Review Article

ROS-1 NSCLC therapy resistance mechanism

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Abstract: *ROS1*-rearrangements occur in 1–2% of advanced non-small cell lung cancer (NSCLC) and define a distinct molecular subgroup. In the last decade, the development of molecularly targeted therapy has changed the standard of care for NSCLC patients harboring *ROS1* and other driver mutations. The multitargeted MET/ALK/ROS1 tyrosine kinase inhibitor (TKI) crizotinib significantly improves overall survival for these patients; however, resistance to crizotinib invariably occurs, leading to disease progression. Several resistance mechanisms to crizotinib have been reported, including pharmacokinetic/dynamic failure, biological acquired resistance by secondary point mutations in the *ROS1* kinase domain, bypass tracks, and phenotypic changes. Next-generation TKIs can be clinically active against *ROS1*-rearranged NSCLC after progression to crizotinib, depending on the acquired resistance mechanism, or have substantial intracranial activity compared to crizotinib. Understanding the mechanisms of resistance to ROS1 TKI is critical to develop selective therapies. Thus, contemporary approaches are essential to improve outcomes with subsequent treatments in patients with *ROS1*-rearranged lung cancer. Tissue or blood-based next generation sequencing (NGS) can help to identify the resistance mechanism to ROS1 TKI and may be considered after tumor progression in order to better select further lines of targeted therapy, although prospective validation of this therapeutic approach is needed. In this review, we perform a comprehensive analysis of *ROS1* fusions in NSCLC, resistance mechanism to ROS1 targeted therapies, and future strategies.

Keywords: Non-small cell lung cancer (NSCLC); ROS1 fusion; targeted therapy; resistance mechanism; liquid biopsy

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Introduction

ROS1 function

ROS proto-oncogene 1, receptor tyrosine kinase gene (c-ROS-1 or *ROS1*) was originally identified in 1982 as the oncogenic part of the avian sarcoma RNA virus UR2 (1-3). *ROS1* gene is located on chromosome 6q22.1 (4,5); the unaltered gene encodes a transmembrane protein with intracellular C-terminal tyrosine kinase domain (6) that shares marked sequence homology and structural similarities to anaplastic lymphoma kinase (ALK), leukocyte tyrosine kinase (LTK), and the insulin receptor families (7). In animals, *ROS1* expression has been detected in the epithelial cells of several organs. Interestingly, a *Ros1*-deficient male mouse was found to be sterile, due to defective differentiation of epididymal epithelial cells; while, female *Ros1*-deficient mice developed no anomalies (8-10). Recently, Kiyozumi *et al.* observed that the extracellular domain of the mouse ROS1 receptor attaches to neural epidermal growth factor-like like 2 (NELL2), a testicular germ cell-secreted lumicrine factor (11).

In humans, ROS1 is highly expressed in the lungs.

Functional activation of human ROS1 receptor via NELL2 is suspected given the results of the aforementioned studies but has not been proven. Therefore, the role of native ROS1 in humans has not been yet well established (10).

ROS1 *gene fusions in cancer*

ROS1 gene is sensitive to chromosomal rearrangements ending in fusion proteins that involve the expression of active ROS1 tyrosine kinase domain. *ROS1* rearrangements were initially identified in a human glioblastoma cell line (12) and are involved in the pathogenesis of other malignancies including cholangiocarcinoma, ovarian cancer, gastric cancer, colorectal cancer, angiosarcoma, inflammatory myofibroblastic tumor, Spitz tumors, epithelioid haemangioendothelioma and lung cancer (13-21).

The precise mechanism by which ROS1 fusion proteins become constitutively active is unclear. Indeed, many of the common *ROS1* fusion partners do not have dimerization domains. Moreover, subcellular localization varies upon fusion partner and has been described not exclusively as a transmembrane protein but also in the cytoplasmic compartment only as well as in the reticular endoplasmic membrane. Once activated, ROS1 kinase activates the SHP-2 phosphatase and upregulates MAPK/ERK, PI3K/AKT/mTOR and JAK/STAT3 signaling pathways to promote cell growth and survival (6,8,16,22-24).

