Resistant Mechanisms to Targeted Therapies in \( \text{ROS1}^+ \) and \( \text{ALK}^+ \) Non-small Cell Lung Cancer

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Abstract

Purpose: Despite initial benefit from tyrosine kinase inhibitors (TKIs), patients with advanced non–small cell lung cancer (NSCLC) harboring \( \text{ALK}^+ \) and \( \text{ROS1}^+ \) gene fusions ultimately progress. Here, we report on the potential resistance mechanisms in a series of patients with \( \text{ALK}^+ \) and \( \text{ROS1}^+ \) NSCLC progressing on different types and/or lines of \( \text{ROS1/ALK} \)-targeted therapy.

Experimental Design: We used a combination of next-generation sequencing (NGS), multiplex mutation assay, direct DNA sequencing, RT-PCR, and FISH to identify fusion variants/partners and copy-number gain (CNG), kinase domain mutations (KDM), and copy-number variations (CNVs) in other cancer-related genes. We performed testing on 12 \( \text{ROS1}^+ \) and 43 \( \text{ALK}^+ \) patients.

Results: One of 12 \( \text{ROS1}^+ \) (8%) and 15 of 43 (35%) \( \text{ALK}^+ \) patients harbored KDM. In the \( \text{ROS1}^+ \) cohort, we identified RIT1, NOTCH1, NF1, multiple proto-oncogenes genes including GNAS, K/HRAS, RET, NTRK1, MAP2K1, and others. In addition, we identified CNV in multiple proto-oncogenes genes including PDGFRα, KIT, KDR, GNAS, K/HRAS, RET, NTRK1, MAP2K1, and others.

Conclusions: We identified a putative TKI resistance mechanism in six of 12 (50%) \( \text{ROS1}^+ \) patients and 37 of 43 (86%) \( \text{ALK}^+ \) patients. Our data suggest that a focus on KDMs will miss most resistance mechanisms; broader gene testing strategies and functional validation is warranted to devise new therapeutic strategies for drug resistance.

Introduction

Large-scale chromosomal alterations involving c-ros oncogene 1 (\( \text{ROS1} \)) or the anaplastic lymphoma kinase (\( \text{ALK} \)) exhibit oncogenic activity in non–small cell lung cancer (NSCLC, refs. 1–3). \( \text{ROS1} \) and \( \text{ALK} \) gene fusions result in the expression of chimeric proteins that constitutively activate downstream proliferation and survival pathways (2, 4, 5). These fusions can be detected by multiple methods including FISH, RT-PCR with direct sequencing, next-generation sequencing (NGS), and IHC (6–11).

Treatment of patients with NSCLC whose tumors harbor \( \text{ROS1} \) and \( \text{ALK} \) fusions using cognate tyrosine kinase inhibitors (TKI) has allowed for dramatic improvements in response rates, progression-free survival, and overall survival compared with chemotherapy (12–16). Crizotinib, the first FDA-approved TKI for \( \text{ALK}^+ \) and the only FDA-approved TKI for \( \text{ROS1}^+ \) patients, demonstrated a PFS of 9.7 months in \( \text{ALK}^+ \) patients and 19.2 months in \( \text{ROS1}^+ \) patients, both studies enrolled patients with and without prior lines of therapy (3, 16). Ceritinib, alectinib, and brigatinib have also been approved for \( \text{ALK}^+ \) patients who have progressed on crizotinib and these drugs demonstrated response rates in the second-line setting of 56%, 50%, and 54%, respectively (17–19). In \( \text{ROS1}^+ \) patients who progressed on crizotinib, there are no FDA-approved TKIs; however, multiple drugs including ceritinib, brigatinib, lorlatinib, and cabozantinib are being evaluated as post-crizotinib options (20–23). For \( \text{ALK}^+ \) patients, there are also multiple new ALK-targeted TKIs that are currently being investigated, including lorlatinib and ensatinib (24–28).

Our group and others have previously reported on the mechanisms of crizotinib resistance in \( \text{ALK}^+ \) patients, which include somatic mutations in the kinase domain (KDM), gene copy-number gains (CNGs), and alternate oncogenic mutations (29, 30). In addition, series describing the resistance mechanisms to next-generation ALK TKIs have been recently published; however, many \( \text{ALK}^+ \) tumors did not have an identifiable mechanism of resistance (30–34). Currently, little is known about resistance mechanisms in patients.
Translational Relevance

Patients with ROS1+ and ALK+ non–small cell lung cancer treated with oncogene-targeted therapy will inevitably develop drug resistance and disease progression. Two general biological mechanisms of resistance are described: alterations that restore signaling through the original oncogene driver in the presence of the drug or alterations that switch dependence to other signaling pathways. Currently, little is known about ROS1 resistance and the spectrum of ALK resistance mechanisms may be changing with the introduction of new inhibitors. As specific resistance mechanisms could influence subsequent drug treatment choices and future drug combination strategies, data on the type and frequency of different resistance mechanisms for ROS1+ and ALK+ cancers are likely to become increasingly important. Our data suggest comprehensive methods, beyond the identification of kinase domain mutations, are needed to identify the full spectrum of drug resistance in ROS1+ and ALK+ cancer.

with ROS1+ NSCLC after treatment with crizotinib and/or other ROS1 TKIs. Here, we report on the potential resistance mechanisms of cohorts of ROS1+ and ALK+ NSCLC patients treated across multiple lines of therapy and with different TKIs.

