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Review

ROS1-Rearranged Lung Adenocarcinoma: From Molecular Genetics to Target Therapy

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Simple Summary: Receptor tyrosine kinases are present on the cell membrane of some cell types and are responsible for the regulation of cell growth. These receptors are frequently deregulated in tumors through various molecular mechanisms involving structural rearrangements, point mutations or gene amplification. Deregulated receptor tyrosine kinases act as oncogenes driving cancer development. One of these membrane tyrosine kinases is ROS1, constitutively activated in a minority of lung cancers through structural rearrangements generating fusion genes involving the ROS1 gene and a partner gene. The identification of this subtype of lung cancers has determined the development of specific molecular treatments targeting the deregulated ROS1 gene, with an improvement in the survival of these patients.

Abstract: Non-small-cell lung cancer (NSCLC) is a heterogeneous group of diseases accounting for 80–85% of lung cancers. A molecular subset of NSCLC (1–2.5%) harboring molecular rearrangements of the tyrosine kinase gene *ROS1* is defined as ROS1-positive and is almost exclusively diagnosed in patients with lung adenocarcinoma histology, predominantly nonsmokers. ROS1 is constitutively activated by molecular rearrangements and acts as a main driver of lung carcinogenesis. These findings have provided a strong rationale for the clinical use of tyrosine kinase inhibitors that target ROS1; these inhibitors block ROS1-positive NSCLC and provide clinical benefit. Crizotinib was introduced as a first-line treatment for ROS1-positive NSCLCs, with 75–80% of patients responding and a PFS of about 20 months. More recently developed ROS1-TKIs, such as entrectinib, lorlatinib, taletrectinib, repotrectinib and NVL-520, are active against some resistant *ROS1* mutants appearing during crizotinib therapy and more active against brain metastases, frequent in ROS1-positive NSCLC. The development of resistance mechanisms represents a great limitation for the targeted treatment of ROS1-positive NSCLCs with TKIs.

Keywords: lung cancer; non-small-cell lung cancer; lung adenocarcinoma; ROS1 rearrangements; target therapy; tyrosine kinase inhibitors; next-generation sequencing

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1. Introduction

A large diversity of molecular subtypes of lung cancer exists; these molecular subtypes are originated by different genetic alterations such as mutations, fusions and copy number changes. Among these events, fusions are observed in rare molecular subtypes, often involving receptor tyrosine kinase (RTK) genes, such as *ALK, RET, ROS1, NTRK 1/2/3, FGFR 1/2/3, EGFR, ERBB2, ERBB4* and *LTK* [1].

The characterization of the gene fusion events has allowed the development of drugs that specifically target these alterations, providing new therapeutic approaches in the treatment of these cancers. Thus, the identification of oncogenic *ALK, RET* and *ROS1* at diagnosis is important because these alterations make up a part of the 40–50% of lung adenocarcinomas (LUADs) bearing a targetable oncogenic alteration [2,3].

This review was based on a literature search using PubMed and a search of data from the most relevant international meetings on lung cancers, and it screened a period of time from 2000 to the present for publications concerning the biology and clinical treatment of *ROS1*-rearranged LUADs. Case reports or studies based on the analysis of only a few ROS1-rearranged LUADs were usually excluded from the present analysis.

1.1. ROS1 Gene Rearrangements

The *ROS1* gene is located at the level of chromosome 6q22 and encodes for a receptor tyrosine kinase belonging to the insulin receptor family. The exact role of ROS1 protein in normal development, as well as its normal physiological ligand, have not been defined and, accordingly, ROS1 is a so-called orphan receptor; it may function as a growth or differentiation factor receptor. The human *ROS1* gene encodes 2347 aminoacidic residues and ROS1 protein is the largest protein tyrosine kinase receptor. *ROS1* gene rearrangements involve the fusion of the 3′ region of the *ROS1* gene containing the kinase domain with the 5′ region of a partner gene. *ROS1* gene rearrangement was initially discovered in a glioblastoma cell line [4]. Rokova et al. first identified *ROS1* and *ALK* fusion genes in NSCLC using a phosphoproteomic approach in the context of a study aiming to characterize tyrosine kinase signaling in tumor cell lines and tumor samples [5].

