting the RAS-MAPK signaling node (such as *NF1*, *MAP2K1*, *MAP3K1*, and *KRAS*) may mediate resistance to ROS1 inhibitors (34). As an example, *KRAS* G12D mutation has been reported in a patient with crizotinib-resistant *ROS1* fusion-positive lung adenocarcinoma (35). Overall, we observed *KRAS* mutations in 6 time-distinct biopsies (8.6%), two of which were canonical alterations *KRAS* G12C and Q61H (MSKCC51 with acquired *KRAS* G12C which was absent in the treatment-naïve tissue; and UCI0004.B with *KRAS* Q61H, respectively), and four of which involved variants of unknown significance (VUS: V8I in MGH0003.A and MGH0003.B, I24N in MGH0018, V114A in MGH0035) (Supplemental Fig. S2). Additionally, *KRAS* amplification concomitant with *CD74-ROS1* fusion was detected in the lorlatinib-resistant lung and plasma biopsies of MGH0028, whose treatment-naïve biopsy did not harbor *KRAS* amplification. One lorlatinib-resistant case [MSKCC11; previously published (34)] had the *MAP2K1* E41_L54del in-frame deletion mutation, a recently described oncogenic driver mutation. Another lorlatinib-resistant plasma (MGH0038.B) had an acquired pathogenic *NRAS* G60E mutation, which affects the nucleotide binding domain and results in activation of downstream signaling (Figure 2B). Finally, *NF1* alterations were identified in 5 TKI-resistant cases (7%; all crizotinib-resistant), including two cases with loss-of-function mutations [*NF1* Q756Ter (MGH0004) and *NF1* R69fs*7 (UCI0003); Figure 2B], one case with possibly loss-of-function mutation [*NF1* c.7458–1G>C splice acceptor variant (MGH0014 – also with *MET* gain and *MET* L1195V), and two cases with VUS [*NF1* E1516D (MGH0018), P1867L (UCI0007)]. It is not known whether the *NF1* alterations may have existed pre-crizotinib in these cases.

We did not identify canonical pathogenic mutations in other drivers such as *ALK*, *BRAF*, or *ERBB2* (those marked in Supplementary Fig. S2 for these genes were VUS). Oncogenic fusions involving *ALK*, *ROS1*, *NTRK1*-3, *RET, BRAF*, or *NRG1* genes were not detected.

DISCUSSION

Since the initial report of oncogenic *ROS1* fusions in lung cancer in 2007 (36), two TKIs with ROS1 activity, crizotinib and entrectinib, have received approval across multiple countries for the treatment of advanced *ROS1* fusion-positive NSCLC and are considered standard of care (3,4,8). In addition, lorlatinib, a highly brain-penetrant, next-generation ALK/ROS1 TKI, has demonstrated efficacy in patients with advanced *ROS1* fusion-positive lung cancer, including in a subset of patients who received prior crizotinib (9). However, resistance to these TKIs is inevitable causing disease relapse in most patients. While *ROS1* mutations causing resistance to crizotinib have been reported, the studies to date have been limited to isolated case reports or small series. Questions remain regarding the scope of mechanisms of resistance to other ROS1 inhibitors such as lorlatinib in *ROS1* fusion-positive disease, and ultimately regarding how to consider the optimal sequencing of various ROS1 inhibitors currently in clinical development.

In this multi-institutional study, we evaluated a cohort of *ROS1* fusion-positive NSCLC patients treated with crizotinib and/or lorlatinib who had at least one post-treatment biopsy. We identified *ROS1* kinase domain mutations in 38% of crizotinib-resistant biopsies and in 46% of lorlatinib-resistant biopsies. In both instances, *ROS1* G2032R represented the predominant mutation, detected in approximately one-third of cases. This solvent front mutation has previously been described in the setting of crizotinib resistance, where it has been shown to cause steric clash with crizotinib binding (11). In our series, *ROS1* G2032R was identified in 32% of post-lorlatinib samples, and lorlatinib was unable to suppress *ROS1* G2032R in patients known to have this mutation pre-lorlatinib. Furthermore, we observed development of this mutation in a patient on lorlatinib therapy. Thus, in contrast to *ALK* fusion-positive lung cancer in which lorlatinib potently overcomes solvent front resistance mutations (e.g., analogous *ALK* G1202R), the experience with lorlatinib and *ROS1* G2032R in *ROS1* fusion-positive lung cancer appears distinct. This is further validated by our preclinical models which demonstrate the decreased potency of lorlatinib as well as crizotinib, entrectinib, ceritinib, and brigatinib against *ROS1* G2032R. In a phase I/II study of lorlatinib, 6 patients were known to have baseline *ROS1* G2032R from cfDNA or tumor analysis; of these, 5 had stable disease lasting between 2.9–9.6 months, 1 had primary progressive disease, and no objective responses were observed on lorlatinib (9), again consistent with modest potency of this TKI against $ROS1^{G2032R}$.

Outside of *ROS1* G2032R, *ROS1* L2086F was detected as a recurring resistance mutation in the post-lorlatinib biopsies. *ROS1* L2086F is analogous to the *ALK* L1256F mutation which causes steric clash with the fluorobenzene group of lorlatinib thereby conferring resistance to lorlatinib in *ALK* fusion-positive lung cancer (24,25), and this mutation has been described in one case resistant to taletrectinib (37). Our structural modeling predicts that $ROS1^{L2086F}$ causes steric interference with the binding of lorlatinib in addition to other type I ROS1 inhibitors such as crizotinib and entrectinib but may accommodate a type II inhibitor cabozantinib. Indeed, in Ba/F3 models, $ROS1^{L2086F}$ single mutant kinase and $ROS1^{L2086F}$-based compound mutants were highly refractory to the type I ROS1 inhibitors whereas cabozantinib maintained potency against the mutant ROS1 kinases. Furthermore, one patient with prior progression on crizotinib and lorlatinib was able to receive cabozantinib with disease control lasting nearly 11 months. These findings are reminiscent of observations in TRK fusion-positive tumors, wherein TRK xDFG mutations conferred resistance to type I TRK inhibitors through steric hindrance and represented a shared liability, but sensitized tumors to type II TRK inhibitors (38).

Our findings may help inform the approach of sequential TKI therapy using alternative ROS1 inhibitors in *ROS1* fusion-positive lung cancer, similar to the previously modeled paradigm in *EGFR*-mutant and *ALK* fusion-positive disease. For patients with disease progression on prior crizotinib or entrectinib and known *ROS1* G2032R, lorlatinib (as with ceritinib or brigatinib) is unlikely to provide significant durable benefit, and alternative inhibitors such as repotrectinib or taletrectinib may be explored on a clinical trial. Repotrectinib is a next-generation ROS1/TRK inhibitor which has demonstrated preliminary efficacy in ROS1 TKI-naïve and TKI-pretreated NSCLC; responses have been observed in patients with baseline $ROS1^{G2032R}$ (39,40) although the extent and durability of these responses await further determination. In the setting of a known *ROS1* L2086F mutation,

either alone or together with another mutation (e.g., G2032R or S1986F), other investigational inhibitors including lorlatinib, taletrectinib, and repotrectinib are predicted to be ineffective. Instead, a role for switching to a type II ROS1 inhibitor cabozantinib may be studied in this setting and potentially against xDFG *ROS1* mutations (G1201A, G1201C) (38). Cabozantinib as a multikinase inhibitor can notably be associated with significant and at times intolerable toxicities that necessitate dose interruptions and reductions or even treatment discontinuations (41). Therefore, there remains a need to develop novel, selective and tolerable ROS1 TKIs including rationally designed type II inhibitors, which retain potency against the spectrum of known *ROS1* resistance mutations such as the recurring G2032R and L2086F.

