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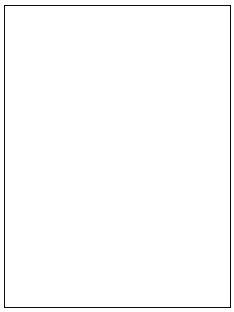

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PII: S1556-0864(17)30668-8

DOI: 10.1016/j.jtho.2017.08.002

Reference: JTHO 672

To appear in: *Journal of Thoracic Oncology*

Received Date: 17 July 2017

Revised Date: 5 August 2017

Accepted Date: 8 August 2017

Please cite this article as: Lin JJ, Shaw AT, Recent Advances in Targeting ROS1 in Lung Cancer, *Journal of Thoracic Oncology* (2017), doi: 10.1016/j.jtho.2017.08.002.

Recent Advances in Targeting ROS1 in Lung Cancer

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Running Title: ROS1 in Lung Cancer

Keywords: ROS1 rearrangement; non-small cell lung cancer; crizotinib; ROS1 inhibitor; resistance

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Disclosures: **JJL** has served as a compensated consultant or received honoraria from Boehringer-Ingelheim and Chugai. **ATS** has served as a compensated consultant or received honoraria from Pfizer, Novartis, Genentech/Roche, Ariad, Ignyta, Blueprint Medicines, KSQ Therapeutics, and Foundation Medicine.

Word Count: 5,582 (excluding abstract, acknowledgements, references, legends)

Figures/Tables: 6

Abstract

ROS1 is a validated therapeutic target in non-small cell lung cancer (NSCLC). In a phase I study, the multi-targeted MET/ALK/ROS1 inhibitor crizotinib demonstrated remarkable efficacy in *ROS1*-rearranged NSCLCs, and consequently gained approval by the United States Food and Drug Administration as well as the European Medicines Agency in 2016. However, similar to other oncogene-driven lung cancers, *ROS1*-rearranged lung cancers treated with crizotinib eventually acquire resistance, leading to disease relapse. Novel ROS1 inhibitors and therapeutic strategies are therefore needed. Insights into the mechanisms of resistance to ROS1-directed tyrosine kinase inhibitors (TKIs) are now beginning to emerge and are helping to guide the development of new ROS1 inhibitors. This review discusses the biology and diagnosis of *ROS1*-rearranged NSCLC, and current and emerging treatment options for this disease. Future challenges in the field are highlighted.

Introduction

Chromosomal rearrangements involving the ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*) gene were first described in non-small cell lung cancer (NSCLC) in 2007 (1). Since then, oncogenic *ROS1* rearrangements have become an established therapeutic target in lung cancer. *ROS1* rearrangements are identified in 1-2% of NSCLC patients (2). While this prevalence may seem low at first glance, the high incidence of lung cancer cases in the United States (U.S.) means that 2,000 to 4,500 patients will be newly diagnosed with *ROS1*-rearranged NSCLC each year (3).

In March 2016, crizotinib, an anaplastic lymphoma kinase (ALK)/ROS1/MET inhibitor, became the first targeted agent approved by the U.S. Food and Drug Administration (FDA) for the treatment of advanced *ROS1*-rearranged NSCLC. This approval was based on the efficacy and safety data from the expansion cohort of the phase I crizotinib study (PROFILE 1001), which demonstrated an objective response rate (ORR) of 72% and median progression-free survival (PFS) of 19.2 months in advanced *ROS1*-rearranged NSCLC (4). Despite often durable responses, the majority of patients ultimately experience disease relapse due to acquired resistance. To date, crizotinib is the only targeted therapy approved for *ROS1*-rearranged NSCLC, underscoring the urgent need to develop novel ROS1-directed therapies.

In this review, we provide an up-to-date overview of the biology and treatment of *ROS1*-rearranged NSCLC, with an emphasis on current and emerging targeted therapy options. We discuss early insights into mechanisms of crizotinib resistance, and how these mechanisms may help guide the development of new therapeutic strategies on the horizon.

ROS1 Function

The *ROS1* gene was originally discovered as a homolog of the transforming sequence of the avian sarcoma RNA virus UR2 (5, 6). Located on chromosome 6q22.1 (7, 8), *ROS1* encodes a receptor tyrosine kinase (RTK) containing a large N-terminal extracellular domain, a hydrophobic single-pass transmembrane region, and a C-terminal intracellular tyrosine kinase domain (9). Phylogenic sequence analysis has revealed that ROS1 is related to the ALK/LTK and insulin receptor RTK families (10). The homology to ALK has been particularly relevant in the development of ROS1-directed therapies; several (but importantly, not all) ALK tyrosine kinase inhibitors (TKIs) harbor dual inhibitory activity against ALK and ROS1 (see discussion below).

ROS1 is an evolutionarily conserved RTK in *C. elegans*, *D. melanogaster*, and vertebrates. In *Drosophila*, the *ROS1* homologue, *Sevenless*, is activated by the binding of its ligand *Bride of sevenless* (*Boss*) and contributes to photoreceptor cell differentiation (9). In chicken, mouse, and rats, *ROS1* expression has been detected in the epithelial cells of the kidneys, male reproductive organs, small intestines, heart, and lungs (11, 12). *ROS1*-deficient male mice are notably healthy but infertile, with defective sperm maturation secondary to the impaired differentiation of epididymal epithelial cells; female *ROS1*-deficient mice develop normally without any detectable abnormalities (13). The biological role of native ROS1 in humans has not yet been defined, and it remains an orphan RTK without a known ligand (9).

***ROS1* Gene Fusions in Cancer**

ROS1 gene fusions were first identified in a human glioblastoma cell line (**Figure 1**) (14). In this cell line, the 3' region of *ROS1* was found fused to the 5' region of the fused in glioblastoma gene (*FIG*; also called golgi associated PDZ and coiled coil motif containing, *GOPC*) via the interstitial deletion of 240 kilobases on chromosome 6q21, resulting in a constitutively active fusion kinase (15, 16). A *CEP85L-ROS1* rearrangement has also been reported in an adult

glioblastoma tumor (17). Since the initial description in glioblastoma, *ROS1* fusions have been detected in a range of malignancies including inflammatory myofibroblastic tumor (18, 19), cholangiocarcinoma (20), ovarian cancer (21), gastric cancer (22), colorectal cancer (23), angiosarcoma (24), spitzoid melanoma (25), and NSCLC (1, 4, 26-38).

NSCLC was the second solid tumor found to harbor *ROS1* rearrangements. In a study of 41 NSCLC cell lines and over 150 NSCLC tumors, ROS1 was identified as a highly activated kinase in one cell line and one tumor sample (**Figure 1**) (1). Sequencing analysis of these samples revealed a novel *SLC34A2-ROS1* fusion in the HCC78 cell line, and a *CD74-ROS1* fusion in the tumor specimen (1). A total of 14 different *ROS1* fusion partner genes have now been reported in lung cancer, including *CD74* (1, 4, 26-29), *SLC34A2* (1, 4, 26-28), *SDC4* (4, 26, 27), *EZR* (4, 26, 30, 31), *FIG* (28, 32), *TPM3* (4, 26), *LRIG3* (26), *KDELR2* (33), *CCDC6* (34), *MSN* (4, 35), *TMEM106B* (36), *TPD52L1* (37), *CLTC* (38), and *LIMA1* (4) (**Figure 2**). Of these, the *CD74-ROS1* occurs most frequently in NSCLC (**Figure 2B**). All *ROS1* fusions retain the entire ROS1 kinase domain (26).