ROS1-*rearranged NSCLC*

Chromosomal rearrangements involving *ROS1* gene were initially described in non-small cell lung cancer (NSCLC) in 2007 by Rikova *et al.* (25). Such rearrangements arise in 1–2% of advanced NSCLC and define a distinct molecular subgroup in NSCLC (8,26,27). Most of break points in *ROS1* occur in a variable region among exons 32 and 35, conserving the kinase domain encoded in 3' (27,28). So far, over 14 different fusion partner genes have been reported in NSCLC patients, including CD74, SLC34A2, SDC4, EZR, FIG, TPM3, LRIG3, KDELR2, CCDC6, MSN, TMEM106B, TPD52L, CLTC, and LIMA1. Of these fusion partners, CD74-*ROS1* is the most common variant in *ROS1*-rearranged NSCLC (~44%), followed by EZR–*ROS1* (16%), SDC4–*ROS1* (14%), SLC34A2–*ROS1* (10%), and TPM3-*ROS1* (8%) (8,10). Contemporary studies suggest that the type of the *ROS1* fusion partner may influence the prognosis and treatment response in patients with advanced *ROS1*-rearranged NSCLC. In fact, patients with CD74-*ROS1* rearrangements had a poorer prognosis than those without CD74-*ROS1* fusions (29,30).

Similar to patients with NSCLC harboring ALK fusions, patients with *ROS1*-rearranged NSCLC are usually younger, light or never smokers, and the predominant histology is adenocarcinoma (8,26). However, compared to patients with ALK-rearranged NSCLC, *ROS1*-rearranged NSCLC patients tend to develop lower rates of extrathoracic and brain metastases at initial diagnosis, and have a lower cumulative incidence of brain metastases (31). Additionally, the incidence of venous thromboembolism (VTE) seems to be higher in patients with NSCLC harboring *ROS1*-fusions than previously observed for the general population with NSCLC (32,33).

ROS1 fusions rarely co-exist with other driver mutations, such as KRAS, EGFR or ALK (30,34). *ROS1* fusions have also been described as a mechanism of resistance to targeted therapies in EGFR mutant lung cancer patients, being this a rare event (35).

Diagnosing *ROS1* rearrangements

Most guidelines recommended ROS1 testing for all advanced-stage lung adenocarcinomas, regardless of clinical characteristics (36-38).

ROS1 fusions can be detected in tumor tissue by different techniques including immunohistochemistry (IHC), reverse transcriptase polymerase chain reaction (PCR), fluorescence in-situ hybridization (FISH), and next generation sequencing (NGS).

FISH

ROS1 FISH has been the standard method for detecting *ROS1* gene fusions, as it was the diagnosis method used in initial clinical trials with crizotinib.

Using fluorescently labelled, paired break-apart probes is considered the "gold standard" in detecting *ROS1* rearrangement. Positivity is defined as >15% of tumor cells containing the classic split 3' and 5', or the atypical isolated 3' signals (8,39).

FISH can be performed on a small amount of tissue, and without previous knowledge of fusion partners. However, it is expensive, technically challenging and hard to interpret,

and both false-positive (non-functional fusions or isolated 3´signals) and false negative results (complex staining patterns or intra-chromosal microdeletions) can occur (39).

IHC

Immunohistochemistry can be used to diagnose *ROS1* fusions, and it is considered a cheaper and faster technique compared with FISH.

However, the ROS1 IHC results can be difficult to interpret. ROS1 staining patterns may vary and IHC may be falsely negative or falsely positive due to several reasons (different intracellular localization of the ROS1 fusions, ROS1 expression from benign pneumocytes and alveolar macrophages, or aneuploidy leading to aberrant expression) (8). Therefore, ROS1 IHC requires verification using a second techniques, such as NGS or FISH.

Reverse transcriptase PCR (RT-PCR)

Combined with NGS or Sanger, RT-PCR allows detection of *ROS1* fusion partners. RNA sequencing needs to be converted to complementary DNA by reverse transcription and PCR amplification, yet potential errors can be easily introduced when manipulating short or very fragmented DNA material. Additionally, RT-PCR requires a previous knowledge of fusions for developing fusion-specific primers; therefore, unknown fusions can be missed, which limits its use (8).

Next generation sequencing (NGS)

Next-generation sequencing allows either DNA, RNA or both DNA and RNA-based nucleic acid sequencing of multiple genes simultaneously to identify any *ROS1* fusion, in addition to other driver mutations.

Tumor DNA enrichment can be completed via amplicon-based or hybrid capture-based approaches. Hybrid capture can sequence wider regions of the genome and better detect the fusion partner gene; therefore, is the preferred method to detect *ROS1* fusion (10).

NGS can be done on tumor tissue-derived DNA or plasma circulating tumor DNA (ctDNA). Liquid biopsy can detect *ROS1* fusions, but this approach can be challenging because of variations of DNA shedding rates that drive the concentration of ctDNA in the blood (10).

However, DNA-based NGS can fail to identify an oncogenic driver due to the lack of capacity of cover intronic breakpoints containing numerous repetitive elements.

RNA-based NGS can overcome this limitation of DNA-based NGS by sequencing coding regions instead of introns (40). Moreover, RNA-sequencing enables detection of any fused partner and the discovery of new ones. These advantages of RNA NGS are limited by the need of high RNA quality, that could be low in clinical samples, particularly in FFPE samples.