In approximately two-thirds and one-half of cases with resistance to crizotinib and lorlatinib, respectively, a *ROS1* kinase domain mutation was not identified, suggestive of ROS1-independent resistance mechanisms. We investigated potential ROS1-independent mechanisms mediating resistance in this series using genetic sequencing. RAS-MAPK pathway activation has previously been implicated in ROS1 inhibitor resistance (34,35,42). In this cohort, pathogenic *KRAS* G12C mutation, *KRAS* amplification, *NRAS* G60E mutation, and *MAP2K1* E41_L54del in-frame deletion mutation were identified in one lorlatinib-resistant case each, and *NF1* alterations were identified in 5 crizotinib-resistant cases, providing further support for the potential role of RAS-MAPK pathway dysregulation in these tumors. We additionally identified high-level *MET* amplification in one lorlatinib-resistant case without concurrent *ROS1* kinase domain mutations. Interestingly, acquired high-level *MET* amplification was also detected in one case resistant to crizotinib, an ALK/ROS1/MET inhibitor. In this crizotinib-resistant case, the *MET* amplification co-occurred with a *MET* L1195V mutation (detected in plasma) which is known to alter MET topology and cause decreased sensitivity to MET inhibitors including crizotinib (33,43). It is therefore plausible that this patient's tumor was able to overcome the constraint on MET and ROS1 imposed by crizotinib via selective expansion of a more highly *MET*-amplified subclone additionally harboring the *MET* resistance mutation. Thus, MET pathway—known to mediate resistance in multiple other lung cancers including *EGFR*-mutant, *ALK* fusion-positive, and *RET* fusion-positive NSCLC (28–32)—may represent another recurring bypass mechanism in *ROS1* fusion-positive lung cancer after treatment with ROS1 inhibitors. Indeed, the true frequency of *MET* amplification in ROS1 TKI-resistant cases may be underestimated in this series as all patients herein (including those who had post-lorlatinib biopsies) received prior crizotinib. Broadly, these cases with RAS-MAPK or MET alterations—including a *KRAS* G12C mutation which is now druggable with covalent inhibitor of $KRAS^{G12C}$ (44,45)—support further investigation of potential combination strategies after resistance to ROS1 TKI(s) develops. It is additionally worth noting that cabozantinib as a multikinase inhibitor could be considered not only for certain *ROS1* kinase domain mutations but also for acquired MET dependency in ROS1 fusion-positive tumors (46).

Our study had several important limitations. It was a retrospective study with a relatively small sample size although remains the largest multi-institutional dataset analyzed to date. Second, selection bias cannot be excluded particularly in the lorlatinib-resistant cohort, as patients known to have *ROS1* resistance mutations in the tumor may have been

preferentially directed towards lorlatinib as subsequent therapy; this may have resulted in a higher frequency of *ROS1* mutations in post-lorlatinib biopsies. Third, matched sequencing data from pre-lorlatinib biopsies were generally not available, and therefore, we could not always determine whether gene alterations were pre-existing versus acquired on lorlatinib. We have indicated which cases had prior biopsies available and the corresponding sequencing results in order to inform data interpretation. In certain cases, discrepancies were identified between matched tumor and plasma analyses, likely owing to multiple factors including tumor heterogeneity. Additionally, not all NGS platforms consistently assessed for certain gene alterations including the *ROS1* L2086F mutation (likely because its incidence and consequence were previously not appreciated, and because L2086 resides in a separate exon from the previously known G2032). Recognizing this, in at least a subset of the cases analyzed by NGS platform where L2086 was not covered, we performed Sanger sequencing of the *ROS1* kinase domain. It remains possible that *ROS1* L2086F may mediate resistance in higher proportion of cases than is appreciable from this study. Furthermore, this limitation highlights the importance of elucidating functional and clinical implications of diverse gene alterations—including and extending beyond *ROS1*—in order to ensure that clinical NGS panels incorporate the assessment of these alterations and do not inadvertently miss a finding that could guide treatment recommendations. Finally, approximately 40% of the lorlatinib-resistant cohort had unknown mechanisms of resistance based on gene sequencing alone. Future studies will need to further investigate mechanisms of signaling dysregulation at the epigenetic, RNA, protein, and phospho-protein levels.

In summary, we demonstrated that *ROS1* kinase domain mutations are identified in over one-third of crizotinib-resistant and nearly a half of lorlatinib-resistant cases, and remain an important hurdle to overcome in the treatment of *ROS1* fusion-positive lung cancer. Our clinical and preclinical findings suggest lorlatinib is not sufficiently potent against the known crizotinib-resistant mutation *ROS1* G2032R, and additionally lacks potency against a previously less well-known mutation *ROS1* L2086F which was refractory to all ROS1 inhibitors tested in this study except for the type II, multikinase inhibitor cabozantinib. This work highlights the potential utility of re-biopsy and sequential TKI therapy in *ROS1* fusion-positive lung cancer. Moving forward, it will be important to continue the ongoing efforts to develop novel, selective ROS1 inhibitors with potency against the known spectrum of *ROS1* resistance mutations. In addition, our work adds insights into potential ROS1-independent resistance mechanisms such as MET and RAS-MAPK alterations, which may support the exploration of combination strategies if further validated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: This work was supported by the Targeting a Cure for Lung Cancer Research Fund and by Be A Piece of the Solution at MGH. JJL and BYY are partially supported by funding from R01-CA164273. SY is partially supported by Lung Cancer Research Foundation Annual Grant Program award.

JJL has served as a compensated consultant for Genentech, C4 Therapeutics, Blueprint Medicines, Nuvalent, and Turning Point Therapeutics; received honorarium and travel support from Pfizer; received institutional research

funds from Hengrui Therapeutics, Turning Point Therapeutics, Neon Therapeutics, Relay Therapeutics, Bayer, Elevation Oncology, Roche, and Novartis; received CME funding from OncLive, MedStar Health, and Northwell Health.

VWZ has received honoraria from AstraZeneca, Blueprint, Roche-Foundation Medicine, Roche/Genentech, Takeda, Xcovery; and had stock ownership of TP Therapeutics until May 2020.

TWJ is an employee of Pfizer.