Three studies in 2012 reported the first analyses on the frequency and on the molecular characterization of *ROS1* fusions occurring in NSCLC. Takeuchi et al. performed a screening of kinase fusions in a large cohort of 1528 Japanese NSCLC patients and identified *ROS1* fusions in 0.9% of the NSCLCs and 1.2% of the LUADs [6]. A second study by Bergethon and coworkers provided evidence that *ROS1* rearrangements define a subset of NSCLC with distinct clinical features comparable to those observed in *ALK*-rearranged NSCLCs [7]. Particularly, through FISH analysis carried out in 1073 NSCLC patients, these authors reported an ROS1 rearrangement by FISH in 1.7% of these tumors, preferentially occurring in younger and never-smoker patients [7]. All *ROS1*-rearranged NSCLCs were LUADs and showed a prognosis comparable to that observed for LUADs without *ROS1* rearrangement [7]. Davies et al. evaluated 428 NSCLC samples for *ROS1* rearrangement by FISH and observed a positivity in 1.2% of cases [8]. The fusion partners were *CD74* in two cases, *SLC34A2* in two cases and *SDC4* in one case [8].

Zhang et al. have explored the prevalence of *ROS1* fusions in a large cohort of 6066 Chinese NSCLC patients and observed a frequency of 2.59%, preferentially in younger patients, never-smokers and those with advanced node stages [9]. Kim et al. explored the frequency and the clinical impact of *ROS1* rearrangements in a cohort of 208 LUAD never-smokers and reported a frequency of 3.4%; in *KRAS/EGFR/ALK*-negative patients, the frequency of *ROS1* rearrangements was 5.7% [10]. The ORR was higher and the PFS was longer in *ROS1*-rearranged LUADs than in those without *ROS1* rearrangements; in contrast, the PFS in response to EGFR inhibitors in patients with *ROS1* fusions was shorter compared to those without *ROS1* rearrangements [10].

It Is important to note that, in addition to *ROS1* gene rearrangements, *ROS1* gene amplifications were also detected in NSCLC patients; thus, Jin et al. reported *ROS1* gene rearrangements in 0.8% of NSCLC patients; ROS1 protein overexpression was observed in 5% of cases [11]. *ROS1* copy number gain is an independent poor prognostic factor [11].

A fundamental study by Lin and coworkers explored the mutational profile of NSCLC for the presence of other driver mutations (*EGFR, KRAS* and *ALK* fusions) in two cohorts of 62 and 166 *ROS1*-rearranged NSCLCs and observed that in the first cohort of patients, non-concomitant *EGFR* mutations or *ALK* fusions were detected;; in the second cohort of patients, 1/166 displayed *EGFR* mutations, 3/166 displayed *KRAS* mutations and there were none with *ALK* rearrangements [12]. A more extensive gene sequencing carried out for 44 patients showed *TP53* mutations in 25% of cases, *CTNNB1* mutations in 7% of cases and *CDKN2A* or *CDKN2B* gene loss in 13% of cases [12]. Zhuang et al. confirmed these observations through the analysis of 3774 NSCLC patients for driver mutations of *EGFR, KRAS, ROS1* and *BRAF*: 2.1% displayed *ROS1* rearrangements and only a minority of these

patients had concomitant *ROS1* fusions and *KRAS* mutations or *EGFR* mutations or both *EGFR* and *KRAS* mutations [13].

Huang et al. reported the largest clinicopathologic and genomic characterization of *ROS1*-rearranged solid tumors, involving the analysis of 356 (275 of these tumors were NSCLC) cases [14]. At the level of the fusion partners, 49.8% were *CD74-ROS1*, 23.6% *EXR-ROS1*, 9.1% *SDC4-ROS1*, 5.1% *SLC34A2-ROS1*, 2.9% *TPM3-ROS1*, 1.5% *SLCA4-ROS1* and 7.6% rare fusion partners [14].