Additionally, there are some limitations of NGS. It is slower and more expensive than IHC or FISH, and tissue requirements are higher. Nevertheless, because of its capacity to detect both known and unknown fusions, and sequence multiple genes at the same time, NGS is increasingly being used and will likely become the standard tool for *ROS1* fusion detection in the future (39).

Molecules targeting *ROS1* in NSCLC: crizotinib

The identification of oncogenic driver mutations in NSCLC involved the development of molecularly targeted therapy, changing the standard of care for patients harboring these driver mutations (8,41). Tyrosine kinase inhibitors (TKIs) with activity against ROS1 fusion proteins have allowed for dramatic improvements in objective response rate (ORR), progression-free survival (PFS), overall survival (OS) and quality of life compared with chemotherapy (42,43).

Crizotinib

Crizotinib is a multi-targeted kinase inhibitor with affinity for ALK, ROS and MET (44).

There is structural equivalence between the ALK and ROS1 kinase domains, resulting in cross-inhibition between current therapies targeted against these kinases (45,46).

Crizotinib is a strong ROS1 inhibitor (44) which blocks ATP dependent cellular processes, developing a complex with the respective protein kinase domains, and inhibiting of ROS1 phosphorylation, leading to cell apoptosis with dose-dependent efficacy showed in cells with SLC34A2-*ROS1* translocations (39,44).

In a phase I study (PROFILE 1001; NCT00585195 (47) enrolling 50 patients with *ROS1*-rearranged advanced NSCLC treated with crizotinib, ORR was 70%. Median progression-free survival (PFS) was 19.2 months and the disease control rate (DCS) was 90%. Responses to crizotinib were detected regardless of the *ROS1* fusion partner. Median OS was reported to be 51.4 months, one of the most impressive median overall survival found in any

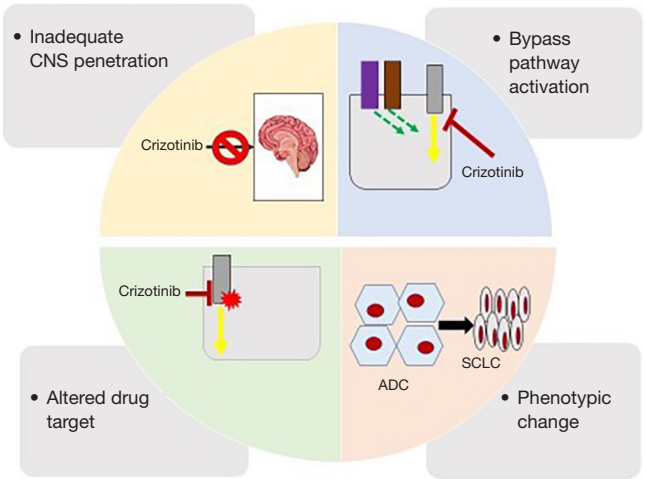

Figure 1 Mechanism of acquired resistance to ROS1 TKIs. Acquired resistance can occur through either pharmacological or biological mechanism.

prospective trial of targeted therapy for advanced NSCLCs. The safety profile of crizotinib was favorable, similar to the observed for patients with ALK positive NSCLC.

After the efficacy and safety results from PROFILE-1001 study, crizotinib became the first targeted therapy approved by both the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2016 for the treatment of advanced *ROS1*-rearranged NSCLC. Moreover, in 2017, both the National Comprehensive Cancer Network (NCCN) and European Society of Medical Oncology (ESMO) guidelines recommend crizotinib treatment for patients with advanced/metastatic lung cancer with known *ROS1* rearrangements (38,48).

Other studies subsequently confirmed the efficacy of crizotinib in *ROS1*-rearranged NSCLC, including two phase II studies in European and East Asian population (49-51). Interestingly, the median PFS duration of patients receiving crizotinib is longer for patients harboring *ROS1* NSCLC than for patients with ALK fusions, possibly owing to more-potent inhibition of ROS1 with crizotinib compared with ALK (31). However, most patients treated with crizotinib invariably relapse. The management of crizotinib-resistant *ROS1*-rearranged NSCLC represents a significant challenge.

Resistance mechanisms to crizotinib

Mechanisms of crizotinib resistance share features with other TKIs and include pharmacokinetic/dynamic failure, biological acquired resistance by secondary point mutations in the ROS1 kinase domain, bypass tracks, and phenotypic changes (*Figure 1*) (52-54).

Intracranial failure

Crizotinib has limited blood–brain barrier (BBB) penetration as it is a substrate of P-glycoprotein and human ATP-binding cassette subfamily efflux transporters (55). Therefore, most patients treated with crizotinib frequently relapse with new brain progression or progression of existing intracranial disease (39). CNS progression to crizotinib might reflect a pharmacokinetic failure of therapy rather than true biological resistance. Cerebrospinal fluid (CSF) concentrations of crizotinib are usually low, and the intracranial ORR with this agent was only 33% (10,55,56).