A number of studies have demonstrated the oncogenic potential of *ROS1* fusions. Expression of *ROS1* fusions results in transformation of NIH3T3 and Ba/F3 cells *in vitro*, and tumorigenicity *in vivo* (16, 20, 26, 27, 30, 39). Transgenic mice expressing *EZR-ROS1* in the lung alveolar epithelium develop bilateral lung adenocarcinomas (30). The exact mechanism of ROS1 kinase activation in the fusion proteins has not been established. Interestingly, in contrast to *ALK* rearrangements in which the fusion partners provide dimerization domains that induce constitutive kinase activation, the majority of the known *ROS1* fusion partners do not contain such domains (**Figure 2A**) (26). Furthermore, localization of the ROS1 fusion proteins varies, ranging from the plasma membrane to the Golgi apparatus to the cytoplasm (28, 40). Once activated, ROS1 signals through the MAPK/ERK, PI3K/AKT, JAK/STAT3, and SHP1/2

pathways to promote cell growth and survival (20, 27, 39, 41). Whether distinct *ROS1* fusions confer differential levels of expression, kinase activation and oncogenicity is unknown.

Clinicopathologic Characteristics of *ROS1*-Rearranged Lung Cancer

Across studies, the reported frequency of *ROS1* rearrangements in NSCLC has ranged from 0.9-2% (2, 17, 26-29, 38). Similar to *ALK*, *ROS1* rearrangements are associated with younger age, never or light smoking history, and adenocarcinoma histology (2, 26). Rarely though, *ROS1* rearrangements have been detected in NSCLC with large cell or squamous cell histology (28, 40). Notably, not all clinicopathologic features are shared between *ALK*- and *ROS1*-rearranged NSCLCs. For example, one recent series comparing 39 *ROS1*-rearranged and 196 *ALK*-rearranged NSCLC patients found that *ROS1* rearrangements were associated with a significantly lower rate of extrathoracic and brain metastases at the time of diagnosis, in addition to a lower cumulative incidence of brain metastases (42). Although the number of patients included in this series was limited, particularly for the *ROS1*-rearranged subset, the findings suggest that patterns of metastases may be distinct for *ROS1*- versus *ALK*-rearranged lung cancer. The biological basis for these differences in metastatic patterns has yet to be determined.

Generally, oncogenic drivers in NSCLC, such as KRAS, EGFR, and ALK, are mutually exclusive (43). An early analysis of 1,073 NSCLC tumor specimens demonstrated no overlap between *ROS1* and *ALK* rearrangements (2). However, conflicting findings have subsequently been reported, with some later studies suggesting a co-occurrence of *ROS1* rearrangements and mutations in *EGFR* (28, 44), *KRAS* (44), or *BRAF* (44). In the most recent and largest series to date, a total of 220 cases of *ROS1*-rearranged NSCLCs were examined (45). Amongst these tumors, *ROS1* fusions did not overlap with *ALK* fusions, and rarely co-occurred with oncogenic

EGFR mutations (0.5%; 1/220) or *KRAS* mutations (1.8%; 4/220) (45). Therefore, *ROS1* rearrangements generally define a unique molecular subset of NSCLC.

Clinical Detection of *ROS1* Fusions

Diagnostic methods used for the detection of *ROS1* fusions largely mirror those used for *ALK*, with a few differences. Crizotinib was initially approved in 2011 for the treatment of advanced *ALK*-rearranged NSCLCs, with ALK fluorescence in situ hybridization (FISH) as the companion diagnostic test. Four years later, ALK immunohistochemistry (IHC; Ventana D5F3 antibody) also received FDA approval as a companion diagnostic (46). In contrast to *ALK*, there are currently no approved companion assays for *ROS1*-rearranged NSCLC. Based on experiences with *ALK*, commonly used methods for *ROS1* fusion detection have included FISH, IHC, reverse transcription polymerase chain reaction (RT-PCR), and next-generation sequencing (NGS).

FISH positivity was required in the registration trials of crizotinib, and FISH is commonly utilized as the standard diagnostic assay for *ALK* rearrangement in NSCLC. Early *ROS1* studies also employed this technique for fusion detection (2, 26, 27). ROS1 FISH utilizes a dual break-apart probe design, with red and green fluorescent labels for the 3' and 5' portions flanking the *ROS1* breakpoint (2). A normal *ROS1* gene without a rearrangement yields the fused, yellow signal. A rearranged *ROS1* gene yields the 'classic' split red and green signals, or the 'atypical' isolated 3' red signal. A tumor is considered 'FISH positive' if at least 15% of evaluated tumor cells contain split or isolated 3' signals. While FISH can be performed on a small amount of formalin-fixed, paraffin-embedded (FFPE) tissue, the assay can be challenging to interpret, particularly for fusions arising from small intrachromosomal deletion events. Therefore, false-negative and false-positive FISH results can occur (45).

IHC serves as an alternative diagnostic tool, and is often faster to perform than FISH. The ROS1 D4D6 rabbit monoclonal antibody (Cell Signaling Technology, MA) is commercially available and exhibits sensitivity nearing 100% and specificity ranging between 92-100% (28, 47, 48). However, the ROS1 IHC readout can be more difficult to interpret and operator-dependent compared to ALK IHC for several reasons. ROS1 staining patterns may vary due to different intracellular localization of the ROS1 fusions (28). Moreover, benign pneumocytes and alveolar macrophages, and, in bone metastatic lesions, osteoclast-type giant cells can all express ROS1, generating more background staining than is seen with ALK (47, 48). IHC results may also be falsely positive due to aneuploidy leading to aberrant expression. Thus, albeit useful as a screening tool, ROS1 IHC by itself may be insufficient to diagnose *ROS1*-rearranged NSCLC, and testing typically requires confirmation using an orthogonal method such as FISH or NGS (45).

A unique advantage of NGS is that it enables multiplex testing and allows for the detection of known as well as novel fusions. Indeed, a number of *ROS1* fusions were discovered using NGS (33, 36, 37). This may become particularly relevant if specific *ROS1* variants are found to impact tumor biology and clinical outcomes, as has been proposed in *ALK*-rearranged lung cancers (49-51). By comparison, RT-PCR requires *a priori* knowledge of fusions for the design and incorporation of fusion-specific primers, and will thus miss the detection of previously unknown fusions. NGS is being increasingly utilized because of these advantages, although there are limitations including higher cost than FISH or IHC, the need for more tissue, and slower turnaround time. Further studies are needed to assess the performance of different NGS platforms; however, in one study, an anchored multiplex PCR enrichment method to detect gene rearrangements in 319 FFPE samples achieved 100% sensitivity and 100% specificity, compared to FISH reference assays (35).

ROS1-Targeted Therapies in Lung Cancer

ROS1-rearranged lung cancers are dependent on (i.e., "addicted" to) ROS1 for growth and survival. Rikova et al. demonstrated that the HCC78 lung cancer cells bearing the *SLC34A2-ROS1* fusion undergo apoptosis upon short interfering RNA-mediated knockdown of *ROS1* (1). Subsequently, pharmacologic inhibition of ROS1 was shown to induce growth inhibition in a number of *ROS1*-rearranged cell line models (2, 27, 28, 52). This finding was later validated in patients (2, 4), spurring efforts to develop ROS1-directed TKIs.

Crizotinib

One year after the initial description of *ROS1* fusions in NSCLC, McDermott et al. fortuitously discovered that HCC78 was the only non-*ALK*-rearranged cell line (in a screen of 602 human cancer cell lines) sensitive to the ALK inhibitor compound TAE684 (52). The authors speculated that this sensitivity could potentially arise from the inhibition of ROS1 by TAE684, based on the homology between ROS1 and ALK (52). Indeed, these two RTKs share a 49% amino acid sequence identity in the kinase domain and 77% identity in the adenosine triphosphate (ATP)-binding site (4), providing a structural basis for the activity of ALK TKIs against ROS1.