The most frequent genetic alterations associated with *ROS1* fusions were at the level of *TP53* (32.4%), *CDKN2A* (31.3%), *CDKN2B* (20.7%), *SETD2* (8.7%), *ARID1A* (5.5%), *RBM10* (4.4%), *CTNNB1* (4.4%) and *U2AF1* (4%); *EGFR* and *PIK3CA* mutations were observed in 1.4% and 2.2% of cases, respectively [14] (Figure 1).

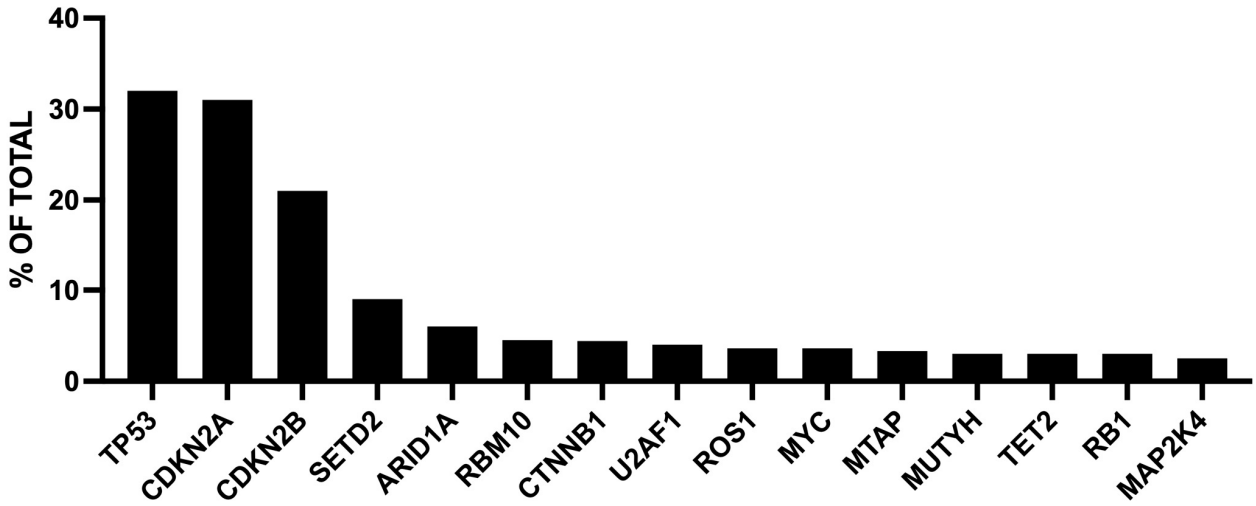

Figure 1. The most recurrent genetic alterations observed in *ROS1*-rearranged NSCLCs. The data were reported by Huang et al. [14].

Interestingly, the co-alteration profile of *ROS1*-rearranged NSCLC was like that observed in *ROS1*-rearranged non-NSCLC, with the only exception being a higher frequency of co-alterations at the level of *TERT, PTEN* and *APC* genes in non-NSCLC cases [14]. In contrast, the *ROS1* fusion partners in non-NSCLC cases are different from those observed in NSCLC cases, with the *GOPC-ROS1* (65.4%) fusions being frequent and the *CD74-ROS1* (4.5%) fusions rare [14].

Sato et al. showed that 11% of patients with an *ROS1* fusion had concurrent MAPK alterations and this correlated with poor survival [15]. The MAPK pathway alterations involved *NF1* gene loss and *MAP3K1, MAP2K1, MAP2K4, KRAS* and *BRAF* gene mutations [15]. The clinicopathologic features of patients with or without MAPK alterations were similar [15]. Furthermore, some patients acquired novel activating mutations in the MAPK pathway following treatment with an ROS1-TKI [15]. These observations supported the conclusion that aberrant MEK-ERK pathway activation caused by alterations of the MAPK pathway can confer resistance to ROS1-TKIs [15].