IDJ has received honoraria from Foundation Medicine, consulting fees from Boehringer Ingelheim, AstraZeneca, Pfizer, Xcovery, Catalyst, Novocure, and Syros; research support from Array, Genentech, Novartis, Pfizer, and Guardant Health; and travel support from Array and Pfizer.

SRD provides independent image analysis for hospital contracted clinical research trials programs for Merck, Pfizer, Bristol Mayer Squibb, Novartis, Roche, Polaris, Cascadian, Abbvie, Gradalis, Clinical Bay, Zai laboratories, Jenssen. Received honorarium from: Siemens and research grant from Lunit Inc, not related to work.

LVS has received consulting fees from AstraZeneca, Genentech and Janssen, and has received institutional research support from Boehringer-Ingelheim, Novartis, AstraZeneca, and Genentech.

BYY received institutional research support from Taiho Oncology Inc.

ATS has served as a compensated consultant or received honoraria from Achilles, Archer, Ariad/Takeda, Bayer, Blueprint Medicines, Chugai, Daiichi-Sankyo, EMD Serono, Foundation Medicine, Guardant, Ignyta, KSQ Therapeutics, Loxo Oncology, Natera, Novartis, Pfizer, Roche-Genentech, Servier, Syros, Taiho Pharmaceutical, and TP Therapeutics; received institutional research funding from Ariad, Ignyta, Novartis, Pfizer, Roche-Genentech, and TP Therapeutics; received travel support from Genentech and Pfizer; and is currently employed by and owns stock in Novartis.

SIO has received personal fees from Pfizer, Merck, Takeda, AstraZeneca, Roche/Genentech, Daiichi Sankyo, Blueprint Medicines, and Janssen JNJ; and is a member of the Scientific Advisory Board of Elevation Oncology, and has stock ownership in Turning Point Therapeutics and Elevation Oncology.

ANH has received research support from Amgen, Pfizer, Novartis, Blueprint Medicines, Eli Lilly, Roche/Genentech, and Relay Therapeutics.

AD has received personal fees/honoraria for advisory boards from Ignyta/Genentech/Roche, Loxo/Bayer/Lilly, Takeda/Ariad/Millenium, TP Therapeutics, AstraZeneca, Pfizer, Blueprint Medicines, Helsinn, Beigene, BergenBio, Hengrui Therapeutics, Exelixis, Tyra Biosciences, Verastem, MORE Health, Abbvie, 14ner/Elevation Oncology, Remedica Ltd., ArcherDX, Monopteros, Novartis, EMD Serono, Melendi, Liberum, Repare RX, associated research paid to Memorial Sloan Kettering from Pfizer, Exelixis, GlaxoSmithKlein, Teva, Taiho, PharmaMar, royalties from Wolters Kluwer and other from Merck, Puma, Merus, Boehringer Ingelheim.

JFG has served as a compensated consultant or received honoraria from Bristol-Myers Squibb, Genentech, Ariad/Takeda, Loxo/Lilly, Blueprint, Oncorus, Regeneron, Gilead, AstraZeneca, Pfizer, Incyte, Novartis, Merck, Agios, Amgen, and Array; research support from Novartis, Genentech/Roche, and Ariad/Takeda; institutional research support from Bristol-Myers Squibb, Tesaro, Moderna, Blueprint, Jounce, Array Biopharma, Merck, Adaptimmune, Novartis, and Alexo; and has an immediate family member who is an employee of Ironwood Pharmaceuticals.

REFERENCES

1. Lin JJ, Shaw AT. Recent Advances in Targeting ROS1 in Lung Cancer. J Thorac Oncol 2017;12(11):1611–25 doi 10.1016/j.jtho.2017.08.002. [PubMed: 28818606]
2. Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. ROS1-dependent cancers - biology, diagnostics and therapeutics. Nat Rev Clin Oncol 2020 doi 10.1038/s41571-020-0408-9.
3. Shaw AT, Ou SH, Bang YJ, Camidge DR, Solomon BJ, Salgia R et al. Crizotinib in ROS1-rearranged non-small-cell lung cancer. N Engl J Med 2014;371(21):1963–71 doi 10.1056/NEJMoa1406766. [PubMed: 25264305]
4. Shaw AT, Riely GJ, Bang YJ, Kim DW, Camidge DR, Solomon BJ et al. Crizotinib in ROS1-rearranged advanced non-small-cell lung cancer (NSCLC): updated results, including overall survival, from PROFILE 1001. Ann Oncol 2019;30(7):1121–6 doi 10.1093/annonc/mdz131. [PubMed: 30980071]