Crizotinib was originally developed as a MET inhibitor and subsequently approved for the treatment of advanced *ALK*-rearranged NSCLCs (53, 54). Further preclinical studies demonstrated that crizotinib also potently inhibits ROS1 (2, 4, 27, 28). In biochemical assays, crizotinib had an IC_{50} of 8 nM against MET, 40-60 nM against ALK, and 60 nM against ROS1 (55). Based on the available preclinical data, the phase I PROFILE 1001 study of crizotinib was amended to include *ROS1*-rearranged NSCLC patients in the expansion cohort. Early responses to crizotinib were marked and reminiscent of responses in *ALK*-rearranged patients (**Figure 3**). Among 50 patients with *ROS1*-rearranged NSCLCs in this trial cohort, the ORR to crizotinib was 72%, with disease control rate (DCR) of 90%. The median PFS reached 19.2

months (4). Based on the efficacy and safety demonstrated in this study, crizotinib was granted full approval by the FDA for the treatment of advanced *ROS1*-rearranged NSCLC in March 2016. Crizotinib has also received approval by the European Medicines Agency (EMA) for metastatic *ROS1*-rearranged NSCLC.

It is worth noting that subsequent studies have suggested a shorter PFS estimate for crizotinib in *ROS1*-rearranged NSCLC. In the French phase II study (56) and in the EUROS1 retrospective study (57) of crizotinib for *ROS1*-rearranged NSCLC, median PFS was 9-10 months, although both of these studies enrolled only ~30 patients. In a larger East Asian phase II study of crizotinib, median PFS among 127 *ROS1*-rearranged lung cancer patients was 13.4 months (58). Each study included patients who had received varying numbers of prior lines of systemic therapy, although for all of these patients, crizotinib remained the first ROS1-directed TKI. A number of phase II studies of crizotinib are currently underway and will help generate more efficacy data for this group of patients.

Resistance to Crizotinib

TKI resistance represents a major hurdle to achieving durable responses to targeted therapy in virtually every context, including *EGFR*-mutant and *ALK*-rearranged NSCLC (59). Crizotinib resistance in *ROS1*-rearranged NSCLC is no exception, and causes the vast majority of patients to eventually progress on therapy. We are still in the early stages of elucidating clinical patterns of crizotinib resistance [e.g., frequency of oligoprogression or central nervous system (CNS)-only progression] as well as molecular mechanisms of resistance, but emerging data offer helpful insights to guide drug development efforts and inform the clinical use of ROS1 inhibitors beyond crizotinib.

Broadly speaking, acquired resistance to crizotinib arises secondary to “on target” (e.g., secondary acquired mutations in the ROS1 kinase domain) and “off target” (e.g., bypass signaling track activation or phenotypic change) mechanisms. Mutations within the ROS1 kinase domain occur in ~50-60% of crizotinib-resistant tumors (42). This is higher than the frequency of ALK kinase domain mutations (~20-25%) observed in crizotinib-resistant *ALK*-rearranged lung cancers (60, 61), and may reflect differences in crizotinib binding characteristics or its higher potency against ROS1 versus ALK (55).

The most frequently observed resistant mutation has been the ROS1 G2032R mutation in the solvent front (i.e., solvent-exposed region of the kinase), analogous to ALK G1202R (**Figure 4A**) (42, 62, 63). G2032R was the first crizotinib-resistant mechanism reported in a patient with *ROS1*-rearranged lung adenocarcinoma (62). Multiple tumor metastatic sites examined at autopsy all harbored this mutation, suggesting it arose early in the evolution of resistance (62). Based on structural and cellular analyses, G2032R causes steric hindrance to the drug binding and does not alter the oncogenic kinase activity (62). In a recent series examining 16 *ROS1*-rearranged NSCLC patients with 17 post-crizotinib tumor biopsies, G2032R was identified in as many as 41% of the resistant biopsies (42), underscoring the importance of developing ROS1 inhibitors with potent activity against G2032R.

Another solvent front mutation, D2033N (analogous to ALK D1203N), has also been detected in crizotinib-resistant tumors (**Figure 4A**) (42, 64). This mutation affects the key electrostatic interaction between the D2033 residue and the piperidine moiety of crizotinib and also affects the neighboring residues at the surface of the ATP-binding pocket (64). As demonstrated in **Figure 4A**, additional mutations reported in clinical samples include: S1986Y/F (a mutation affecting the αC helix of the kinase domain which causes steric interference with drug binding; analogous to ALK C1156Y) (42, 65), L2026M (a ‘gatekeeper’ mutation in the ATP-binding

pocket which hinders drug binding; analogous to ALK L1196M) (66), and L1951R (a solvent front mutation; no known analogous mutation in ALK) (66). The L1951R resistance mutation also emerged in an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen with *CD74-ROS1*-transformed Ba/F3 cells (67). Interestingly, along with G2032R, L1951R conferred the highest level crizotinib-resistant phenotype *in vitro* compared to other mutations including L2026M (67).

Off-target mechanisms of crizotinib resistance have been reported for ROS1, but are thus far (in the clinic) limited to isolated case reports. Tumor cells may activate an alternative signaling pathway ("bypass pathway") and acquire resistance. As an example, an activating KIT mutation, D816G, was detected in a crizotinib-resistant *ROS1*-rearranged lung tumor, but not in the treatment-naïve tumor (68). In this resistant tumor specimen, other genes including *ROS1*, *EGFR*, *KRAS*, and *BRAF* did not have detectable alterations (68). Upregulation of EGFR signaling—not mediated by activating *EGFR* mutations at the DNA level—has been reported as a bypass mechanism in HCC78 cells made resistant to crizotinib (63, 69), although not yet validated in the clinic. Interestingly, in *ROS1*-rearranged and other fusion kinase-driven cell line models, EGFR activation and signaling appears to serve as an important early adaptive survival response to TKI exposure (70). Another preclinical study has suggested a KRAS G12C mutation as a potential crizotinib resistance mechanism in *ROS1*-rearranged lung cancer (71).

Finally, phenotypic changes such as epithelial mesenchymal transition (EMT) may contribute to crizotinib resistance. In one crizotinib-resistant, *ROS1*-rearranged lung tumor, no alterations were detected in *ROS1*, *EGFR*, *ALK*, *KRAS*, or *MET*. Instead, EMT-like changes were observed with decrease in E-cadherin and increase in vimentin (63). Similar changes were noted in two crizotinib-resistant clones derived from HCC78 cells with the acquisition of spindle-shaped morphology. However, these clones contained a concomitant ROS1 L2155S mutation, shown to confer crizotinib resistance in cell lines (63) [but not identified in mutagenesis screens (65) or

patient samples (42, 62-66)]. Histologic transformation from adenocarcinoma to small-cell lung cancer (SCLC)—a known phenomenon in EGFR and ALK TKI resistance (59)—has not yet been reported in the context of ROS1. Nonetheless, we anticipate that SCLC transformation may eventually be observed as more patients are treated with sequential, increasingly potent ROS1 inhibitors. Further studies are needed to comprehensively characterize the landscape of TKI resistance mechanisms in *ROS1*-driven lung cancer.

Other ROS1 Inhibitors in Development

Table 1 lists additional ROS1 inhibitors in development. These TKIs each inhibit a different spectrum of kinase targets and *ROS1* resistance mutations (**Figure 4B**), and exhibit unique toxicity profiles and activity in the central nervous system (CNS). Collectively, all of these factors will influence which TKI is used, and in what context, in the clinic. Below, we summarize the available data for the ROS1 inhibitors in development, and then discuss our approach to the sequential use of ROS1 TKIs in the clinic at the present time.