Materials and Methods

Patient population

Patients with advanced ROS1+ or ALK+ NSCLC were considered for rebiopsy following progression on specific ROS1 or ALK TKI therapy. All patients gave informed consent for collection of clinical correlates, tissue collection, research testing, and cell line derivation under Institutional Review Board (IRB)-approved protocols. Formalin-fixed paraffin-embedded (FFPE), frozen (placed in liquid nitrogen), and/or fresh tissue samples were obtained according to the safety standards of the institutional review board, pulmonologist, or surgeon. Prior therapies and days until progression for each patient were obtained from chart review. Days until progression were determined on the basis of imaging studies, which demonstrated disease progression.

NGS

We analyzed samples from 10 ROS1+ and 29 ALK+ patients with a custom capture-based NGS panel of 48 genes (NimbleGen SeqCap EZ Choice Library, Roche). See Supplementary Table S1 for gene list. Samples were run on a NextSeq sequencer (Illumina). We used approximately 100 ng (range, 35–150 ng) of DNA for each NGS assay. Total genomic DNA was isolated from tissue using the QIAamp DNA Mini Kit (Qiagen) and/or fresh tissue samples were obtained according to the safety standards of the Institutional Review Board. Formalin-fixed paraffin-embedded (FFPE) or frozen tissue was used for these studies. Bacterial colonies were miniprepped (Qiagen) and used to transform competent cloning vector (Invitrogen) and used to transform competent.

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DNA Sequencer (37). Mutation analysis was performed with the Mutation Surveyor software v3.97–4.0.0 from Soft Genetic. The reference sequence used for ROS1 was NM_002944.2, for ALK NM_004304.4, and for EML4 NM_019063.3. Anchored-multiplex PCR (Archer FusionPlex assay) was utilized in three tumor samples to further characterize ALK fusions, but also detected other gene fusions.

The SNapshot assay for evaluation of multiple oncogenic mutations in APC, AKT1, BRAF, CTNNB1, EGFR, FLT3, JAK2, KIT, KRAS, MAP2K1 (MEK1), NOTCH1, NRAS, PIK3CA, PTEN, and TP53 was performed by amplification using 13 multiplexed PCR reactions followed by single nucleotide base extension reactions. The products were separated by capillary electrophoresis and analyzed using GeneMapper 4.0 (38).

Subcloning and sequencing of ROS1

DNA was isolated from frozen tissue sample via Rnasey Mini Kit (Qiagen) which then underwent first-strand synthesis using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. The resulting cDNA was subjected to 30 rounds of PCR amplification targeting exons 35 to exon 39 of ROS1. The 387 bp-PCR product was then inserted into a TA cloning vector (Invitrogen) and used to transform competent TOP10 bacteria (Invitrogen) and subsequently plated on LB amp plates. Bacterial colonies were miniprepped (Qiagen) and DNA sequenced using the T7 primer.
ALK and ROS1 FISH positivity was determined using break-apart probes (Vysis LSI ALK 2p23) Dual Color, Break Apart Rearrangement Probe and Vysis LSI ROS1 (Cen) SpectrumGreen and Vysis LSI ROS1 (Tel) SpectrumOrange (Abbott Molecular). The FISH assays and analyses were performed as described previously with minor modifications (39). Specimens were considered positive for rearrangement when ≥15% of cells carried split 3' and 5' signals, which were physically separated by ≥2 signal diameters for ALK or ≥1 signal diameter for ROS1, or single 3' signals. Copy-number of the rearranged genes was based on determination of the mean of split and isolated red signals per tumor cell (40). At least 50 tumor cells were analyzed per specimen. CNG was defined as a more than twofold increase in the mean of the rearranged gene per cell in the posttreatment specimen compared with the pretreatment specimen.

### Results

**Demographics**

Twelve ROS1+ and 43 ALK+ patients underwent biopsies after demonstrating radiographic progression while on treatment with a ROS1+ or ALK-directed TKI. Three of 12 (25%) ROS1+ patients and 18 of the 43 (42%) ALK+ patients had more than one line of ROS1/ALK–targeted therapy prior to the biopsy that was evaluated in this study. Five patients (all ALK+) had >1 repeat biopsy following progression on different treatments. Fifteen of the ALK+ patients were reported previously, with updated sequencing using NGS (Supplementary Table S3; refs. 29, 43). Table 1 shows the characteristics of both cohorts. A majority of ROS1+ and ALK+ patients were males with adenocarcinoma and were never or light smokers. The median ages were 47 and 53 for the ROS1+ and ALK+ patients, respectively. All patients received crizotinib as their initial TKI therapy against ROS1 or ALK with median time transfecting using TansIT-X2 (Mirus) with this construct and then cultured in the presence of 1.5 μmol/L of crizotinib for 4 weeks. After 4 weeks, the surviving cells were assessed for the genomic rearrangement using primers that flank RALGAPA1 and NRG1 breakpoint.