Coexistent genetic alterations involving *ROS1* and *ALK* fusions and *ROS1* fusions and *MET* amplification are extremely rare in LUAD patients [16].

Experimental studies have shown that *ROS1* fusions act as drivers of lung oncogenesis. *ROS1* fusions display ligand-independent, constitutive activation of ROS1 kinase catalytic activity. *ROS1* fusions, as well as *ALK* fusions, activate signaling pathways that promote cell proliferation and survival. These signaling pathways are mainly represented by RAS-MEK-ERK, JAK-STAT3, PI3-AKT-mTOR and SHP2 [17]. Several studies have investigated the specific signaling pathways activated by ROS1 fusion proteins. Jun and coworkers showed that CD74/ROS1-induced phosphorylation of E-Syt1 (Extended Synaptotagmin-like protein 1) promotes invasive in vitro and metastatic in vivo properties of tumor cells; elimination

of E-Syt1 expression drastically reduced the invasive properties of *CD74-ROS1*-expressing tumor cells [18].

The mechanisms though which the fusion ROS1 protein displays constitutive kinase activation are unclear in that, while some fusion partners like EZR and TMP3 possess coiled-coil domains, suggesting that dimerization leads to the constitutive activation of ROS1 fusion proteins, the amino terminal domains of other fusion partners such as CD74 lack the ability to induce dimerization [19].

The subcellular localization, conferred by the 5′ fusion partners, seems to have some relevant consequences for downstream signaling; thus, the SDC4-ROS1 and SLC342-ROS1 fusions possess a greater capacity to activate MAPK signaling pathways compared to ROS1 fusion proteins such as CD74-ROS1 localized at the level of endoplasmic reticulum; all these ROS1 fusion proteins activate STAT3 signaling at a similar level [20].

Other studies have shown that three different ROS1 fusion proteins, CD74-ROS1, EZR-ROS1 and SCL34A2-ROS1 have the capacity to interact with GRB2-SOS1 complex and, through this mechanism, to activate MAPK signaling [15].

Although the various ROS1 fusion proteins observed in NSCLC may activate cell signaling through different mechanisms, all these fusions have oncogenic activity in both in vitro and in vivo animal models. Transgenic mice expressing *CD74-ROS1, SDC4-ROS1* or *EZR-ROS1* in *TP53*-WT type II alveolar cells display numerous lung adenomas and adenocarcinomas [21]. These ROS1-fusion-positive mouse lung cancers were a useful tool to evaluate multikinase inhibitors [22].

1.2. Target Therapy of ROS1-Rearranged NSCLC

Preclinical studies have strongly supported the clinical evaluation of multikinase drugs with inhibitory activity on ROS1.

1.2.1. Crizotinib

Crizotinib was the first ROS1-TKI approved by the FDA and EMA for first-line treatment of *ROS1*-rearranged NSCLCs. The phase I PROFILE 1001 trial showed the efficacy and an acceptable safety profile of crizotinib in the treatment of metastatic *ROS1*-positive LUAD patients with ORR of 72%, a DCR of 90%, an mDoR of 24.7 months, an mPFS of 19.3 months and an moS of 51.4 months [23,24]. Other prospective phase II clinical studies have confirmed the efficacy of crizotinib in the first-line treatment of *ROS1*-positive LUAD patients [25–27]. Particularly, Wu et al. reported a single-arm study of 127 *ROS1*-rearranged LUAD patients who had received three or fewer lines of systemic therapy; ORR was 76.17% (with 17 CR and 74 PR), DoR 19.7 months and mPFS 15.9 months [27]. The ORR in patients with CNS metastases was like that observed in patients without CNS (73.9% vs. 71.2%, respectively); however, PFS was shorter in patients with brain metastases than patients without baseline brain metastases (10.2 months vs. 18.8 months, respectively), thus showing that CNS metastases are a negative prognostic factor in *ROS1*-positive LUAD patients [27]. The final results of this study showed a median duration of follow-up of 56.1 months and a median OS of 44.2 months [28] (Table 1).