Ceritinib. Ceritinib is a potent and selective ALK inhibitor, active in crizotinib-naïve and -pretreated *ALK*-rearranged NSCLC (72-75). Ceritinib also inhibits ROS1 with a cellular IC_{50} of 180 nM in Ba/F3 cells expressing *ROS1* rearrangement (as compared to cellular IC_{50} of 27-35 against *ALK*), and 50 nM in HCC78 cells harboring *SLC34A2-ROS1* (76).

In a Korean phase II study, 32 patients with *ROS1*-rearranged advanced NSCLC were treated with ceritinib, dosed 750 mg daily fasting. Of note, in the two patients who had received prior crizotinib, no clinical response was observed. The trial was subsequently amended to include only crizotinib-naïve patients. Among crizotinib-naïve patients, the ORR was 67%, with DCR of 87%. The median PFS was 9.3 months for the entire cohort, and reached 19.3 months for

crizotinib-naïve patients (76). Eight patients in the study had known brain metastases; intracranial ORR was 25% with intracranial DCR of 63% (76).

While ceritinib is clinically active in crizotinib-naïve *ROS1*-rearranged NSCLC, these findings also suggest that the role of ceritinib may be much more limited in the crizotinib-resistant setting. Indeed, ceritinib can inhibit the ROS1 L2026M gatekeeper mutation *in vitro* (68), but not G2032R (62, 67), D2033N (64), L1951R (67), or S1986Y/F (65) (**Figure 4B**). Furthermore, the efficacy of ceritinib must be weighed against its toxicities. Consistent with prior experiences (72-75), the most common ceritinib-associated adverse events (AEs) in the phase II study were diarrhea (78%), nausea (59%), anorexia (56%), and vomiting (53%) (76)—at higher frequencies overall than observed with crizotinib (4). Alternative ceritinib dosing with food may help ameliorate these side effects going forward (77). In the ASCEND-8 study, the 450 mg daily dosing of ceritinib with meals was much better tolerated as compared to 750 mg daily dosing fasting, yet reached similar steady-state plasma levels based on pharmacokinetic studies (77). Confirmation that antitumor efficacy is comparable between the two dosing regimens remains to be established. Larger, global studies will help better assess the systemic and intracranial efficacy of ceritinib in *ROS1*-rearranged lung cancers, particularly in crizotinib-naïve patients.

Brigatinib. Brigatinib recently received accelerated FDA approval for use in advanced *ALK*-rearranged NSCLC (78, 79). Like crizotinib and ceritinib, it also has anti-ROS1 activity based on preclinical studies, with an IC_{50} of 7.5 nM (versus anti-ALK IC_{50} of 9.8 nM) in *CD74-ROS1*-expressing Ba/F3 cells (80). In a recently reported phase 1/2 study of brigatinib, 3 patients with *ROS1*-rearranged NSCLC were enrolled. Two crizotinib-pretreated patients had stable disease and progressive disease, whereas a crizotinib-naïve patient had a partial response (78). The activity of brigatinib against resistant *ROS1* mutants has thus far appeared comparable to ceritinib based on Ba/F3 models. *In vitro*, brigatinib inhibits the L2026M mutation, but not

G2032R, D2033N, or L1951R (64, 67, 80, 81), raising the concern that similarly to ceritinib, it likely has limited activity against crizotinib-resistant *ROS1*-driven tumors (**Figure 4B**). The most common treatment-emergent AEs for brigatinib have consisted of nausea, diarrhea, headache, and cough; however, it has notably been associated with early pulmonary events including radiographic changes consistent with pneumonitis (78, 79).

Lorlatinib. Lorlatinib is a highly potent, small-molecule oral TKI optimized for selective ALK/ROS1 inhibition and robust CNS penetration (82). *In vivo* experiments in rats suggest a 30-40% drug exposure in the brain as compared to plasma; consistent with this, lorlatinib inhibits *ROS1*-driven glioblastoma tumor growth in mice (82). Moreover, in patients treated at the standard 100 mg daily dosing, lorlatinib achieves an even higher cerebrospinal fluid to plasma ratio ranging 61-96% (compared to the 30-40% seen in rats), consistent with excellent CNS penetration (83).

Recently, preliminary results from the phase I study of lorlatinib in *ALK*- and *ROS1*-rearranged NSCLCs were presented. Among 12 patients with *ROS1*-rearranged lung adenocarcinomas, the ORR was 50% with median PFS of 7 months (84). Among 5 patients with intracranial target lesions, 4 (80%) achieved intracranial responses (84). Lorlatinib was generally well tolerated, with the most frequent AEs of hypercholesterolemia (72%), hypertriglyceridemia (39%), peripheral neuropathy (39%), and peripheral edema (39%) (81).

Of note, lorlatinib has *in vitro* activity against several crizotinib-resistant mutations including L2026M (65, 82), S1986Y/F (65), and D2033N (64) (**Figure 4B**). Facchinetti et al. reported on a patient with *EZR-ROS1*-rearranged, crizotinib-resistant NSCLC with the S1986Y/F mutation, who achieved a dramatic and durable disease response to lorlatinib (65). However, lorlatinib's activity against the ROS1 G2032R mutation may be limited in the clinic. Based on preclinical

studies, G2032R appears to significantly reduce the cellular potency of lorlatinib, with IC_{50} ranging from 177 nM to 508 nM in *ROS1*-rearranged Ba/F3 models (as compared to cellular IC_{50} of 0.5-1 nM against wild-type ROS1) (65, 80, 81). Therefore, lorlatinib could be clinically active in select patients post-crizotinib, but may have limited efficacy in the subset of patients with tumors known to harbor G2032R. A global phase I/II trial of this agent in *ALK*- and *ROS1*-rearranged NSCLC has completed accrual (NCT01970865).

Entrectinib. Entrectinib is an oral inhibitor with low nanomolar potency against ALK, ROS1, and TRK kinases in enzymatic assays (85), and is able to effectively penetrate the blood-brain barrier (86). Its cellular IC_{50} against ROS1 in Ba/F3 models is ~5 nM (86). Notably, entrectinib has failed to demonstrate preclinical activity against the ROS1 L2026M or G2032R resistance mutations (81), suggesting it likely has little role in treating crizotinib-resistant *ROS1*-rearranged NSCLC. In two phase I studies of entrectinib (ALKA-372-001 and STARTRK-1), 14 patients with crizotinib-naïve *ROS1*-rearranged tumors (13 NSCLCs, 1 melanoma) were evaluated. The ORR to entrectinib was 86% (intracranial ORR, 63%), and median PFS was 19 months (87). Of note, 6 patients with prior crizotinib did not respond to entrectinib, consistent with the aforementioned preclinical studies. An ongoing phase II basket trial of entrectinib (NCT02568267) is enrolling patients with *ROS1*-rearranged NSCLCs; prior crizotinib is allowed only if patients have CNS-only disease progression.

Cabozantinib. Cabozantinib is an inhibitor of multiple tyrosine kinases including MET, VEGFR2, RET, and KIT. This multitargeted TKI is approved for use in medullary thyroid cancer and advanced renal cell carcinoma after prior anti-angiogenic therapy. Recent studies have demonstrated that cabozantinib additionally harbors anti-ROS1 activity (67, 80-82). In particular, cabozantinib has been shown across multiple studies to be active against solvent front resistance mutations in ROS1, including G2032R and D2033N (**Figure 4B**) (67, 80-82). The

IC_{50} for cabozantinib in Ba/F3 cells expressing G2032R ranges from 13.5 nM (67) to 26 nM (81), and the IC_{50} in Ba/F3 expressing D2033N is 0.8 nM (64). Cabozantinib induced a near complete response in a patient with *ROS1*-rearranged NSCLC, who progressed on crizotinib with an acquired D2033N mutation (64).