5. Michels S, Massuti B, Schildhaus HU, Franklin J, Sebastian M, Felip E et al. Safety and Efficacy of Crizotinib in Patients With Advanced or Metastatic ROS1-Rearranged Lung Cancer (EUCROSS): A European Phase II Clinical Trial. J Thorac Oncol 2019;14(7):1266–76 doi 10.1016/j.jtho.2019.03.020. [PubMed: 30978502]
6. Wu YL, Yang JC, Kim DW, Lu S, Zhou J, Seto T et al. Phase II Study of Crizotinib in East Asian Patients With ROS1-Positive Advanced Non-Small-Cell Lung Cancer. J Clin Oncol 2018;36(14):1405–11 doi 10.1200/JCO.2017.75.5587. [PubMed: 29596029]
7. Moro-Sibilot D, Cozic N, Perol M, Mazieres J, Otto J, Souquet PJ et al. Crizotinib in c-MET- or ROS1-positive NSCLC: results of the AcSe phase II trial. Ann Oncol 2019;30(12):1985–91 doi 10.1093/annonc/mdz407. [PubMed: 31584608]
8. Drilon A, Siena S, Dziadziuszko R, Barlesi F, Krebs MG, Shaw AT et al. Entrectinib in ROS1 fusion-positive non-small-cell lung cancer: integrated analysis of three phase 1–2 trials. Lancet Oncol 2020;21(2):261–70 doi 10.1016/S1470-2045(19)30690-4. [PubMed: 31838015]
9. Shaw AT, Solomon BJ, Chiari R, Riely GJ, Besse B, Soo RA et al. Lorlatinib in advanced ROS1-positive non-small-cell lung cancer: a multicentre, open-label, single-arm, phase 1–2 trial. Lancet Oncol 2019;20(12):1691–701 doi 10.1016/S1470-2045(19)30655-2. [PubMed: 31669155]
10. NCCN NCCN. Non-Small Cell Lung Cancer (Version 2.2021). 2021.
11. Awad MM, Katayama R, McTigue M, Liu W, Deng YL, Brooun A et al. Acquired resistance to crizotinib from a mutation in CD74-ROS1. N Engl J Med 2013;368(25):2395–401 doi 10.1056/NEJMoa1215530. [PubMed: 23724914]
12. Gainor JF, Tseng D, Yoda S, Dagogo-Jack I, Friboulet L, Lin JJ et al. Patterns of Metastatic Spread and Mechanisms of Resistance to Crizotinib in ROS1-Positive Non-Small-Cell Lung Cancer. JCO Precis Oncol 2017;2017 doi 10.1200/PO.17.00063.
13. Dagogo-Jack I, Rooney M, Nagy RJ, Lin JJ, Chin E, Ferris LA et al. Molecular Analysis of Plasma From Patients With ROS1-Positive NSCLC. J Thorac Oncol 2019;14(5):816–24 doi 10.1016/j.jtho.2019.01.009. [PubMed: 30664990]
14. Drilon A, Somwar R, Wagner JP, Vellore NA, Eide CA, Zabriskie MS et al. A Novel Crizotinib-Resistant Solvent-Front Mutation Responsive to Cabozantinib Therapy in a Patient with ROS1-Rearranged Lung Cancer. Clin Cancer Res 2016;22(10):2351–8 doi 10.1158/1078-0432.CCR-15-2013. [PubMed: 26673800]
15. McCoach CE, Le AT, Gowan K, Jones K, Schubert L, Doak A et al. Resistance Mechanisms to Targeted Therapies in ROS1(+) and ALK(+) Non-small Cell Lung Cancer. Clin Cancer Res 2018;24(14):3334–47 doi 10.1158/1078-0432.CCR-17-2452. [PubMed: 29636358]
16. Facchinetti F, Loriot Y, Kuo MS, Mahjoubi L, Lacroix L, Planchard D et al. Crizotinib-Resistant ROS1 Mutations Reveal a Predictive Kinase Inhibitor Sensitivity Model for ROS1- and ALK-Rearranged Lung Cancers. Clin Cancer Res 2016;22(24):5983–91 doi 10.1158/1078-0432.CCR-16-0917. [PubMed: 27401242]
17. Zhou Y, Jiang W, Zeng L, Mi J, Song L, Lizaso A et al. A novel ROS1 G2032 K missense mutation mediates lorlatinib resistance in a patient with ROS1-rearranged lung adenocarcinoma but responds to nab-paclitaxel plus pembrolizumab. Lung Cancer 2020;143:55–9 doi 10.1016/j.lungcan.2020.03.019. [PubMed: 32208297]
18. Zheng Z, Liebers M, Zhelyazkova B, Cao Y, Panditi D, Lynch KD et al. Anchored multiplex PCR for targeted next-generation sequencing. Nat Med 2014;20(12):1479–84 doi 10.1038/nm.3729. [PubMed: 25384085]
19. Cheng DT, Mitchell TN, Zehir A, Shah RH, Benayed R, Syed A et al. Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT): A Hybridization Capture-Based Next-Generation Sequencing Clinical Assay for Solid Tumor Molecular Oncology. J Mol Diagn 2015;17(3):251–64 doi 10.1016/j.jmoldx.2014.12.006. [PubMed: 25801821]
20. Wagle N, Berger MF, Davis MJ, Blumenstiel B, Defelice M, Pochanard P et al. High-throughput detection of actionable genomic alterations in clinical tumor samples by targeted, massively parallel sequencing. Cancer Discov 2012;2(1):82–93 doi 10.1158/2159-8290.CD-11-0184. [PubMed: 22585170]

21. Gainor JF, Dardaei L, Yoda S, Friboulet L, Leshchiner I, Katayama R et al. Molecular Mechanisms of Resistance to First- and Second-Generation ALK Inhibitors in ALK-Rearranged Lung Cancer. Cancer Discov 2016;6(10):1118–33 doi 10.1158/2159-8290.CD-16-0596. [PubMed: 27432227]
22. Lin JJ, Liu SV, McCoach CE, Zhu VW, Tan AC, Yoda S et al. Mechanisms of resistance to selective RET tyrosine kinase inhibitors in RET fusion-positive non-small-cell lung cancer. Ann Oncol 2020;31(12):1725–33 doi 10.1016/j.annonc.2020.09.015.
23. Katayama R, Kobayashi Y, Friboulet L, Lockerman EL, Koike S, Shaw AT et al. Cabozantinib overcomes crizotinib resistance in ROS1 fusion-positive cancer. Clin Cancer Res 2015;21(1):166–74 doi 10.1158/1078-0432.CCR-14-1385. [PubMed: 25351743]
24. Okada K, Araki M, Sakashita T, Ma B, Kanada R, Yanagitani N et al. Prediction of ALK mutations mediating ALK-TKIs resistance and drug re-purposing to overcome the resistance. EBioMedicine 2019;41:105–19 doi 10.1016/j.ebiom.2019.01.019. [PubMed: 30662002]
25. Yoda S, Lin JJ, Lawrence MS, Burke BJ, Friboulet L, Langenbucher A et al. Sequential ALK Inhibitors Can Select for Lorlatinib-Resistant Compound ALK Mutations in ALK-Positive Lung Cancer. Cancer Discov 2018;8(6):714–29 doi 10.1158/2159-8290.CD-17-1256. [PubMed: 29650534]
26. Song A, Kim TM, Kim DW, Kim S, Keam B, Lee SH et al. Molecular Changes Associated with Acquired Resistance to Crizotinib in ROS1-Rearranged Non-Small Cell Lung Cancer. Clin Cancer Res 2015;21(10):2379–87 doi 10.1158/1078-0432.CCR-14-1350. [PubMed: 25688157]
27. Davare MA, Vellore NA, Wagner JP, Eide CA, Goodman JR, Drilon A et al. Structural insight into selectivity and resistance profiles of ROS1 tyrosine kinase inhibitors. Proc Natl Acad Sci U S A 2015;112(39):E5381–90 doi 10.1073/pnas.1515281112. [PubMed: 26372962]
28. Schoenfeld AJ, Chan JM, Kubota D, Sato H, Rizvi H, Daneshbod Y et al. Tumor Analyses Reveal Squamous Transformation and Off-Target Alterations As Early Resistance Mechanisms to First-line Osimertinib in EGFR-Mutant Lung Cancer. Clin Cancer Res 2020;26(11):2654–63 doi 10.1158/1078-0432.CCR-19-3563. [PubMed: 31911548]
29. Piotrowska Z, Isozaki H, Lennerz JK, Gainor JF, Lennes IT, Zhu VW et al. Landscape of Acquired Resistance to Osimertinib in EGFR-Mutant NSCLC and Clinical Validation of Combined EGFR and RET Inhibition with Osimertinib and BLU-667 for Acquired RET Fusion. Cancer Discov 2018;8(12):1529–39 doi 10.1158/2159-8290.CD-18-1022. [PubMed: 30257958]
30. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316(5827):1039–43 doi 10.1126/science.1141478. [PubMed: 17463250]
31. Dagogo-Jack I, Yoda S, Lennerz JK, Langenbucher A, Lin JJ, Rooney MM et al. MET Alterations Are a Recurring and Actionable Resistance Mechanism in ALK-Positive Lung Cancer. Clin Cancer Res 2020;26(11):2535–45 doi 10.1158/1078-0432.CCR-19-3906. [PubMed: 32086345]
32. Rosen EY, Johnson ML, Clifford SE, Somwar R, Kherani JF, Son J et al. Overcoming MET-Dependent Resistance to Selective RET Inhibition in Patients with RET Fusion-Positive Lung Cancer by Combining Selpercatinib with Crizotinib. Clin Cancer Res 2020 doi 10.1158/1078-0432.CCR-20-2278.
33. Recondo G, Bahcall M, Spurr LF, Che J, Ricciuti B, Leonardi GC et al. Molecular Mechanisms of Acquired Resistance to MET Tyrosine Kinase Inhibitors in Patients with MET Exon 14-Mutant NSCLC. Clin Cancer Res 2020;26(11):2615–25 doi 10.1158/1078-0432.CCR-19-3608. [PubMed: 32034073]
34. Sato H, Schoenfeld AJ, Siau E, Lu YC, Tai H, Suzawa K et al. MAPK Pathway Alterations Correlate with Poor Survival and Drive Resistance to Therapy in Patients with Lung Cancers Driven by ROS1 Fusions. Clin Cancer Res 2020;26(12):2932–45 doi 10.1158/1078-0432.CCR-19-3321. [PubMed: 32122926]
35. Zhu YC, Lin XP, Li XF, Wu LX, Chen HF, Wang WX et al. Concurrent ROS1 gene rearrangement and KRAS mutation in lung adenocarcinoma: A case report and literature review. Thorac Cancer 2018;9(1):159–63 doi 10.1111/1759-7714.12518. [PubMed: 28971587]
36. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 2007;131(6):1190–203 doi 10.1016/j.cell.2007.11.025. [PubMed: 18083107]