The AcSé prospective phase II clinical trial showed a lower efficacy of crizotinib compared to that observed in the two previous studies, but these differences could be related to the recruitment in this study of patients more heavily pretreated than in the two previous studies [25].

Some retrospective studies compared the outcomes of *ROS1*-positive patients following treatment with crizotinib and with chemotherapy. Shen et al. compared 77 *ROS1*-positive patients treated with crizotinib to 47 treated with platinum-pemetrexed chemotherapy; after a median follow-up of 28.1. months, the ORR of crizotinib was higher than that of chemotherapy (86% vs. 44.7%, respectively) and the mPFS was longer for crizotinib than for chemotherapy (18.4 months vs. 8.6 months, respectively) [29]. However, OS was similar in the crizotinib and in the chemotherapy groups [29]. In line with these observations, Xu et al. evaluated 102 *ROS1*-rearranged LUADs and observed an mPFS of 14.9 months in those

treated with crizotinib compared to 8.5 months in those treated with chemotherapy [30]. In patients bearing *CD74-ROS1* fusion variants, the mPFS with crizotinib, but not with chemotherapy, was significantly longer than in those harboring non-CD74 fusion variants (20.1 months vs. 12.0 months and 8.6 months vs. 4.3 months, respectively) [30] (Table 2).

In a group of 32 patients with either ALK- or ROS1-rearranged LUADs, the mOS was significantly longer among patients treated with crizotinib compared to those treated with chemotherapy [31].

Table 1. Frequency of different ROS1 fusion proteins observed in NSCLC and their cellular location. Data on the frequency of various ROS1 fusion variants are reported in [14].

Fusion Protein	Frequency in NSCLC (%)	Cellular Location
CD74-ROS1 (Cluster of differentiation 74)	49.8	Endoplasmic reticulum
EZR-ROS1 (Ezrin)	23.6	Cytoskeleton
SDC4-ROS1 (Syndecan)	9.1	Endosomes
SLC34A2-ROS1 (Solute carrier family 34 member 2)	5.1	Endosomes
TPM3-ROS1 (Tropomyosin)	2.9	Cytoplasm
SLC4A4-ROS1 (Solute carrier family 4 member 4)	1.5	Endosomes

Zhang and coworkers reported the retrospective analysis of 235 *ROS1*-positive patients treated either with first-line crizotinib or chemotherapy; the PFS for the crizotinib group was 18.0 months compared to 7.0 months for the chemotherapy group [32]. Two factors seem to negatively affect the effectiveness of crizotinib treatment: (i) patients with brain metastases had a significantly shorter PFS compared to those without CNS baseline metastases; (ii) patients with tumor suppressor mutations (*TP53*, *RB1* or *PTEN*) or who harbor concomitant driver mutations (*EGFR* or *KRAS*) have a significantly shorter PFS compared to those without these mutations (9.5 months vs. 24.0 months and 11.0 months vs. 24 months) [32].

1.2.2. Entrectinib

The small-molecule TKI entrectinib is a potent inhibitor of ROS1 as well as of ALK, designed to cross the blood–brain barrier and to remain active in the CNS. Preclinical models have supported the high efficacy of entrectinib in brain tumor models.

An integrated analysis of three phase I and II trials of entrectinib explored the safety and the efficacy of this RSO1-TKI in a population of patients with advanced or metastatic *ROS1*-positive NSCLC. All patients received previous anticancer treatment except for ROS1-TKIs. A total of 161 patients were enrolled; the ORR was 67% and responses were durable (12-month DoR rate 63%), the median PFS was 15.7 months, and 12-month OS was 81% [33,34]. In patients with measurable CNS disease, the intracranial ORR was 79% and the 12-month rate of DoR was 55% [33,34]. A recent updated analysis of these studies, with a median follow-up extended to 29.1 months, showed an ORR of 68%, an mDoR of 20.5 months, an mPFS of 15.7 months and an mOS of 47.8 months; in patients with measurable baseline CNS metastases, the intracranial ORR was 80%, mDoR 12.9 months and median intracranial PFS was 8.8 months [35] (Table 2).