Cabozantinib could thus represent a therapeutic option for crizotinib-pretreated patients with a G2032R mutation against which other available ROS1-targeted agents described above have limited activity. However, the significant toxicities seen with this agent due to its lack of selectivity—including palmar-plantar erythrodysesthesia, gastrointestinal toxicities and cardiovascular toxicities such as hypertension—will likely hinder development of this TKI for *ROS1*-rearranged NSCLC (88). In a phase II study of cabozantinib in 26 patients with *RET*-rearranged lung adenocarcinomas, 73% required dose reductions because of intolerable drug-related toxicities, consistent with experiences in other solid tumors (88). Alternative dosing regimens may need to be explored in order to mitigate toxicities. A phase II trial of cabozantinib is ongoing in patients with NSCLC harboring *RET*/*ROS1*/*NTRK* fusions or increased MET/AXL activity, and will help to define its activity in the ROS1 subset (NCT01639508).

DS-6051b. DS-6051b is an oral ROS1/TRK inhibitor currently in phase I testing (NCT02279433). Preliminary results from a Japanese phase I study of DS-6051b were recently presented (89). Among 13 patients with *ROS1*-rearranged NSCLCs, of whom 8 were evaluable, the ORR was 62.5% with DCR of 100%. Among the 3 crizotinib-pretreated patients in this cohort, no objective responses were seen. The most common treatment-emergent AEs included transaminitis, diarrhea, nausea, and constipation (89). More data is needed in order to evaluate the safety and activity of DS-6051b in *ROS1*-rearranged NSCLC.

TPX-0005. TPX-0005 is a potent ALK/ROS1/TRK inhibitor specifically designed to overcome the gatekeeper and solvent front resistance mutations in the respective kinases (90). Preliminary preclinical data suggest that it is active against the solvent front mutations including ROS1 G2032R, and also inhibits kinases implicated in bypass signaling such as SRC and FAK (90). A phase I/II study of TPX-0005 in advanced solid tumors with *ALK*/*ROS1*/*NTRK1*-3 rearrangements (TRIDENT-1) is now enrolling (NCT03093116).

Our current approach. Deciding which ROS1 inhibitor to use, at what juncture in the course of a patient's treatment, is complex—particularly given the limited data available for some of the newer agents. **Figure 5** summarizes our current evidence-based approach to the treatment of *ROS1*-rearranged NSCLC. An important caveat is that this approach will evolve over time as additional data emerge on new TKIs and resistance mechanisms.

For now, crizotinib remains the standard of care first-line TKI for the treatment of advanced *ROS1*-rearranged NSCLC. Once patients progress on or after crizotinib, we strongly recommend a repeat tumor biopsy if feasible and safe, in order to characterize the mechanism(s) of resistance and determine the presence of any *ROS1* resistance mutations. If a tumor biopsy is not feasible, a "liquid biopsy" using a circulating tumor DNA (ctDNA) NGS-based assay may serve as an alternative, albeit less sensitive than the former. Of note, liquid biopsies have not yet been validated for the detection of *ROS1* mutations; and furthermore, it can be more technically challenging to detect gene fusions as compared to point mutations using ctDNA.

The presence of a *ROS1* resistance mutation in the repeat biopsy specimen suggests that the tumor may still be ROS1-dependent, and the patient should be directed toward clinical trials of new ROS1 inhibitors such as lorlatinib or TPX-0005, with activity against the detected

mutations. In the particular case of G2032R, lorlatinib may be less effective; therefore, trials of other agents such as TPX-0005, or off-label use of cabozantinib (albeit with concerns regarding its toxicities), may be needed. In the absence of a *ROS1* resistance mutation post-crizotinib, novel ROS1 TKIs may still be tried; however, these patients may derive greater benefit from chemotherapy or a combination strategy (e.g., a trial of a ROS1 TKI combined with another targeted agent or chemotherapy). A number of studies have suggested that patients with *ROS1*-rearranged lung cancer may be particularly responsive to pemetrexed-based therapies, similar to what has been observed in *ALK*-rearranged lung cancer (91-94).

The potential role of immunotherapy in the treatment of *ROS1*-rearranged NSCLC post-ROS1 TKIs is unclear. At least in *EGFR*-mutant and *ALK*-rearranged NSCLC, there appears to be limited benefit derived from checkpoint inhibitor monotherapy (61, 95-97). Further studies are needed to evaluate the role of immunotherapy in *ROS1*-rearranged lung cancer, and to assess the presence of potential biomarkers of response to immunotherapy—including the level of PD-L1 expression, inflammation in the tumor microenvironment, and tumor mutational burden—in this subset of patients.

Conclusions and Future Directions

Ten years have passed since the initial report of *ROS1* rearrangements in NSCLC (**Figure 1**). *ROS1*-rearranged lung cancers are dependent on ROS1 for survival, and are thus sensitive to treatment using ROS1-targeted TKIs. Today, the National Comprehensive Cancer Network (NCCN) guidelines recommend testing for ROS1—along with EGFR, ALK, and PD-L1—at the time of diagnosis of metastatic NSCLC (98). Systematic diagnostic testing for ROS1 in metastatic NSCLC is not yet recommended by the European Society of Medical Oncology (ESMO) guidelines, although it is suggested (99). While crizotinib is currently the only FDA- and EMA-approved agent for the treatment of *ROS1*-rearranged NSCLC, a number of additional

ROS1 inhibitors are undergoing clinical testing, with several showing early signals of clinical activity in the post-crizotinib setting.

As more ROS1 inhibitors are developed, further research will be critical to defining the role of each TKI in the clinic. Paramount to addressing this question will be the rigorous evaluation of each agent's (1) CNS activity, (2) potency and clinical activity against resistant ROS1 mutant kinases, particularly G2032R, and (3) toxicity profile. Current data suggest that G2032R is the most frequent *ROS1* resistance mutation emerging post-crizotinib (42). Therefore, development of agents that effectively target the G2032R mutation and are safe/tolerable in patients should be of highest priority in the ROS1 field.

In *ALK*-rearranged NSCLC for which multiple FDA-approved TKIs are available, the question of optimal sequencing of ALK TKIs has taken center stage. The recently reported data from J-ALEX and the global ALEX studies comparing alectinib with crizotinib in the front-line setting demonstrate that alectinib—a more potent and CNS-penetrant ALK TKI—is superior to crizotinib in advanced *ALK*-rearranged NSCLC (100, 101). The large magnitude of benefit seen with alectinib suggests that upfront use may be superior to the current sequential approach of crizotinib followed by alectinib. Extrapolating to *ROS1*-rearranged lung cancer, upfront use of a more potent and CNS-penetrant ROS1 TKI may confer greater benefit than crizotinib and possibly sequential treatment, although this remains to be evaluated. Future preclinical and clinical studies will help inform the optimal first-line therapy for *ROS1*-rearranged NSCLC.

Similar to what we have observed in *EGFR*-mutant and *ALK*-rearranged NSCLC (59), combination strategies (e.g., combining a ROS1 TKI with another targeted agent or chemotherapy) need to be developed and evaluated as potential strategies to overcome resistance to ROS1 inhibitors. In the case of resistance driven by off-target mechanisms such

as bypass signaling activation, the use of a ROS1 inhibitor as monotherapy may be ineffective. There are currently no actively enrolling combination trials for *ROS1*-rearranged NSCLC. However, the knowledge of bypass resistance mechanisms outlined above suggests potential therapeutic avenues one could pursue. For instance, an approach of co-targeting ROS1 and EGFR could be explored given the preclinical evidence for the role of EGFR in driving acquired resistance as well as adaptive survival response to ROS1-targeted therapy (70, 71). Additionally, an entrectinib-based regimen may soon enter early-phase clinical testing, combining the blockade of ALK/ROS1 and MEK, a critical downstream survival and proliferation pathway (71, 102). For any new combination regimen, safety and dosing schedule will need to be carefully evaluated, as additive and/or unexpected toxicities may arise. Further advances in ROS1-based combination strategies will require an enhanced understanding of the bypass and downstream signaling tracks involved in ROS1 TKI resistance. Ultimately, upfront use of combination regimens may help delay and even prevent TKI resistance from emerging, and hence have a more transformative impact on the natural history of the cancer.