37. Papadopoulos KP, Borazanci E, Shaw AT, Katayama R, Shimizu Y, Zhu VW et al. U.S. Phase I First-in-human Study of Taletrectinib (DS-6051b/AB-106), a ROS1/TRK Inhibitor, in Patients with Advanced Solid Tumors. Clin Cancer Res 2020;26(18):4785–94 doi 10.1158/1078-0432.CCR-20-1630. [PubMed: 32591465]

38. Cocco E, Lee JE, Kannan S, Schram AM, Won HH, Shifman S et al. TRK xDFG Mutations Trigger a Sensitivity Switch from Type I to II Kinase Inhibitors. Cancer Discov 2020 doi 10.1158/2159-8290.CD-20-0571.

39. Drilon A, Ou SI, Cho BC, Kim DW, Lee J, Lin JJ et al. Repotrectinib (TPX-0005) Is a Next-Generation ROS1/TRK/ALK Inhibitor That Potently Inhibits ROS1/TRK/ALK Solvent- Front Mutations. Cancer Discov 2018;8(10):1227–36 doi 10.1158/2159-8290.CD-18-0484. [PubMed: 30093503]

40. Cho BC, Drilon AE, Doebele RC, Kim D-W, Lin JJ, Lee J et al. Safety and preliminary clinical activity of repotrectinib in patients with advanced ROS1 fusion-positive non-small cell lung cancer (TRIDENT-1 study). Journal of Clinical Oncology 2019;37(15_suppl):9011- doi 10.1200/JCO.2019.37.15_suppl.9011.

41. Drilon A, Rekhtman N, Arcila M, Wang L, Ni A, Albano M et al. Cabozantinib in patients with advanced RET-rearranged non-small-cell lung cancer: an open-label, single-centre, phase 2, single-arm trial. Lancet Oncol 2016;17(12):1653–60 doi 10.1016/S1470-2045(16)30562-9. [PubMed: 27825636]

42. Ku BM, Bae YH, Lee KY, Sun JM, Lee SH, Ahn JS et al. Entrectinib resistance mechanisms in ROS1-rearranged non-small cell lung cancer. Invest New Drugs 2020;38(2):360–8 doi 10.1007/s10637-019-00795-3.

43. Engstrom LD, Aranda R, Lee M, Tovar EA, Essenburg CJ, Madaj Z et al. Glesatinib Exhibits Antitumor Activity in Lung Cancer Models and Patients Harboring MET Exon 14 Mutations and Overcomes Mutation-mediated Resistance to Type I MET Inhibitors in Nonclinical Models. Clin Cancer Res 2017;23(21):6661–72 doi 10.1158/1078-0432.CCR-17-1192. [PubMed: 28765324]

44. Hong DS, Fakih MG, Strickler JH, Desai J, Durm GA, Shapiro GI et al. KRAS(G12C) Inhibition with Sotorasib in Advanced Solid Tumors. N Engl J Med 2020;383(13):1207–17 doi 10.1056/NEJMoa1917239. [PubMed: 32955176]

45. Hallin J, Engstrom LD, Hargis L, Calinisan A, Aranda R, Briere DM et al. The KRAS(G12C) Inhibitor MRTX849 Provides Insight toward Therapeutic Susceptibility of KRAS-Mutant Cancers in Mouse Models and Patients. Cancer Discov 2020;10(1):54–71 doi 10.1158/2159-8290.CD-19-1167. [PubMed: 31658955]

46. Salgia R, Sattler M, Scheele J, Stroh C, Felip E. The promise of selective MET inhibitors in non-small cell lung cancer with MET exon 14 skipping. Cancer Treat Rev 2020;87:102022 doi 10.1016/j.ctrv.2020.102022. [PubMed: 32334240]

STATEMENT OF TRANSLATIONAL RELEVANCE

ROS1 inhibitors are standard of care in advanced *ROS1* fusion-positive (ROS1+) lung cancer. Recently, a next-generation ALK/ROS1 inhibitor lorlatinib demonstrated efficacy in this disease. However, mechanisms of resistance to lorlatinib in ROS1+ lung cancer are not well-known, and insights into crizotinib resistance have been limited to small series. We performed molecular profiling of the largest series to date of crizotinib- and lorlatinib-resistant biopsies, finding that *ROS1* kinase domain mutations mediate resistance in one-third to one-half of cases, respectively. Recurrent resistance mutations in *ROS1* included G2032R and less well-characterized L2086F. In Ba/F3 models, type I inhibitors including crizotinib, entrectinib, and lorlatinib were unable to overcome $ROS1^{L2086F}$, whereas type II inhibitor cabozantinib maintained potency. We additionally detected MET and RAS-MAPK alterations in resistant specimens. Our study highlights the importance of developing novel ROS1 inhibitors with potency against recurrent *ROS1* resistance mutations and may inform sequential treatment strategies in ROS1+ lung cancer.