**Probem ligation assay**

This assay, performed on FFPE tumor samples, was described previously using the Duolink In Situ PLA (Sigma; ref. 42). Antibodies used for this assay included ALK (D5F3) and ErbB2 (29D5) from Cell Signaling Technology and GRB2 (clone 81) from BD Biosciences.

### Table 1. Patient demographics

<table>
<thead>
<tr>
<th></th>
<th>ROS1 (n = 12)</th>
<th>ALK (n = 43)</th>
<th>Total (n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
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<tr>
<td>Sex (%)</td>
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<td>8 (67)</td>
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<td>4 (33)</td>
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<td><strong>Histology (%)</strong></td>
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</tr>
<tr>
<td>Adenocarcinoma</td>
<td>11 (92)</td>
<td>42 (98)</td>
<td>53 (96)</td>
</tr>
<tr>
<td>Large cell</td>
<td>1 (8)</td>
<td>1 (2)</td>
<td>2 (4)</td>
</tr>
<tr>
<td><strong>Smoking status (%)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Never/light</td>
<td>11 (92)</td>
<td>37 (86)</td>
<td>48 (87)</td>
</tr>
<tr>
<td>Current/former</td>
<td>1 (8)</td>
<td>6 (14)</td>
<td>7 (13)</td>
</tr>
<tr>
<td><strong>Prior treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crizotinib</td>
<td>12 (100)</td>
<td>43 (100)</td>
<td>55 (100)</td>
</tr>
<tr>
<td>Ceritinib</td>
<td>2 (17)</td>
<td>7 (16)</td>
<td>9 (16)</td>
</tr>
<tr>
<td>Brigatinib</td>
<td>1 (8)</td>
<td>9 (21)</td>
<td>10 (18)</td>
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<tr>
<td>Aleclinib</td>
<td>0</td>
<td>4 (9)</td>
<td>4 (7)</td>
</tr>
<tr>
<td>Lorlatinib</td>
<td>0</td>
<td>1 (2)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>&gt;2 ALK/ROS1 targeted tx pre bx</td>
<td>3 (25)</td>
<td>19 (44)</td>
<td>22 (40)</td>
</tr>
<tr>
<td><strong>Biopsy site (%)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lymph node</td>
<td>1 (8)</td>
<td>22 (41)</td>
<td>23 (35)</td>
</tr>
<tr>
<td>Lung parenchyma</td>
<td>3 (25)</td>
<td>13 (24)</td>
<td>16 (24)</td>
</tr>
<tr>
<td>Liver</td>
<td>1 (8)</td>
<td>6 (11)</td>
<td>7 (13)</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>7 (58)</td>
<td>13 (24)</td>
<td>20 (36)</td>
</tr>
</tbody>
</table>

Abbreviations: bx, biopsy; tx, treatment.

*Never/light was considered to be >10 pack years.

*All patients received crizotinib as the first ALK- or ROS1–targeted therapy. Prior treatments are those targeted treatments the patient received prior to resistance biopsy.

*Percentage based on total number of rebiopsies. Five patients had >1 resistance biopsy.
(range) on therapy before progression of 187 days (106–533) and 206 days (28–1,035). In the ALK+ cohort, two patients received >1 TKI and received crizotinib TKI rechallenge prior to biopsy, including those patients the median time on therapy before progression was 194 days (Table 2). The preprogression biopsy treatments for the ROS1+ patients were crizotinib (9), ceritinib (2), and brigatinib (1; Table 2; Supplementary Table S3). The preprogression biopsy treatments for the ALK+ patients were crizotinib (30), ceritinib (6), brigatinib (7), alectinib (4), and lorlatinib (2).

### Fusion partner and variant frequencies

Ten ROS1+ and 29 ALK+ samples underwent custom-capture NGS. The coverage for ROS1 intron 31 was poor due to repetitive sequences. Coverage for introns 32 and 33 was sufficient for fusion detection (Supplementary Table S2). The fusion partner was identified in eight of 10 patients with ROS1+ tumors who were tested by NGS (Fig. 1A). Notably, one of the CD74-ROS1 fusions was only detected through the baiting of the CD74 gene and would not have been detected if only the ROS1 gene had been baited in the NGS assay (data not shown). In the remaining two patients, we were unable to identify the fusion partners using NGS; however, both patients were FISH positive and responded to ROS1 TKI therapy. The ROS1 fusion partners we identified in this cohort have all been described previously (1, 10).