Finally, efforts are needed to standardize the diagnosis and treatment of *ROS1*-rearranged lung cancer at the global level. Clinicians, researchers, regulatory agencies, and patient advocates will need to come together in this endeavor, in order to ensure that patients across communities have access to the diagnostic tools and emerging therapy options. It is our hope that with continued research into the biology of *ROS1*-driven lung cancers, development of new therapeutic strategies, and enhanced patient access to these advances, we can further extend and improve the lives of patients with *ROS1*-rearranged lung cancer.

Grant Support

This work was supported by a grant from the National Cancer Institute (5R01CA164273, to ATS), by Be a Piece of the Solution, and by LungStrong.

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Figure Legends

Figure 1. Timeline of key advances in targeting ROS1 in lung cancer. Abbreviations: NSCLC, non-small cell lung cancer; ALK, anaplastic lymphoma kinase; FDA, Food and Drug Administration.

Figure 2. *ROS1* rearrangements in non-small cell lung cancer (NSCLC). (A) Schematic representations of ROS1 fusion proteins described to date. Note that KDELR2-ROS1 is not shown, as the genomic structure of this fusion has not been published. Blue, ROS1 tyrosine kinase domain; purple, ROS1 transmembrane domain; green, coiled-coil domain. (B) Distribution of ROS1 fusion proteins by the reported frequencies in NSCLC. Each fusion protein listed under the 'other' category likely occurs in <1% of *ROS1*-rearranged NSCLCs, unless otherwise indicated. [*Figure updated/modified from ref. 103.*]

Figure 3. Clinical response of a *ROS1*-rearranged patient to crizotinib. (A) Axial (top) and coronal (bottom) computed tomography images of the chest before crizotinib. (B) Axial (top) and coronal (bottom) computed tomography images of the chest after 6 weeks of crizotinib, demonstrating a dramatic improvement in the left lung mass, bilateral pulmonary nodules and pleural effusions.

Figure 4. Crizotinib-resistant *ROS1* mutations. (A) Crizotinib-resistant secondary *ROS1* mutations reported to date, mapped on the structure data of ROS1 kinase domain (left) in complex with crizotinib (PDB:3ZBF). Analogous *ALK* resistance mutations are mapped on the ALK kinase domain in complex with crizotinib (PDB:2XP2) on the right, revealing structural similarities. Note: The ALK mutation analogous to ROS1 L1951R has not been reported and is therefore not shown. (B) The activity of ROS1-directed tyrosine kinase inhibitors against known

crizotinib-resistant *ROS1* mutations. This table is based on the available preclinical data, not all of which have been validated in the clinic.

Figure 5. Our approach to the treatment of advanced *ROS1*-rearranged lung cancer. After progression on first-line crizotinib, a repeat tumor biopsy is strongly recommended if feasible and safe, in order to determine the mechanism of crizotinib resistance. Liquid biopsy may be an alternative option if tumor biopsy is not feasible. The detection of a secondary *ROS1* resistance mutation can inform the selection of next-line therapy. For example, in the case of a G2032R mutation, ROS1 inhibitors that have limited activity against G2032R based on preclinical data (see Figure 4B) should be avoided. In the absence of a *ROS1* resistance mutation, options could include combination regimen trials or chemotherapy. Progression on/after crizotinib limited only to the central nervous system (CNS) may be effectively treated with a ROS1 inhibitor that has improved CNS penetration, such as entrectinib or lorlatinib. Of note, in the case of "oligoprogression" (i.e., progression in a limited number of metastatic sites) on first-line crizotinib, local ablative therapy could be considered with the continuation of crizotinib (see ref. 61).

Table 1. Ongoing clinical trials for *ROS1*-rearranged NSCLC.

Study drug	Company	Phase	Target kinases	Primary outcome	Study location	ClinicalTrials.gov identifier
Crizotinib	Pfizer	II	ROS1, ALK, MET	ORR	France	NCT02034981
Crizotinib	Pfizer	II	ROS1	ORR	Europe	NCT02183870
Crizotinib	Pfizer	II	ROS1, ALK, MET	ORR	UK	NCT02664935
Crizotinib	Pfizer	II	ROS1, MET	ORR	Italy	NCT02499614
Ceritinib	Novartis	II	ROS1	ORR	ROK	NCT01964157
Ceritinib	Novartis	II	ROS1, ALK	ORR	China	NCT02276027
Lorlatinib	Pfizer	II	ROS1, ALK	ORR	Global	NCT01970865*
Lorlatinib	Pfizer	II	ROS1, ALK	Intracranial DCR	USA	NCT02927340
Entrectinib	Ignyta	II	ROS1, ALK, TRK A/B/C	ORR	Global	NCT02568267
Cabozantinib	Exelixis	II	ROS1, TRK A/B/C, RET, MET, AXL	ORR	USA	NCT01639508
DS-6051b	Daiichi Sankyo	I	ROS1, TRK A/B/C	Toxicity profile, ORR	USA	NCT02279433
DS-6051b	Daiichi Sankyo	I	ROS1, TRK A/B/C	Toxicity profile	Japan	NCT02675491
TPX-0005	TP Therapeutics	I	ROS1, ALK, TRK A/B/C	Toxicity profile, ORR	USA, ROK	NCT03093116

*This trial is now closed to accrual for ROS1 patients.
Note: While there are currently no open clinical trials of brigatinib in *ROS1*-rearranged NSCLC, brigatinib is also known to have anti-ROS1 activity.
Abbreviations: NSCLC, non-small cell lung cancer; ORR, objective response rate; DCR, disease control rate; UK, United Kingdom; USA, United States of America; ROK, Republic of Korea.