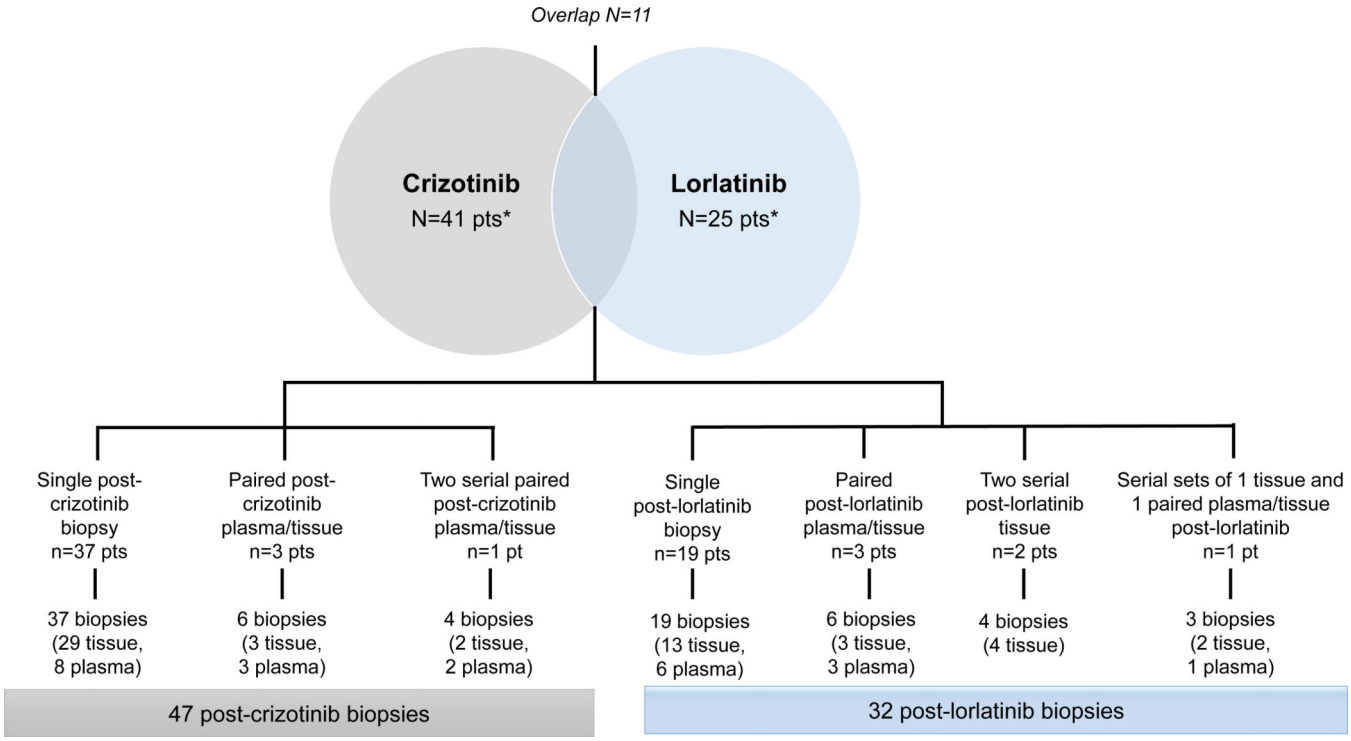
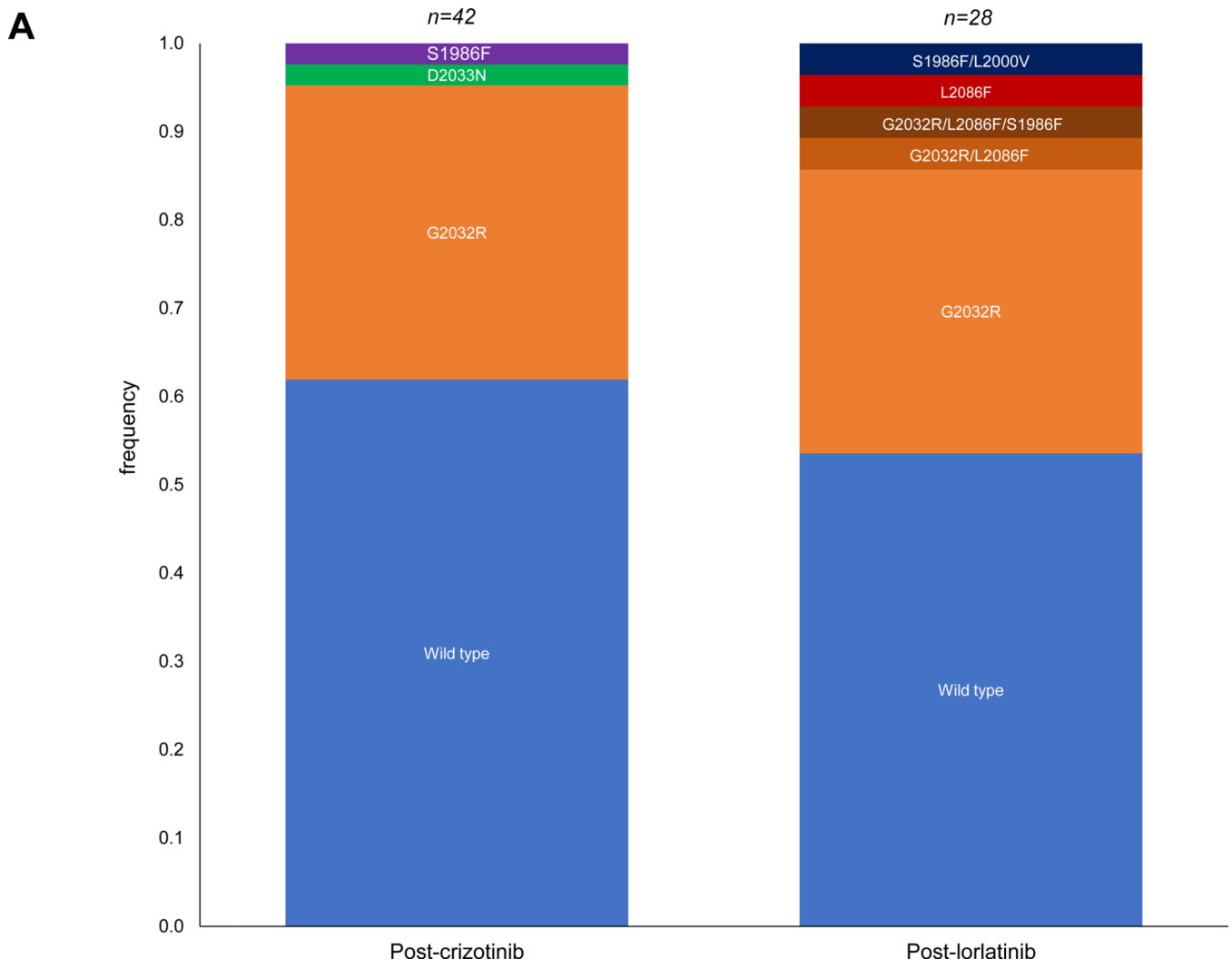


Figure 1. Schematic of resistant biopsies from the *ROS1* fusion-positive NSCLC cohort.

The schematic summarizes a total of 47 post-crizotinib and 32 post-lorlatinib biopsies analyzed from 55 patients included in the cohort. The timing of these biopsies for each patient are further delineated in Supplementary Figure S1. Pt, patient.