The estimated incidence of brain metastases in advanced NSCLC is between 20% and 40% prior to start treatment, and can increase to up to 30–50% in patients pre-treated with TKIs in *ROS1*+ lung cancer patients (57).

Novel ROS1 inhibitors have been designed to better penetrate the BBB and achieve a higher drug CNS concentration, obtaining higher efficacy for patients with brain metastasis. In metastatic patients in first-line setting, entrectinib (intracranial ORR 55%), lorlatinib (iORR 64%) and repotrectinib (iORR 100%) showed substantial intracranial activity in patients with treatment naïve *ROS1*-

Table 1 ROS1 mutations conferring crizotinib resistance and ROS1 inhibitor capable of combating mutations

Mutation	Protein location	Proposed mechanism of resistance	ROS TKI with activity against resistance mutation
G2032R	Solvent front of the kinase hinge	Glycine-to-arginine substitution at codon 2031 in the ROS1 kinase domain causes resistance to RO1 kinase inhibition through steric interference with drug binding	Cabozantinib (61) Repotrectinib (62) Taletrectinib (63)* Lorlatinib (64)*
D2033N	Solvent front of the kinase hinge	Aspartic acid-to-asparagine substitution at codon 2033 leads to a modification of electrostatic forces at the exterior surface of the ATP-binding site and reorientation of surrounding residues	Lorlatinib (65)* Cabozantinib (66) Repotrectinib (62)
L2026M	Gatekeeper position of inhibitor binding pocket	Leucine-to-methionine substitution at codon 2026	Lorlatinib (59)* Ceritinib (24)* Brigatinib (24)* Cabozantinib (61,67)* Repotrectinib (68)*
S1986F/Y	Not reported	Serine to tyrosine (S1986Y)/serine to phenylalaline (S1986F) substitution leads to an obstruction in the path to the enzyme active site and an increase in kinase activity	Lorlatinib (24)*

*, *in vitro* only.

rearranged NSCLC (58-60). Entrectinib is now approved by both the FDA and EMA in ROS-1 rearranged NSCLC in first line and is a treatment to consider in patients with brain metastases at diagnosis.

Notably, the FDA has granted repotrectinib fast-track designation for the treatment of *ROS1*-rearranged NSCLC patients previously treated with one prior ROS1 TKI and a platinum-doublet, being a potential second-line treatment option after CNS or systemic progression to crizotinib.

On-target resistance (ROS1-intrinsic mechanisms) (Table 1)

Secondary point mutations within the ROS1 kinase domain have been identified in both clinical and preclinical studies, occurring approximately in 50–60% of crizotinib resistant tumors (31).

Several of these mutations are analogous to ALK secondary mutations that arise with ALK inhibitors, although secondary point mutations occur more frequently in crizotinib resistant *ROS1* rearranged NSCLC, probably due to a higher inhibitory potency against ROS1 (10,39,47). These acquired mutations significantly reduce the potency of the inhibitor against the active ROS1 kinase by steric hindrance and it can also affect the sensitivity to other ROS1 TKIs. Thus, one strategy to overcome crizotinib-resistance could be the identification of the type of secondary mutation in order to select an effective targeted therapy in second line capable of inhibiting the mutated target (69).

G2032R

The first documented and most reported *ROS1* secondary mutation observed in patients to date is the *ROS1* G2032R mutation. It was the first crizotinib-resistant mechanism discovered in a patient with *ROS1*-rearranged NSCLC (70). Several small series had identified G2032R as one of the most frequent resistance mutations (31).

The G2032R mutation, a glycine-to-arginine substitution at codon 2032 in the solvent-front, causes resistance to crizotinib through steric interference with the drug binding at ROS1-kinase residues exposed to solvent (39,69,70). It is structurally similar to the ALK-G1202R mutation, making it difficult to inhibit pharmacologically (46,70).

Additionally, *ROS1* G2032R can lead to epithelial-mesenchymal transition (EMT) and to enhance the invasive capacities of *ROS1* fusion-driven cancer cells through upregulation of Twist1 (10,71).

D2033N

D2033N mutation, a substitution of an aspartic acid-to-asparagine at *ROS1* 2033 codon, is analogous to ALK D1203N (52,69). As the G2032R mutation previously described, D2033N mutation arises at the solvent-front region of the ATP-binding site of ROS1, affecting key electrostatic interaction between the D2033 residue and the piperidine moiety of crizotinib, and the surrounding residues at the surface of the ATP-binding pocket (39,66).