Figure 1

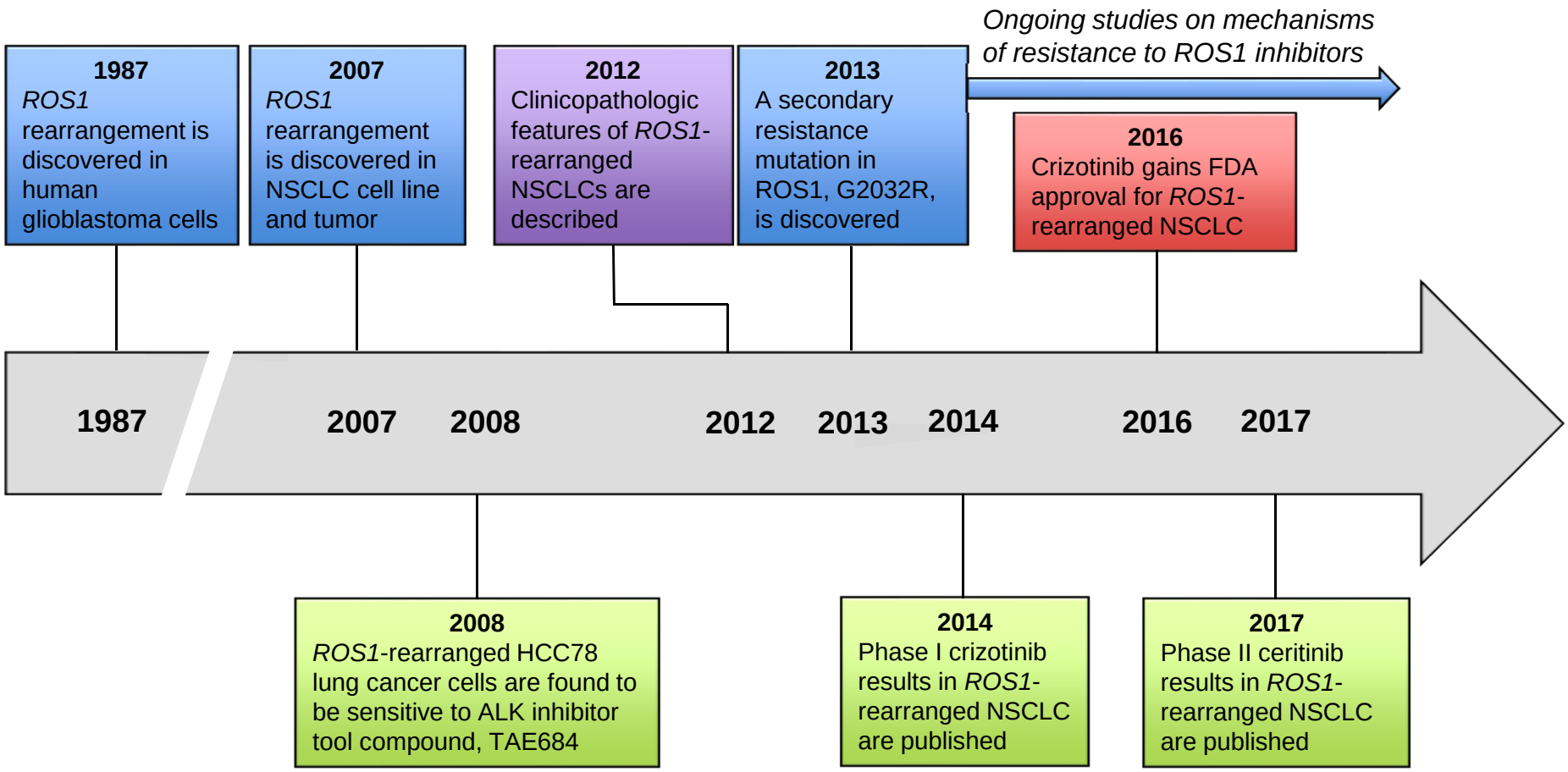

Ongoing studies on mechanisms of resistance to ROS1 inhibitors
1987
ROS1 rearrangement is discovered in human glioblastoma cells
2007
ROS1 rearrangement is discovered in NSCLC cell line and tumor
2012
Clinicopathologic features of ROS1-rearranged NSCLCs are described
2013
A secondary resistance mutation in ROS1, G2032R, is discovered
2016
Crizotinib gains FDA approval for ROS1-rearranged NSCLC
1987
2007
2008
2012
2013
2014
2016
2017
2008
ROS1-rearranged HCC78 lung cancer cells are found to be sensitive to ALK inhibitor tool compound, TAE684
2014
Phase I crizotinib results in ROS1-rearranged NSCLC are published
2017
Phase II ceritinib results in ROS1-rearranged NSCLC are published

Figure 2

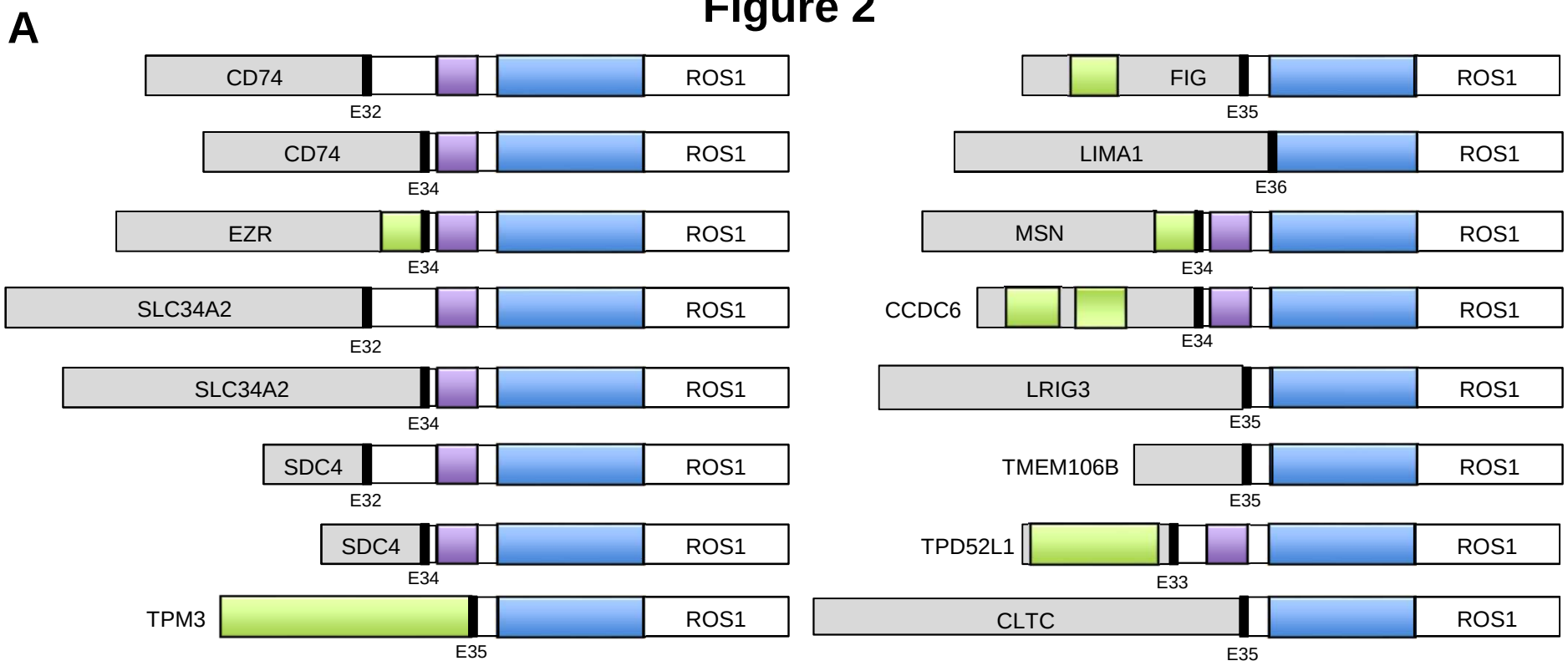

A
CD74
ROS1
E32
CD74
ROS1
E34
EZR
ROS1
E34
SLC34A2
ROS1
E32
SLC34A2
ROS1
E34
SDC4
ROS1
E32
SDC4
ROS1
E34
TPM3
ROS1
E35
FIG
ROS1
E35
LIMA1
ROS1
E36
MSN
ROS1
E34
CCDC6
ROS1
E34
LRIG3
ROS1
E35
TMEM106B
ROS1
E35
TPD52L1
ROS1
E33
CLTC
ROS1
E35
B
SLC34A2 12%
SDC4 6%
Unknown 10-15%
EZR 14-15%
Other 7-13%
CD74 40-45%
TPM3 2-3%
FIG 2%
LIMA1 (<1%)
MSN (<1%)
KDELR2 (<1%)
CCDC6 (<1%)
LRIG3 (<1%)
TMEM106B (<1%)
TPD52L1 (<1%)
CLTC (<1%)