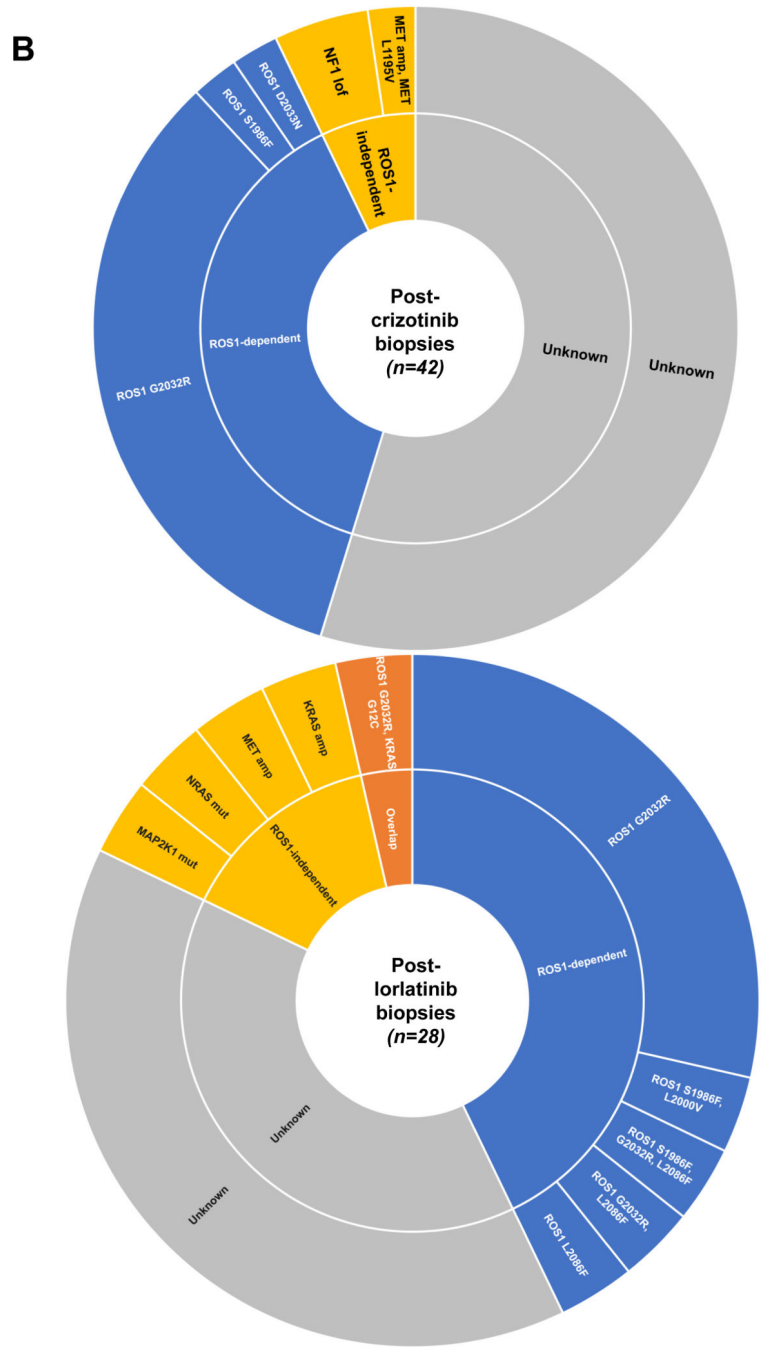


Figure 2. Distribution of ROS1-dependent and ROS1-independent resistance.

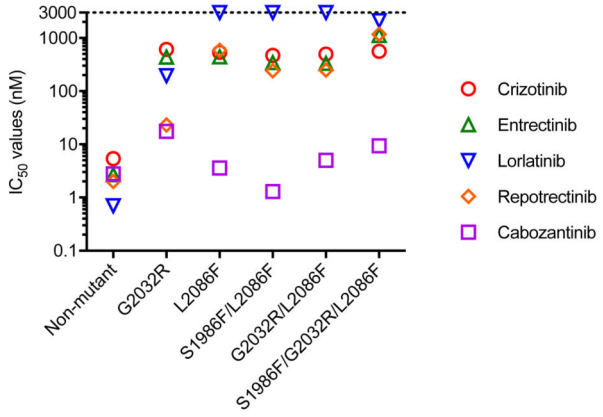
(A) The frequency of *ROS1* mutation(s) detected in each distinct crizotinib- or lorlatinib-resistant biopsy is demonstrated. If *ROS1* mutation(s) were detected in only one of the paired plasma and tissue specimens, these mutation(s) were included. Of note, not all samples were tested for the presence of the *ROS1* L2086F mutation. (B) The distribution of presumed resistance mechanisms identified in the post-crizotinib and post-lorlatinib biopsy cohort. amp, amplification; mut, mutation; lof, loss-of-function.

A

IC_{50} (nmol/L)	Crizotinib	Entrectinib	Lorlatinib	Repotrectinib	Cabozantinib	Ceritinib	Brigatinib	Taletrectinib	Alectinib
Parental	840.5	1801.0	>3000	1218.0	>3000	1117.0	>3000	>3000	1207.0
Non-mutant	5.4	2.7	0.7	2.0	2.8	16.4	9.4	2.6	995.4
G2032R	609.6	436.3	196.6	23.1	17.5	346.4	472.7	53.3	1091.0
L2000V	37.1	25.9	2.5	10.1	7.6	124.9	78.9	29.8	985.0
L2086F	536.8	440.0	>3000	587.9	3.6	226.9	159.3	1265.0	672.5
S1986F/L2000V	159.4	36.1	2.4	7.2	5.1	86.9	62.5	20.3	1080.0
S1986F/L2086F	469.7	344.2	>3000	241.2	1.3	154.8	48.5	662.6	919.9
G2032R/L2086F	498.6	335.4	>3000	248.9	5.0	573.9	450.9	744.2	1254.0
S1986F/G2032R	594.4	718.5	990.6	65.1	70.1	614.7	717.0	105.4	1137.0
S1986F/G2032R/L2086F	562.8	1111.0	2131.0	1178.0	9.4	1116.0	1341.0	2432.0	1150.0

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

B



C

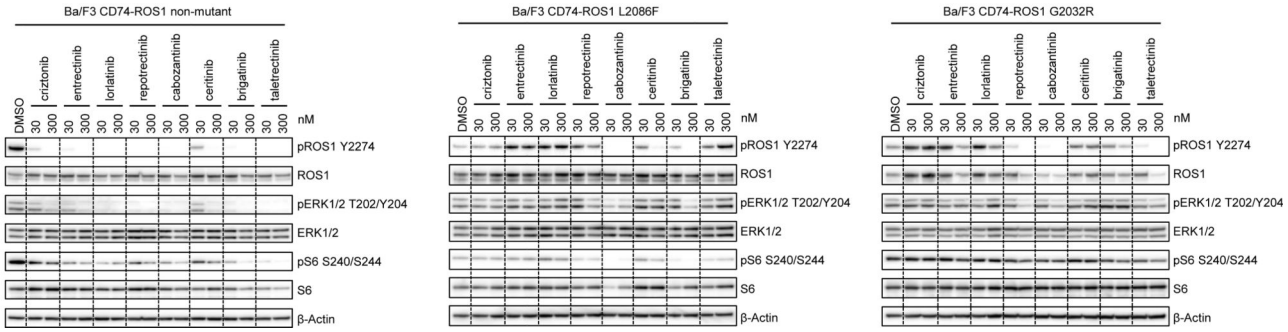


Figure 3. Preclinical activity of ROS1 inhibitors against mutant ROS1 kinases.