L2026M

L2026M involves leucine and methionine and it is analogous to ALK L1196M. It is located at the "gatekeeper" position of the ATP-binding pocket which hinders drug binding, conferring crizotinib resistance (53).

S1986Y/F

S1986Y/F mutations (substitution of the serine at 1986 ROS1 position by either tyrosine (S1986Y) or phenylalanine (S1986F) induces a positional change in the αC helix of the kinase domain which causes steric interference with drug binding. It is analogous to ALK C1156Y (52).

Other secondary mutations reported in crizotinib resistant *ROS1*-rearranged NSCLC include E1935G, L1947R, L1951R, G1971E, L1982F, C2060G, V2098I, and L2155 (10).

*Off-target resistance (***ROS1-** *extrinsic mechanisms)*

By-pass tracks

Resistance to TKIs can develop via ROS1-extrinsic resistance mechanisms.

Activation of bypass-signaling have been reported for crizotinib resistance in *ROS1* rearranged NSCLC (34). Tumor cells under the pressure of the targeted TKI can become resistant through upregulation of either downstream or parallel cell signaling pathways like EGFR, MET, HER2, KRAS, KIT, BRAF and MEK (10,31,52,53,66).

Retrospective studies have reported resistance to crizotinib driven by one of these bypass-signaling pathways in 42–44% of crizotinib-resistant *ROS1*-rearranged NSCLC tumors (31,39,72).

In a series of 75 patients with *ROS1* rearranged NSCLCs treated with ROS1 inhibitors, eight (11%) were shown to have concurrent MAPK alterations. Increased HER2 phosphorylation has been identified in a crizotinib-resistant CD74-*ROS1* NSCLC patient (73). KRAS G12D, BRAF V600E or KIT D816G mutations have been detected in the clinical setting after progression to crizotinib (10,74-76). EGFR mutations rarely co- occur with *ROS1* fusions in the clinical setting (77,78). However, EGFR pathway activation is a relevant mechanism of resistance to ROS1 inhibition (54,79,80).

Combination therapies to overcome this kind of off-target resistance is a promising strategy already tested preclinically (81), although clinical responses have yet to be reported. In addition, it may be challenging to achieve maximum drug levels for each drug in combination therapy due to the potential additive side effects or resulting in too toxic combinations (10). Novel treatment methods such as multiple low dose therapy (MLD) and adaptive dose escalation can be an effective strategy to overcome TKI-resistant NSCLC tumors without significantly increasing toxicity, with encouraging results (82,83).

Phenotypical changes

Phenotypic changes, like epithelial to mesenchymal transition (EMT), can emerge as a resistance mechanism to crizotinib. In one patient with crizotinib-resistant, *ROS1*-fusion positive NSCLC, no alterations were identified in ROS1, ALK, MET, EGFR, or KRAS, but EMT-like alterations (reduced E-cadherin and increased vimentin) was reported as the potential acquired resistance mechanism (8,79).

The most extreme phenotypic change is the histologic transformation from adenocarcinoma to small-cell lung cancer (SCLC), a known phenomenon in TKI acquired resistance in EGFR-mutant NSCLC (8,84). Recently, Lin *et al.* reported histologic transformation to SCLC in TKI-resistant *ROS1*+ NSCLC (84). Currently, these patients should receive standard platinum-etoposide based chemotherapy and be treated like a SCLC, as ROS1 dependance has not been proven for these tumors. Increased knowledge on the fundaments of phenotypic changes in lung adenocarcinoma harboring *ROS1* fusions can be critical for the development of more accurate targeted therapies.

Other agents targeting **ROS1***—first line*

Although crizotinib has been so far the standard-of care for patients with *ROS1*-rearranged NSCLC, other TKI have been tested in the first-line. Resistance mechanism to different TKIs used in the front-line treatment other than

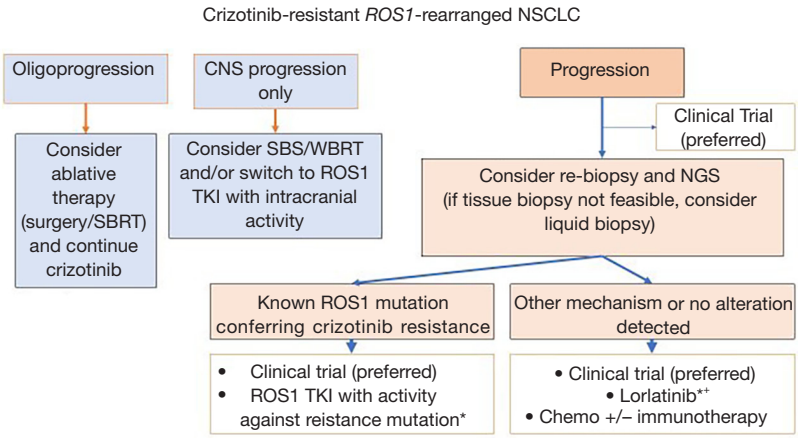

Figure 2 Suggested approach in crizotinib-resistant *ROS1*-rearranged NSCL. *, if available; +, neither entrtectinib or ceritinib have demonstrated clinical activity after crizotinib failure. NSCLC, non-small cell lung cancer; CNS, central nervous system; SBRT, sterotactic body radiotherapy; SRS, sterotactic radiosurgery; WBRT, whole brain radiotherapy; NGS, next-generation sequencing.

crizotinib for *ROS1*-positive cancer patients can modified subsequent therapies.