**Figure 1.**

ROSI1 and ALK fusion partner and variant frequencies. A, ROS1 fusion partner frequencies in the 12 patients with tumor samples. Two patients' tumor samples were positive by FISH but negative by NGS. Two patient samples did not undergo NGS. B, ALK fusion variant frequencies in the 29 patients whose tumor samples underwent NGS. All NGS negative and NGS not done patients responded to crizotinib. Although fusion partner not identified, one patient found to have two ROS1 kinase domain mutations, illustrated in Fig. 2. **C**, One patient whose NGS and FISH was negative for ALK fusion; however, the patient's pretreatment tumor sample was positive for ALK fusion indicating loss of ALK during treatment.
gene rearrangements that required additional testing to confirm the presence of an in-frame gene fusion. In the first case, the genomic breakpoint was in intron 17 of EML4 and exon 20 of ALK (E17;A20). Inspection of the genomic sequence did not support an in-frame transcript between EML4 exon 17 and ALK exon 20. However, further testing using RT-PCR with direct sequencing and anchored-multiplexed PCR (AMP) revealed that the fusion transcript contains EML4 exon 17 and sequences derived from EML4 intron 17 and is lacking the first 79 nucleotides from exon 20 of ALK (Supplementary Fig. S1A). This patient demonstrated a tumor response to alectinib (Supplementary Fig. S1B–S1E). We identified three additional ALK fusions using the capture-based NGS assay in which there was evidence of a rearrangement within intron 19 of ALK, but in which the 5’ gene partner could not be positively identified; one of these was also further characterized by AMP and identified as E2;A20, whereas another had a complex rearrangement of E16;A20. In patients for whom we had biopsy samples at more than one treatment time point, we did not see any evidence of change in the fusion variant. Finally, one acquired resistance sample was negative for an ALK fusion using both NGS and FISH, indicating likely loss of the ALK fusion gene, a finding we have previously reported in resistance samples (29).

**ROSI1 kinase domain mutations**

Among our ROS1+ patients, exons 36–42 were sequenced by NGS and/or direct sequencing to evaluate for the presence of KDM. The average read-depth for these exons was greater than 3,000X for each exon using NGS (Supplementary Table S2). We identified only one patient whose resistance biopsy demonstrated ROS1 KDMs (Fig. 2). The patient had been treated with crizotinib for approximately 17 months prior to the
postprogression biopsy. The postprogression sample harbored dual ROS1 mutations generating a L2026M substitution, the gatekeeper position, and L1951R, which is located at the solvent front (Fig. 2A). The mutations occurred at different variant allele frequencies (VAF), 19% and 9%, respectively. We confirmed that these mutations were in trans by subcloning the ROS1 gene and demonstrating that no clones contained both treatments (data not shown). To further explore the clinical significance of these cooccurring mutations we tested sensitivity to treatment with crizotinib and ceritinib in an early live culture (CUTO16) derived from this patient sample by evaluating phosphorylation of ROS1, SHP2, and ERK1/2 (Fig. 2B). The patient was treated with ceritinib 750 mg daily orally given only partially decreased ROS1 activation as well as partially decreased downstream signaling of SHP2 and ERK1/2 (Fig. 2B). The protein and showed markedly increased pHER2 compared with not shown). CUTO23 displayed high level of total HER2 (Fig. 2F). We were unable to detect EGFR or pEGFR in these samples with detectable ALK rearrangement (51). Among the six patients with ALK CNG demonstrated both an increase in the number of rearranged copies per cell and in the number of cells with detectable ALK rearrangement (51). Among the six patients with biopsies with CNG, patient 7 also had a KDM (G1269A, VAF 26%) and 24% (S1206C) (Supplementary Fig. S3B). The crystal structure of the ALK kinase domain with the position of these two mutations is illustrated in Supplementary Fig. S3C. S1206 and E1210 are in the solvent-exposed region, adjacent to the adenosine triphosphate-binding pocket (47, 48). Thus, cooccurring mutations in this region may interfere with TKI activity.

Another of the ROS1 samples, (ROS1–8), harbored no KDM. A cell line, CUTO23, was derived from the malignant pleural effusion of this patient following progression on crizotinib. This cell line, confirmed to harbor CD74–ROS1 by NGS, showed resistance to crizotinib in cell proliferation assays (Fig. 2D). Although ROS1 protein was detectable at the predicted molecular weight for CD74–ROS1, pROS1 could not be detected (Fig. 2E; Supplementary Fig. S2). Crizotinib failed to inhibit pERK1/2, even at high concentrations, but did inhibit pSHP2, a known SHP1 signaling adaptor, suggesting that a bypass signaling pathway that did not utilize SHP2 was the cause of resistance (Fig. 2E). Given prior evidence by our group that HER family RTK members can mediate resistance to ROS1 inhibitors, we queried whether a pan-HER inhibitor, afatinib, might overcome the ROS1 resistance in this cell line (45, 46). Addition of afatinib partially restored crizotinib sensitivity in cell proliferation assays (Fig. 2D). Furthermore, afatinib inhibited critical downstream signaling via AKT and ERK1/2 (Fig. 2E). Another of the ROS1 samples, (ROS1–6), harbored two KDMs is illustrated by preprogression TKI in Fig. 3 and Supplementary Table S3.