1.2.3. Lorlatinib

Lorlatinib is a brain-penetrant, third-generation, ATP competitive, reversible TKI of ALK and ROS1, retaining activity in vitro against several crizotinib-resistant *ROS1* mutations.

A phase I/II clinical study enrolled 69 *ROS1*-positive patients with advanced disease NSCLC; 30% were TKI-naïve, 58% treated in first-line with crizotinib, 12% treated with another ROS1-TKI or two or more TKIs [36]. A 62% ORR was observed in the TKI-naïve patients and 35% among those previously treated with crizotinib; intracranial responses were observed in 64% of TKI-naïve patients and in 50% of those pretreated with crizotinib [36] (Table 2).

In the IFCT-1803 LORLATU study, Girard and coworkers explored 80 advanced refractory *ROS1*-rearranged NSCLC patients (all pretreated with at least one ROS1-TKI, 69% with chemotherapy and 64% with brain metastases) treated with lorlatinib; ORR was 45%, DCR was 82%, PFS 7 months and OS 19.6 months [37]. The CNS response rate was 72% and the median duration of CNS response was 16.7 months; among the patients who did not receive brain radiotherapy before lorlatinib initiation, the CNS response rates were 68% and 20.6 months [37] (Table 2).

1.2.4. Taletrectinib

Taletrectinib is a next-generation, selective, CNS-penetrant, ROS1 inhibitor. Two initial phase I studies have shown that taletrectinib possesses a pronounced clinical activity in patients with advanced *ROS1*-positive LUADs who are ROS1-TKI-naive (PFS of 29.1 months) or crizotinib-refractory (PFS of 14.2 months) and has an acceptable safety profile [38].

The results of the TRUST-1 trial were recently shown; this trial involved two stages: stage 1 involved just six patients and stage 2 had two groups of patients, one TKI-naïve and another one previously treated with crizotinib. In patients who were TKI-naïve, taletrectinib induced an ORR of 92.5%, with a disease control rate of 95.5%, and with a median duration of response and PFS that were not reached; in the group of patients who were pretreated with crizotinib, taletrectinib induced an ORR of 52.6%, with a median duration of response that was not reached, with a disease control rate of 81.6% and the median PFS of 9.8 months [39,40]. The overall response rate in patients with intracranial disease was 90%. Importantly, taletrectinib induced responses in 80% of patients whose tumors harbored the G2032R resistance mutation [39,40] (Table 2).

Table 2. Rearranged NSCLC patients. Abbreviations: Cr, crizotinib; Pl, platinum-based chemotherapy; CN, crizotinib-naïve; CR, crizotinib-resistant; TN, TKI-naïve; TT, TKI-treated; CT, chemotherapy-treated; BM, brain metastases.

TKI	Clinical Trial Phase	Number of Patients	ORR (%)	DCR (%)	mPFS (mo)	mDOR (mo)	mOS (mo)	Grade $\frac{3}{4}$ Adverse Events	Reference
Crizotinib	PROFILE 1001 I/II	53	72	90	19.3	24.7	51.4	36	[23,24]
Crizotinib	East Asian NCT01945021 II	127	72	90	16	19.7	44.2	25	[27,28]
Crizotinib	Ac Sé I/II	36	47	83	6	-------	17	-------	[25]
Crizotinib	EUCROSS II	34	70	90	20	-------	NR	20	[26]
Crizotinib Platinum (PL)	Retrospective Study	104 56 (Cr) 46 (Pl)	84(Cr) 57 (Pl)	96 (CR) 100 (Pl)	14.9 (Cr) 8.5 (Pl)	--------	NR (Cr) NR (Pl)	--------	[29]
Entrectinib	ALKA-372-001 STRATRK-1 STRATRK-2 I/II	161	68	90	16	20.5	47.8	31	[33–35]