Entrectinib

Entrectinib is a multikinase inhibitor targeting TRK, ROS1 and ALK. Notably, entrectinib can effectively cross the blood-brain barrier (85,86) acting on preexisting CNS lesions and preventing or delaying the onset of metastases to the brain (87). However, entrectinib has little to no role in treating crizotinib-resistant *ROS1*-positive NSCLC. The results of three large multicenter trials (the phase I ALKA-372-001 trial, NCT02097810; the phase I STARTRK-1 study, NCT02097810; and the phase II STARTRK-2 trial; NCT02568267) for entrectinib in *ROS1*-positive NSCLC were reported in 2018 leading to FDA approval for the first-line treatment of advanced *ROS1*-rearranged lung cancer.

Fifty-three *ROS1*-rearranged and ROS1-inhibitor-naïve NSCLC patients were evaluated for entrectinib with an ORR of 77%, an intracranial ORR of 74%, a median duration of response (mDOR) of 24.6 months, and median PFS of 19 months (86). Outcomes were similar to those reported with crizotinib, despite the much higher proportion of patients with baseline CNS metastases in entrectinib trials (>40% *vs.* 18%, respectively). Although cross-trial indirect comparisons should be avoided, and no face to face comparative trials are expected, entrectinib might be a better option than crizotinib in those patients with CNS metastases at diagnosis and could potentially delay the development of new brain metastases in those without intracranial disease (10).

Substitutions engendering resistance to entrectinib include F2004C/I and G2032R (10).

Entrectinib has no activity against the *ROS1* resistance mutations L2026M, G2032R, and D2033N (86,87) and seems to be ineffective in treating crizotinib resistant *ROS1*-rearranged tumors (8,30).

Ceritinib

Ceritinib is a strong and selective ALK and ROS1 inhibitor with efficacy in *ROS1*-rearranged NSCLC and BBB penetration (88). However, its use is limited by the lack of regulatory approval in this setting and severe gastrointestinal toxicities (diarrhea, nausea, anorexia, and vomiting) at the recommended full dose (10).

In a phase II study, 30 patients with crizotinib-naïve *ROS1*-positive metastatic lung cancer were treated with ceritinib, showing an ORR of 62%, a disease control rate (DCR) of 81% and a median PFS of 19.3 month. In eight patients with CNS metastases, the intracranial ORR was 25% with an intracranial DCR of 63%. No responses in the two crizotinib-resistant patients were reported.

Ceritinib shows no activity against most *ROS1* resistant mutations, including *ROS1* G2032R, so its use is limited to crizotinib-naïve patients. *ROS1* E1990G and F1994L substitutions have been reported as resistance mechanism to ceritinib (10).

Brigatinib

Brigatinib is approved in metastatic ALK-fusion positive

lung cancer and, like crizotinib and ceritinib, it also has activity against ROS1-fusion tumors based on preclinical studies (8). A phase 1/2 study of brigatinib included 3 patients with *ROS1*-rearranged NSCLC. One patient was crizotinib- naïve and had a partial response. Of the two crizotinib-pretreated patients, one had stable disease and the other developed progressive disease. The activity of brigatinib against acquired *ROS1* mutations seems to be comparable to ceritinib, based on Ba/F3 models. In vitro, brigatinib inhibits the L2026M mutation, but not other common resistance mutations as G2032R, L1951R, D2033N. Therefore, it likely has limited activity against crizotinib-resistant *ROS1*-driven tumors. Toxicity related to brigatinib includes nausea, diarrhea, headache, and cough. Additionally, it has been associated with early pulmonary toxicity including with pneumonitis, so a two-step dosing has been recommended (8).

Overcoming crizotinib-resistance in *ROS1*-rearranged NSCLC

Other treatment strategies are available upon crizotinib progression and include the use of chemotherapy, immunotherapy or targeted therapies (*Figure 2*). Targeted therapies against ROS1 kinase domain can be selective (type I) or multikinase inhibitors (type II) with off-target effects. In some cases, solitary progressions can be managed with local ablative treatments to prolong the benefit of crizotinib. The use of selective treatments based on the mechanism of resistance remains experimental and the indication of rebiopsy either by tissue biopsy or liquid biopsy is not currently validated and recommended outside of clinical trials.