(A) IC_{50} values of crizotinib, entrectinib, lorlatinib, repotrectinib, cabozantinib, ceritinib, brigatinib, and taletrectinib, or ALK (not ROS1) inhibitor alectinib, in parental Ba/F3 cells and Ba/F3 cells expressing nonmutant or mutant *CD74-ROS1.* Data are from three replicates. (B) IC_{50} values of crizotinib, entrectinib, lorlatinib, repotrectinib, and cabozantinib against nonmutant ROS1, $ROS1^{G2032R}$, or $ROS1^{L2086F}$-based mutant kinases. (C) Suppression of phospho-ROS1 and its downstream targets in Ba/F3 cells expressing nonmutant ROS1, $ROS1^{G2032R}$, or $ROS1^{L2086F}$ treated with ROS1 inhibitors.

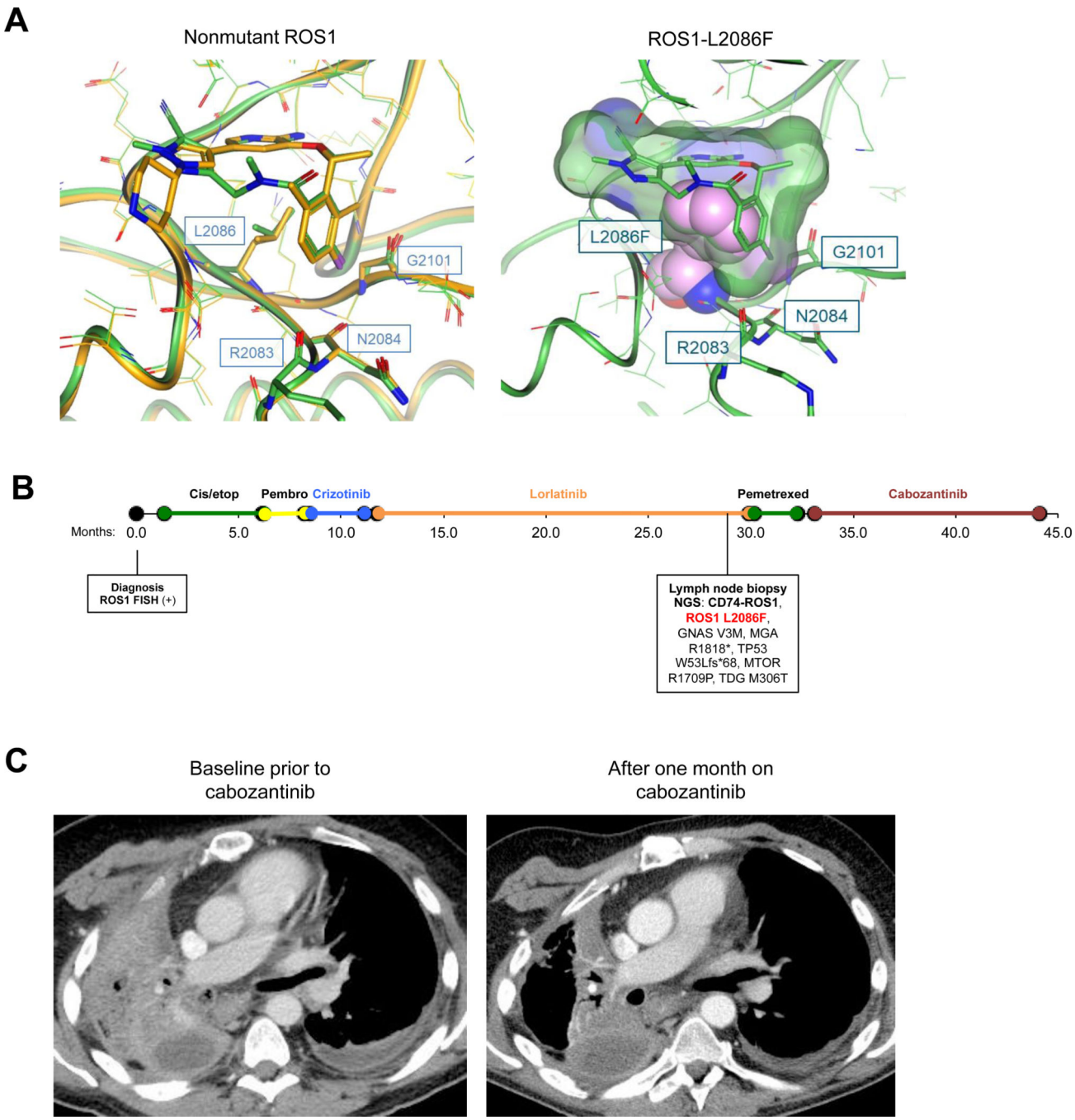


Figure 4. Resistance to lorlatinib with a *ROS1* L2086F mutation and subsequent treatment with cabozantinib.

(A) Left panel, X-ray co-crystal structure of the nonmutant ROS1 kinase domain bound to crizotinib (colored yellow) or lorlatinib (colored green). Right panel: Structural modeling of $ROS1^{L2086F}$ mutant (phenylalanine colored pink with Connolly surface) bound to lorlatinib. (B) Treatment course of MGH0026. The patient had disease progression on lorlatinib. A lorlatinib-resistant lymph node was biopsied and analyzed by NGS (results shown below the timeline). Patient received a brief course of pemetrexed, discontinued for intolerability, before starting cabozantinib. (C) Representative computed tomography images before and

after one month on cabozantinib, showing improved aeration but persistent right lung consolidation and loculated effusion, and slightly decreased left pleural effusion.

TABLE 1

Baseline characteristics of the *ROS1* fusion-positive lung cancer cohort evaluated in this study.

Characteristic	n (%), N=55
Age at diagnosis, median (range)	50 (22–81)
Female	41 (75)
Never or light smoker	53 (96)
Adenocarcinoma	55 (100)
ROS1 fusion	
CD74-ROS1	24 (44)
SDC4-ROS1	8 (15)
SLC34A2-ROS1	5 (9)
EZR-ROS1	5 (9)
TPM3-ROS1	1 (2)
Other	4 (7)
Unknown	8 (15)
Biopsies	
Post-crizotinib only	30 (55)
Post-lorlatinib only	14 (25)
Post-crizotinib and post-lorlatinib	11 (20)