**FISH identified copy-number gain in ALK** but not **ROS1** resistance samples

ALK fusion CNG has been previously implicated in TKI resistance (29, 47, 49, 50). We further characterized a subset of patients with available matched biopsies before and after treatment with TKIs to identify those with evidence of copy-number change by FISH. Biopsy specimens were available for comparative FISH for two ROS1 samples, but no CNG was identified. Of the 28 ALK patients in whom biopsy specimens were available for comparative FISH, we identified CNG in six patients (21%) (Table 3). Patients 7 and 8 were reported previously (29). Overall, all biopsies with ALK CNG demonstrated both an increase in the number of rearranged copies per cell and in the number of cells with detectable ALK rearrangement (51). Among the six patients with biopsies with CNG, patient 7 also had a KDM (G1269A, VAF 29%) and patient 31 was found to have a mutation in an alternate cancer-related gene (EGFR exon 19 deletion). In addition, patient 24 acquired a CNG after treatment with ceritinib when compared with a prior, post-crizotinib sample.

**Identification of potential ROS1- and ALK-independent mechanisms of resistance**

We and others have previously identified bypass signaling pathways as a mechanism of drug resistance that obviates the need for the normal dominant oncogene such as ROS1 and ALK (29, 45). The NGS panel was thus designed to evaluate alterations in additional oncogenes beyond ALK and ROS1. Among the 12 ROS1 patients, we identified a mutation in the kit proto-oncogene tyrosine kinase (KIT) (D816G) that we have previously characterized in vitro as an acquired resistance...
We also found a mutation in β-catenin (CTNNB1 S45F), which has been identified as a potential oncogenic driver in lung cancer, although we were not able to demonstrate its absence prior to therapy due to lack of available tissue (53).

Among the 43 ALK+ patients, we identified 13 oncogenic alterations in six different genes that may have resulted in new oncogenic activity or resistance to treatment (two fusions, one incidence of loss of ALK fusion gene, and 10 oncogenic mutations; Supplementary Table S3).

**Figure 3.**
Kinase domain mutation distribution in ALK+ resistance samples. A, Distribution of kinase domain mutations across ALK+ samples. One sample harbored G1202’ (VAF 19%) in combination with G1202R (VAF 25%). Three patients demonstrated compound mutation after multiple ALK-targeted TKIs. B, Distribution of kinase domain mutations in those patient who had a postprogression biopsy after crizotinib, first-line therapy for all patients. C, Distribution of kinase domain mutations in patients who had postprogression biopsy after >1 TKI. D, Distribution of kinase domain mutations in patients who had postprogression biopsy after crizotinib. E, Distribution of kinase domain mutations in patients who had postprogression biopsy after brigatinib. F, Distribution of kinase domain mutations in patients who had postprogression biopsy after ceritinib. G, Distribution of kinase domain mutations in patients who had postprogression biopsy after alectinib. H, One patient had a postprogression biopsy after treatment with lorlatinib. Prior treatments for each patient listed in subscript. Patients with serial biopsies on different treatments indicated with asterisk. If multiple biopsies KDM listed for biopsy on which it appeared. Abbreviations: A, alectinib; B, brigatinib; C, crizotinib; X, stop codon.
Gene fusions identified as ALK resistance mechanisms

Patient ALK-22 demonstrated a RALGAPA1–NRG1 fusion on the post-alectinib tumor sample (Supplementary Fig. S4A and S4B). Although NRG1 fusions have been described, RALGAPA1 has not been described as a partner with NRG1. To demonstrate this fusion was both functional and could induce resistance to an ALK inhibitor we used CRISPR to engineer this fusion into the H3122 cell line to (H3122-NRG1). The presence of the NRG1 fusion in H3122 was confirmed by genomic sequencing (not shown). H3122-NRG1 cells demonstrated marked resistance to crizotinib and sensitivity was restored by the pan-HER inhibitor afatinib (Fig. 4A). Western blot analysis demonstrated increased pHER3 (ERBB3), the receptor for the ligand neuregulin 1 (Fig. 4B). H3122-NRG1 cells showed persistent pAKT and pERK1/2 in the presence of crizotinib. Afatinib inhibited pHER3 and inhibited pAKT and pERK1/2 and the addition of afatinib to crizotinib similarly inhibited AKT and ERK1/2 signaling. Notably the induction of the NRG1 fusion in H3122 cells led to the loss of phosphorylation of SHP2, a known signaling adaptor for ALK. Analysis of the ALK-22_2 sample by proximity

Table 3. FISH analysis to evaluate for copy-number alterations

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>ALK FISH % cells positive</th>
<th>ALK FISH pattern</th>
<th>Abnormal ALK copy-number/cell</th>
<th>ALK FISH</th>
<th>ALK FISH % cells positive</th>
<th>ALK FISH pattern</th>
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<tr>
<td>7</td>
<td>28%</td>
<td>Split</td>
<td>0.5 × sR, sG</td>
<td>Positive</td>
<td>82%</td>
<td>Split</td>
<td>1.5 × sR, sG</td>
<td>Positive</td>
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<tr>
<td>8</td>
<td>48%</td>
<td>Split</td>
<td>0.5 × sR, sG</td>
<td>Positive</td>
<td>66%</td>
<td>Split</td>
<td>2.2 × sR, sG</td>
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<tr>
<td>20</td>
<td>76%</td>
<td>sR</td>
<td>0.78 × sR</td>
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<td>sR</td>
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<td>22</td>
<td>76%</td>
<td>sR</td>
<td>1.32 × sR</td>
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<td>sR</td>
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<td>sR</td>
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<td>80%</td>
<td>sR</td>
<td>1.84 × sR</td>
<td>Positive</td>
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Abbreviations: ID, patient ID; mix, split red/green and single red; split, split red/green; sG, single green; sR, single red.