Table 2. *Cont.*

TKI	Clinical Trial Phase	Number of Patients	ORR (%)	DCR (%)	mPFS (mo)	mDOR (mo)	mOS (mo)	Grade $\frac{3}{4}$ Adverse Events	Reference
Lorlatinib	NCT01970845 I/II	69 21 (TN) 48 (TT)	41 (overall) 62 (CN) 35 (CR)	---	25.3 (CN) 13.8 (CR)	---	---	43	[36]
Lorlatinib	LORLATINU	80 100% (TT) 69% (CT) 64% (BM)	45	82	7.1	6.9	19.6	33	[37]
Taletrectinib	TRUST-I	109 67 (TN) 42 (TT)	92.5 (TN) 52.6 (TT)	95.5 (TN) 81.6 (TT)	33.2 (TN) 9.8 (TT)	NR (TN) NR (TT)	--- ---	29	[39,40]

Based on the clinical data accumulated to date, taletrectinib has been granted breakthrough therapy designation by the FDA for the treatment of adult patients with advanced or metastatic *ROS1*-positive NSCLC who are either TKI-treatment naïve or were previously pretreated with crizotinib.

Given the favorable results observed in the TRUST-1 trial, a phase II, multicenter, open-label, single-arm study was proposed for the treatment of *ROS1*-positive NSCLC patients with advanced/metastatic disease [41]. This study will involve the treatment with taletrectinib of 119 patients subdivided into four cohorts: cohort 1 involving patients chemotherapy-naïve or pretreated with one prior line of chemotherapy; cohort 2 involving patients pretreated with one ROS1-TKI (crizotinib or entrectinib) and chemotherapy-naïve or pretreated with one prior line of chemotherapy; cohort 3 involving patients pretreated with two or more ROS1-TKIs and chemotherapy-naïve or pretreated with two lines of chemotherapy; and cohort 4 involving patients with *ROS1*-positive solid tumors other than SCLC [41].

1.2.5. Repotrectinib

Repotrectinib (TPX-0005) is a novel next-generation ROS1/TRK/ALK-TKI designed to overcome refractory solvent-front mutations (SFMs) such as $ROS1^{G2032R}$ with efficient central nervous system penetration [41]. Preclinical studies have supported the clinical evaluation of repotrectinib as first-line and after progression to prior ROS1-TKIs [42].

The TRIDENT-1 phase I/II study evaluated the safety and the efficacy of repotrectinib in TKI-naïve and TKI-pretreated patients with advanced *ROS1, ALK* or *TRK* fusion-positive tumors. Preliminary results on 11 TKI-naïve *ROS1*-positive NSCLC patients showed an ORR of 82% with a duration of response not reached; in 18 TKI-pretreated patients, an ORR of 39% was observed; all patients with *ROS1 G2032R* showed tumor regression [43].

Recently, the results observed in 171 patients were reported and subdivided into ROS1-TKI-naïve (12 mo PFS 80%), ROS1-TKI and no chemotherapy (12 mo PFS 44%), ROS1-TKI and one chemotherapy (12 mo PFS 15%) and ROS1 two TKIs and no chemotherapy (12 mo PFS 7%) [44,45]. In ROS1-TKI-naïve patients without or with CNS metastases, there was a 6 mo ORR of 76% and 100%, respectively; in ROS1 one TKI and no chemotherapy, there was a 6 mo ORR of 41% and 33%, respectively; in one TKI and one chemotherapy, there was a 6 mo ORR of 44% and 40%, respectively; in two TKIs and no chemotherapy, there was an ORR of 40% and 13%, respectively [42,43]. These observations support a durable clinical activity of repotrectinib in ROS1-TKI-naïve and pre-treated patients with or without baseline CNS metastases [43,44] (Table 3).

Table 3. Results of the phase I–II clinical trial TRIDENT-1 involving the treatment of ROS1-rearranged NSCLC patients with advanced disease. The patients were subdivided into four subgroups: TKI-naïve (TN), treated with 1 TKI and chemotherapy-naïve (1T-CN), treated with 1 TKI and 1 chemotherapy treatment (1T-1C) and treated with 2 lines of TKIs and chemotherapy-naïve (2T-CN). For each of these four subgroups, the patients were subdivided into one group with CNS metastases and one group without CNS metastases. Data were reported in [43,44].