Liquid biopsies in NSCLC

Liquid biopsies are simple, non-invasive blood tests to detect circulating tumor DNA (ctDNA) and have been shown to be non-inferior to up-front tumor tissue genotyping in NSCLC (89). Prior clinical data have demonstrated the sensitivity of plasma ctDNA testing for the detection of common driver mutations in NSCLC, including *ROS1* fusions (89-92). A recent review by Drilon et colleagues brought up this unanswered clinical question for discussion (10).

After progression to first-line targeted TKI, a wide spectrum of molecular changes and resistance mechanism can arise. Tissue biopsy can be difficult to obtain in some cases (due to initial response, intracranial disease, oligoprogression) and intratumor heterogeneity must be also taken into account (10). Genomic heterogeneity directly impacts therapeutic choices; depending on the type of the resistance mechanisms in an individual patient, different treatments should be considered.

Analysis of ctDNA can show an integrative view of molecular alterations that can be missed by only tissue biopsy. The non-invasive nature of liquid biopsy allows serial monitoring for acquired resistance mechanisms. However, liquid biopsies have also limitations, as may be influenced by the low ctDNA shedding by the tumor or low allelic fraction, for example in low-tumor burden or solitary brain metastasis, but sensitivity can vary depending on the detecting methodology. In addition, off-target resistance by bypassing signaling or phenotypical changes are also missed in liquid biopsies based on ctDNA. The clinical implications of liquid biopsy findings for the optimal selection of therapy, particularly before radiographic progression, remain to be prospectively assessed (10).

Serial tumor biopsy

Given the rarity of *ROS1*-rearranged NSCLC and the limited preclinical models or *ROS1* fusion lung cancer there is a collective effort to analyze tissue and generate cell lines or patient-derived xenografts (PDX). This is of particular interest in the case of acquired resistance to ROS1 inhibitors as crizotinib. In this regard, the ROS1 Cancer Model Project aims to generate valid research models from patients to understand ROS1 biology, resistance mechanisms, and biomarker testing (93).

Second and subsequent lines of therapy in ROS1-*rearranged NSCLC*

Crizotinib is highly effective in patients with *ROS1* TKI- naive disease, but most tumors invariably acquire one of the resistance mechanisms previously reviewed, limiting their long-term clinical benefit. Additionally, intracranial activity is poor. Therefore, novel agents have been developed to overcome crizotinib resistance and CNS progression disease. Next-generation TKIs can be clinically active against *ROS1*-rearranged NSCLC after progression to crizotinib, depending on the acquired resistance mechanism, or have substantial intracranial activity compared to crizotinib (10).

Lorlatinib

Lorlatinib (PF-06463922) is an elegantly designed, highly potent small-molecule oral TKI designed for selective ALK/ROS1 inhibition, and robust CNS penetration (94).

In patients treated at the standard 100 mg daily dosing, lorlatinib had a cerebrospinal fluid to plasma ratio ranging 61–96%, indicating an excellent CNS penetration (59,95). Notably, lorlatinib has in vitro activity against several crizotinib-resistant mutations, including L2026M (64), S1986Y/F (96), and D2033N (66).

In a phase II trial of *ROS1*-rearranged NSCLC patients, 70% of which had progressed to crizotinib, lorlatinib achieved an ORR of 36.2%, intracranial ORR of 56%, and a median PFS of 9.6 months (94). Lorlatinib was overall well tolerated, and the most frequent adverse events were hypercholesterolemia (72%), hypertriglyceridemia (39%), peripheral neuropathy (39%), and peripheral edema (39%) (94).

However, lorlatinib has limited efficacy against the *ROS1* G2032R in preclinical models. Thus, lorlatinib could be clinically active in selected patients after crizotinib but may have limited efficacy in the subset of patients with tumors known to harbor G2032R (21).

Although lorlatinib has been recommended by the NCCN Guidelines for the treatment of advanced *ROS1*-rearranged NSCLC that progressed after crizotinib, entrectinib or ceritinib, the approval by the US FDA and EMA in this indication is still pending.

Given its higher intracranial ORR, lorlatinib can reestablish durable disease control in *ROS1*-rearranged cancers with pharmacokinetic intracranial failure and/or a limited spectrum of resistance mutations (10). However, its activity against the ROS1 G2032R mutation might be limited in the clinic; Based on preclinical studies, G2032R appears to significantly decrease the cellular potency of lorlatinib. Performing biomarker studies with tissue or blood-based NGS test after progression to crizotinib could identify patients who would benefit more from this treatment.