Figure 4. RALGAPA1-NRG1 fusion induces drug resistance in ALK+ cancer. A, Cellular proliferation of the EML4-ALK cell lines, H3122 (blue) or its derivative H3122-NRG1 (red), which harbors a CRISPR induced RALGAPA1-NRG1 fusion using increasing doses of crizotinib demonstrating marked resistance crizotinib resistance. Addition of afatinib (100 nmol/L) resensitized the H3122-NRG1 (black) to crizotinib. IC50 for crizotinib was 187 ± 3.7 nmol/L, for H3122-NRG1 was 1182 ± 5.3 nmol/L, and for H3122-NRG1 with afatinib was 200 ± 10.5 nmol/L. Error bars, mean ± SEM for three triplicate experiments (n = 9). B, Western blot analysis of H3122 or H3122-NRG1 cells treated with crizotinib 500 nmol/L and/or afatinib 100 nmol/L as indicated for 2 hours. H3122-NRG1 cells demonstrated increased levels of phosphorylated and total ERBB3, pAKT and pERK1/2 compared with H3122 cell lines, but lack of pSHP2. Crizotinib inhibits SHP2, AKT, and ERK1/2 in H3122 cells. AKT and ERK are not inhibited by crizotinib alone in H3122-NRG1, but are inhibited by afatinib as is ERBB3 indicating oncogene switch from ALK to HER3, the receptor for neuregulin. C, Proximity ligation assay (PLA) of ALK and GRB2 from ALK-22_2 FFPE tumor sample showing functional ALK signaling. D, PLA of ERBB2 and GRB2 from ALK-22_2 sample demonstrating functional ERBB2 signaling.

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ROS1 and ALK Resistance Mechanisms
ligation assay demonstrated the presence of both ALK-GRB2 and HER2 (ERBB2)-GRB2 complexes consistent with functional signaling by both of the ALK and NRG1 gene fusions (Fig. 4C and D). HER2 is known to heterodimerize with HER3 upon activation (10). The tumor sample ALK-44_2 was found to have a CCDC6-RET gene fusion on the post-brigatinib biopsy in addition to the known EML4-ALK fusion (not shown). This gene fusion, which fuses exon 1 of CCDC6 to exon 12 of RET has been described as a primary oncogenic driver in NSCLC and other cancers (10). This RET gene fusion was not identified on the prior biopsy, which occurred post-alecromitin (ALK-44_1).

Two patients were found to have EGFR mutations (L858R and exon 19 deletion). Three KRAS mutations were also observed (G12C, G12V, and G13D). The clinical details of the patients with G12C and G12V have been previously reported (29); however, we were previously unable to validate the presence of a functional ALK fusion (other than ALK FISH positivity) in the sample also harboring KRAS G12C. Using NGS, we demonstrated both an EML4-ALK (E6;A20) fusion as well as a KRAS G12C mutation in the same biopsy specimen. Notably, KRAS G12C was present in the tumor sample prior to crizotinib therapy and heralded primary progression as best response to therapy. This is in contrast to the patient with KRAS G12D who did not demonstrate this mutation in the pre-TKI clinical sample and had a much longer response to crizotinib.

We also identified mutations in isocitrate dehydrogenase 1 (IDH1) (R132C) and neurofibromatosis 1 (NF1) (Q642X) in different patients. Interestingly, the NF1 mutation was found in a patient (ALK-28) with a clinical syndrome consistent with neurofibromatosis and this patient demonstrated short-lived responses to both crizotinib (139 days) and alecromitin (110 days). In another patient (ALK-46), we identified a previously unreported RIT1 mutation, K139N, although the significance of this mutation is unknown. Mutations in RIT1 have been associated with lung adenocarcinoma via activation of the RAS/RAF/MEK and PI3K pathways (54). Finally, we identified mutations in notch homolog 1 (NOTCH1) D1533 and D1538) in patients ALK-8 and ALK-42. Mutations in NOTCH1 have been identified in hematologic, head and neck and lung squamous cell carcinomas (55, 56), although their clinical significance in this setting is unknown.

We performed analysis of CNV using our target capture NGS data (ref. 57; Fig. 5 and Supplementary Fig. S5). In ROS1+ cohort, three patient samples demonstrated evidence of CNV of cancer-related genes by NGS ROS1-1 (SRC, ERBB2, STK11, and NOTCH1), ROS1-9 (PDGFR A, KIT, and KDR) and ROS1-11 (FGFR3, RET, and ERBB2).

We also observed increases in CNV in several proto-oncogenes within our ALK dataset including KRAS (ALK-10): EGFR (ALK-21 and -27_2), FGFR1 (ALK-35 and -47), GNAS (ALK-40), DDR2 (ALK-10 and -41), HRAS (ALK-25 and -37), and NTRK1 and RIT1 in sample ALK-25. These genes may have a role in bypass pathway activation and resistance to targeted therapy. Notably, patient ALK-10 with evidence of KRAS CNV also harbored the preexisting KRAS G12C mutation, which is notable given that mutant KRAS alleles frequently demonstrate CNG (58).