Figure 3

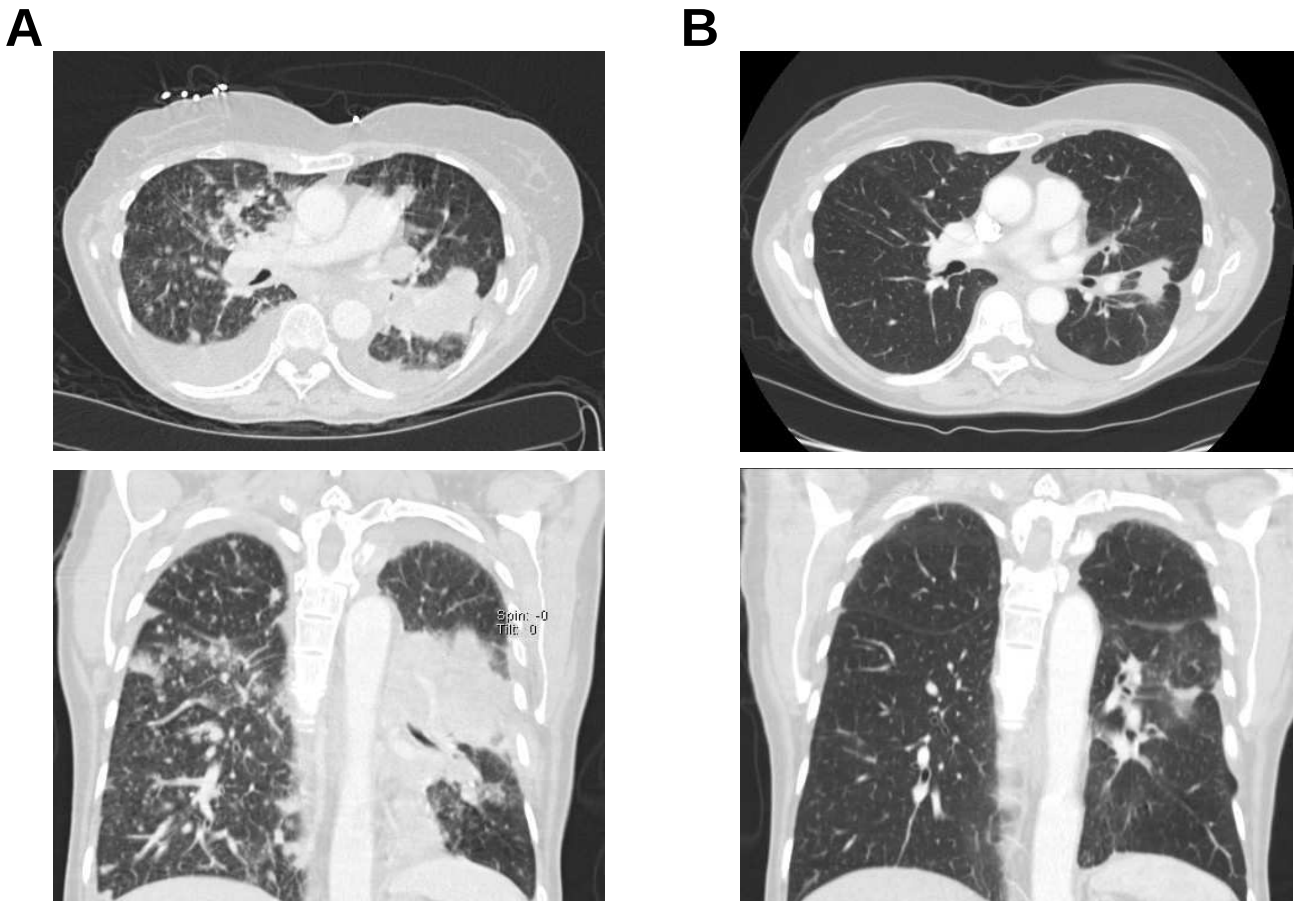
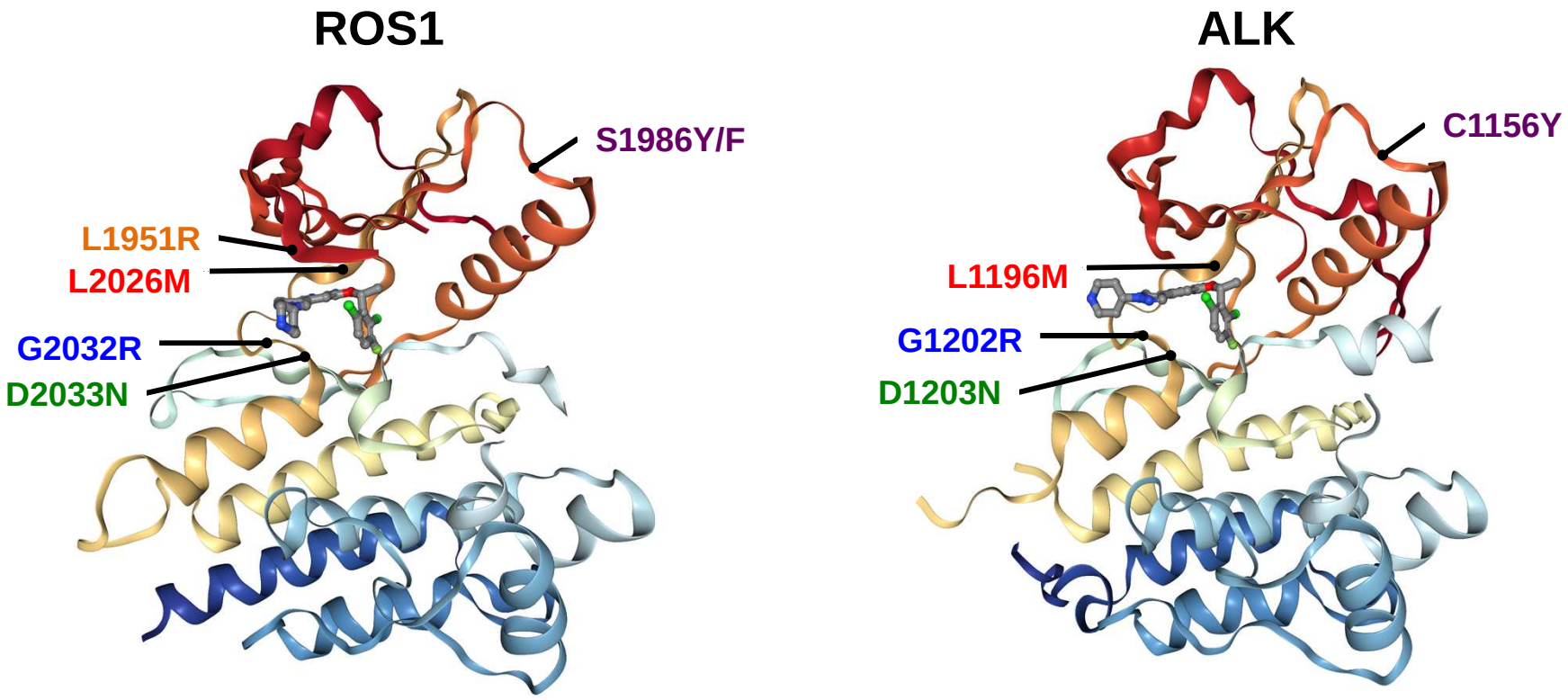


Figure 4

A



B

	Gatekeeper	αC helix		Solvent front	
	L2026M	S1986Y/F	G2032R	D2033N	L1951R
Crizotinib	No	No	No	No	No
Ceritinib	Yes	No	No	No	No
Brigatinib	Yes	Unknown	No	No	No
Lorlatinib	Yes	Yes	No	Yes	Unknown
Entrectinib	No	Unknown	No	Unknown	Unknown
TPX-0005	Yes	Unknown	Yes	Yes	Unknown
Cabozantinib	Yes	Unknown	Yes	Yes	Yes

Figure 5