Repotrectinib

Repotrectinib (TPX-0005) is a potent ALK/ROS1/TRK inhibitor that has shown promising clinical activity in patients with *ROS1*-rearranged NSCLC and in patients with acquired resistant mutations resistant to prior TKI (30). Repotrectinib is active in CNS and against several *ROS1*- point resistance mechanisms, including *ROS1* G2032R (65,97).

The phase I/II trial (TRIDENT-1, NCT03093116) assessing repotrectinib enrolled 11 TKI-naïve *ROS1*-rearranged NSCLC patients, showing an ORR of 82%. In 18 TKI pre-treated patients, ORR was 57% at the recommended dose (60). The intracranial ORR was 75%. The responders included patients who received two or more prior TKIs and had tumors harboring the *ROS1* G2032R resistance mutation with an ORR 43%, and disease regression occurred in all patients.

Repotrectinib is active both intracranially and against several *ROS1*- intrinsic resistance mechanisms, including *ROS1* G2032R (65). Based in these results, repotrectinib has been granted FDA Fast Track Designation in patients with *ROS1*-rearranged NSCLC who have progressed on one ROS1 TKI and a platinum-doublet chemotherapy (10).

Taletrectinib

Taletrectinib (DS-6051b) is an oral, small molecule TKI with high affinity for ROS1 and NTRK kinases. In preclinical models, taletrectinib overcomes resistance to *ROS1* G2032R mutation (98). In a phase 1 trial (63) taletrectinib showed an ORR of 33% in crizotinib-resistant patients. Regrettably, *ROS1* mutation status was not determined, so the efficacy of this agent in the setting of *ROS1* on-target resistance is yet unknown (10).

The most common treatment-related AEs were transaminitis (80%), diarrhea (53%), nausea (46%), and constipation (33%) (63).

Cabozantinib

Cabozantinib is a multi-kinase inhibitor with activity against RET, MET, VEGFR2, ALX, TIE2, KIT, and ROS1 (66). Cabozantinib is already approved for use in medullary thyroid cancer and advanced renal cell carcinoma after prior anti-angiogenic therapy. Moreover, cabozantinib has also demonstrated clinical activity against *ROS1* fusions, particularly against solvent front resistance mutations in *ROS1* including G2032R and D2033N (99).

Within a phase II study, cabozantinib achieved a major partial response in a patient with CD74-*ROS1*-rearranged NSCLC, who had progressed on crizotinib with an secondary solvent-front D2033N mutation (66). Moreover, a case series of four patients with crizotinib and ceritinib-resistant *ROS1*-rearranged NSCLC reported by Sun *et al.* showed 1 partial response and 3 stable disease, including patients with intracranial response, with PFS durations ranged from 4.9 to 13.8 months (61).

Cabozantinib as a multi-targeted TKI, and due to its lack of selectivity, causes significant off-target toxicity not observed with more selective ROS1 TKIs, including

gastrointestinal toxicities (diarrhea), cardiovascular toxicities such as hypertension, and palmar-plantar erythrodysesthesia. As a consequence, most patients require dose reductions because of severe adverse events (8).

The spectrum of acquired resistance to type II TKI can differ from type I TKIs. Substitutions observed with type II ROS1 TKIs as cabozantinib include E1974K, F2004V/C, E2020K, V2089M, D2113N/G, M2134I, and F2075V none of which involves solvent- front or gatekeeper residues (10).

Chemo-immunotherapy

After the failure of targeted therapies, conventional cytotoxic chemotherapy remains a standard treatment. Several studies have shown that pemetrexed-based chemotherapy for *ROS1* fusion-positive tumors is associated with better responses rates and longer PFS compared with patients with NSCLC harboring other driver mutations (100).

Finally, although prior data supports the lack of response to checkpoint blockade in actionable mutation-driven NSCLC, several case reports have demonstrated the potential role of ICIs either alone or in synergistic combination in *ROS1*-rearranged NSCLC (101,102).

Conclusion

The development of targeted therapies against driver mutations have dramatically improved the prognosis of lung cancer patients harboring these actionable mutations, including patients with *ROS1*-rearranged NSCLC.

At this time, several major resistance mechanisms have arisen to oral TKI that lead to treatment failure and next-generation TKIs have been developed in order to overcome resistances. Intracranial activity, efficacy against resistant *ROS1*-mutant kinases (particularly G2032R), and testing combination therapies for bypass-signaling resistance mechanisms may be keys for prolonging disease control and improving survival.

Tissue or blood-based NGS can help to identify the resistance mechanism to ROS1 TKI and may be considered after tumor progression in order to better select further lines of targeted therapy although prospective validation of this therapeutic approach is needed. In the precision medicine era, a longitudinal assessment of the mechanism of resistance on an individual patient basis may unravel the best treatment strategy and sequence.

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