Discussion

In this study, we evaluated potential mechanisms of resistance at the time of progression on targeted therapy in 12 ROS1+ patients and 43 ALK+ patients across multiple different TKIs and lines of therapy.

Within the ROS1 cohort of 12 patients, a possible mechanism of resistance was identified in 50% of ROS1+ patients (summarized in Fig. 6A). Together with the relatively small number of patients in the ROS1+ cohort, the confidence intervals around the exact frequency of any mechanism must be considered broad. Despite this we found very few KDM in ROS1 patients compared with the frequency, and we and others have identified in ALK-positive patients (29, 30). Previously, KDMs have been identified in patient tumor samples include G2032R, D2033N, and S1986Y/F (59–61). Although the KDM mutations we identified (L2026M and L1951R) have been previously reported, in mutagenesis screens they have not been reported in patient samples (21, 61, 62).

Within the subcategories of ALK+ patients, a mechanism of resistance was identified in 71% of 28 patients evaluated after first-line crizotinib (Fig. 6B). In the 21 patients who received more than one ALK-targeted therapy, 95% of patients demonstrated a possible mechanism of resistance (Fig. 6C). Thus, similar to prior reports, we found that patients treated with >1 TKI were more likely to have developed a KDM compared with patients who had received crizotinib as a first line of targeted therapy (30). While it is possible that this represents a difference in resistance mechanisms to crizotinib versus next-generation ALK inhibitors, an alternative explanation may be patient selection bias. Whereas most patients who progressed on crizotinib as the first ALK TKI were offered biopsy at progression, patients on second- or third-line ALK TKIs who experienced immediate progression were not generally offered rebiopsy at our institution as they had typically undergone biopsy 6 to 8 weeks prior, and it was deemed unlikely that new information would be identified from these tumor samples. Patients with primary progression to an appropriate ALK TKI are the least likely to have an ALK KDM, thus skewing the denominator for this cohort of patients. In the ALK+ cohort who received >1 ALK targeted TKI, the small numbers of patients within each treatment group and frequent lack of sufficient pretreatment tissue for comparison makes it difficult to draw significant conclusions about the relationship between different KDM and prior TKI therapy. For example, one of the two patients who received treatment with lorlatinib had a posttreatment biopsy that demonstrated a D1203A mutation. This may be a mutation that evolved in response to lorlatinib; however, the patient has received several prior TKIs without pretreatment biopsy, so it is difficult to ascribe causality. Despite the small numbers, it is notable that two of seven brigatinib samples harbored compound mutations in cis.

ALK+ patient 17 illustrates the challenges of focusing on KDM characterization as the main source of resistance. This patient had an F1174C mutation in a post-first-line crizotinib biopsy sample. Despite this mutation, which has documented resistance to ceritinib (63), the patient responded to ceritinib for nearly one year. After rechallenges with crizotinib then ceritinib, resistance again demonstrated the F1174C mutation. This case

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illustrates several important points; first, although resistance mechanisms can be inferred on progression after a period of response and known sensitivity patterns, definitive determination of an acquired resistance mechanism requires pre/post-treatment testing demonstrating that the mechanism was acquired during the most recent treatment and/or demonstration of resolution of the resistant clone on new effective treatment. Consideration of alternative oncogenic drivers and...
evaluation of bypass pathway activation may identify clinically informative information. Future efforts may need to rely on multiple genomic and proteomic evaluations to determine the full extent of resistance mechanisms.

With regard to KDM in ROS1$^+$ patients, it is notable that other small clinical series have suggested a much higher rate of ROS1 KDM (45%), with almost all KDM (4 of 5, 80%) being G2032R (64). We did not observe this in our cohort, raising questions about the true frequency of ROS1 KDM. The two KDM in our cohort, L2026M and L1951R, cooccurred in one patient.

Use of early passage live-cell culture from this patient’s tumor allowed us to identify a drug with potential clinical activity that was initially stable on serial CT imaging. The rapid progression observed in this patient was consistent with the lack of in vitro activity of ceritinib against the L1951R subclone, inadequate ceritinib exposures to achieve the potential inhibition of L2026M observed in vitro, or the activity of a bypass signaling pathway not detected by our testing. In tumor sample ROS1–, we were unable to identify any acquired resistance alterations using NGS; however, we demonstrated the role of the HER2 pathway in mediating bypass of a ROS1 TKI using CUTO23, a patient-derived cell line from this tumor sample. This confirms previous preclinical work by our laboratory demonstrating the critical role for the HER family in ROS1$^+$ NSCLC and highlights the difficulty in uncovering mechanisms of resistance not driven by mutations (45, 46). In the ALK cohort, we identified an NRG1 fusion further highlighting the role of the HER family RTK signaling in drug resistance.

Dardei and colleagues recently demonstrated that a SHP2 inhibitor could overcome resistance in ALK$^+$ NSCLC models (65). Importantly, two resistance models generated in this work, H3122-NRG1 (ALK) or CUTO23 (ROS1) did not show significant SHP2 modulation by inhibition of the bypass resistance mechanisms, nor did our prior ROS1 resistance models of HCC78- and CUTO2-KITD816G, suggest that SHP2 inhibition might not be able to overcome some modes of bypass-mediated resistance (52).

We also identified a RET fusion as a mechanism of acquired resistance in ALK$^+$ NSCLC. Recently ALK, RET, FGFR3, and NTRK1 fusions have been described as resistance mechanisms to the

Figure 6.
Resistance mechanisms after TKI in ROS1$^+$ ALK$^+$ patients. Venn diagrams demonstrating the distribution of resistance mechanisms identified in each cohort. A, ROS1 cohort ($n = 12$). B, ALK 1 TKI cohort ($n = 28$). C, ALK >1 TKI cohort ($n = 21$).
that this germline mutation may have played a role in intrinsic patient progression after approximately 4 months on a prior clinical diagnosis of neurotumor suppressor gene

deficiencies, and cholangiocarcinoma (29, 71, 72). Mutation in the mutations encoding substitutions at R132 have been implicated via activation of p21, RAS, and the mTOR pathway (73, 74). This case was particularly compelling as the NF1 mutation was likely a germline mutation for the patient with a VAF 50% and a prior clinical diagnosis of neurofibromatosis. Notably, this patient progressed systemically after approximately 4 months on crizotinib and approximately 3.5 months on alectinib, suggesting that this germline mutation may have played a role in intrinsic drug resistance.

In our ROS1+ cohort, we identified three alternative pathway mutations: KIT, β-catenin, and GNA11. The activating mutation in KIT has been described previously by our lab and is notable for being resistant to most KIT-specific TKIs (52). One patient had a mutation in the gene encoding β-catenin (S45F, VAF 22%). β-Catenin is involved in cell-cell adhesion and is thought to be involved in the wingless/WNT signaling pathway. This mutation has been previously reported in SCLC lung cancer and implicated in its development, and was reported in another cohort of ROS1 patients (53, 64). GNA11 is known to be involved in development of uveal melanoma, although the functional significance of this mutation is unknown (75, 76). In this study, we did not assay for RAS CNG by FISH or WT EGFR activation, which are known mechanisms of resistance in ROS1+ NSCLC, this may have decreased our detection of resistance mechanisms in the ROS1+ cohort (45, 77).

CNG of the dominant oncogene provides another source of resistance that have been described previously (29, 47, 49, 50). We did not identify evidence of ROS1 CNG in the patients evaluated in our ROS1+ cohort; however, ROS1 FISH was performed in a small number of these samples. However, in our ALK+ patient cohort, we reported 6 of 28 evaluated patients (21%) demonstrated ALK fusion CNG. These may be underestimates of incidence, as analysis of CNG requires pre- and posttreatment samples, which were not always available in our series.

CNV evaluation by NGS allows the monitoring of multiple genes simultaneously, where FISH can typically only test one gene at a time. Using CNV by NGS, we identified several putative resistance mechanisms not identified by other analyses. In the ROS1 and ALK cohorts treated with crizotinib, none of the 9 cases with CNV identified had evidence of a KDM, suggestive of alterations that contribute to resistance. Finally, samples ROS1–9 and ALK–33 showed evidence of CNV of genes located at the chromosomal locus of 4q12, which is frequently amplified in lung cancer and may generate drug resistance via bypass signaling mediated by these RTKs (78).

In this report, we describe the patterns of resistance mutations in biopsies from a cohort of patients with ROS1 or ALK rearrangements. As larger datasets are accumulated and we have increased experience with patients on multiple different TKIs, we may be able to identify unique patterns of resistance that develop with individual TKIs. In addition, although KDMs remain an important mechanism of resistance, our data clearly shows that bypass pathway signaling through both genetic alterations and nonmutation–driven pathways are at least equally important and we are likely to benefit from inquiry into these mechanisms for patient care.

Disclosure of Potential Conflicts of Interest

C.E. McCoach is a consultant/advisory board member for Takeda and Guardant Health. A. Le holds ownership interest (including patents) in Molecular Abbott. P. A. Bunn is a consultant/advisory board member for AstraZeneca, Takeda, and Genentech. R. Dziadziuszko reports receiving speakers bureau honoraria from Roche, Pfizer, and Novartis, and is a consultant/advisory board member for Roche. D. L. Aisner reports receiving commercial research grants from Genentech and is a consultant/advisory board member for Abbvie, Bristol, Myres, Squibb, and Genentech. R. C. Doebele reports receiving commercial research grants from Ignyta, holds ownership interest (including patents) in Rain Therapeutics, is an associate/advisory board member for Pfizer, I stratégie, Ariad, Takeda, AstraZeneca, Guardant Health, Trovagene, and Spectrum, and reports other remuneration from Abbott Molecular. No potential conflicts of interest were disclosed by the other authors.

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References


Resistance Mechanisms to Targeted Therapies in \(ROS1^+\) and \(ALK^+\) Non –small Cell Lung Cancer

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