Drug	TRIAL	Patient Number	ORR (with CNS Metastases)	ORR (without CNS Metastases)	DOR at 6 mo (with CNS Metastases)	DOR at 6 mo (without CNS Metastases)
Repotrectinib	TRIDENT-1	171 71(TN) 56(1T-CN) 26(1T-1C) 18(2T-CN)	89(TN) 33(1T-CN) 40(1T-1C) 13(2T-CN)	76(TN) 41(1T-CN) 44(1T-1C) 40(2T-CN)	100(TN) 63(1T-CN) 50(1T-1C) 100(2T-CN)	87(TN) 92(1T-CN) 71(1T-1C) 50(2T-CN)

1.2.6. NVL-520

Although taletrectinib and repotrectinib determine responses in *ROS1*-positive NSCLC patients, the use of these agents is limited to some extent by gastrointestinal and neurologic toxicities. Neurologic toxicity caused by these two TKIs was attributed to their inhibitory activity on tropomyosin-related kinases (TRK), a phenomenon dependent on the similarities occurring between ROS1 and TRKs.

To bypass these limitations, a new ROS1-TKI was developed, endowed with the capacity of a wide inhibitory activity against *ROS1* mutants, brain penetration and avoidance of the dose-limiting inhibition of TRKs. NVL-520 is a macrocyclic small molecule acting as a potent inhibitor of the aminopyridine moiety of ROS1; this compound forms two hydrogen bonds with Glu 2027 and Met 2029 in the hinge region of the ROS1 molecule [43]. NVL-520 possesses a higher inhibitory activity for WT-ROS1 (IC_{50} 0.7 nmol/L) and ROS1 G2032R (IC_{50} 7.9 nmol/L) compared to 30 nmol/L of repotrectinib and 100 nmol/L of taletrectinib. NVL-520 is active against only ROS1 and ALK [45].

NVL-520 inhibits the viability of cancer cell lines with various types of ROS1 fusion proteins, including ROS1 fusions bearing different types of point mutations, including ROS1 G2032R. ROS1 L2086F, an on-target resistance mutation observed in cancers from patients who progressed on lorlatinib, confers a resistance to NVL-520 inhibition, with a shift of IC_{50} from 1.3 nmol/L to 6.8 nmol/L. Importantly, NVL-520, at variance with taletrectinib and repotrectinib, does not exert any inhibitory activity on tropomyosin kinases [45].

Preclinical models supported the efficacy of NVL-520 on *ROS1*-positive xenograft tumors, including G2032R-inclusive intracranial tumors and patient-derived xenograft models [45].

As a clinical proof of concept, NVL-520 was evaluated in three patients with TKI-refractory ROS1 fusion-positive NSCLCs, including two with *ROS1 G2032R* and one with intracranial metastases, resulting in partial responses, with no observed neurological toxicities [45].

1.3. Resistance Mechanisms

Although ROS1-TKIs have contributed to improve the outcomes of *ROS1*-rearranged NSCLCs, the occurrence of resistance mechanisms consistently limit the clinical benefit of these drugs.

1.3.1. Resistance to Crizotinib

A part (10–15%) of *ROS1*-positive patients treated in first-line are constitutively resistant to crizotinib.

Most of the patients responding to crizotinib develop resistance through different mechanisms that can be classified as ROS1-intrinsic and ROS1-extrinsic. The intrinsic mechanisms are related to the appearance of punctual mutations in the ROS1 kinase domain, with consequent modification of the binding site; the extrinsic mechanisms are

mainly represented by activation of other signaling pathways, intracranial failure due to scarce penetration through the blood–brain barrier and phenotypic changes (mesenchymal to epithelial transition, transformation into small cell lung cancer).

Punctual mutations in the kinase-binding domain are responsible for 40–55% of cases of resistance to crizotinib. G2032R is largely the most frequent *ROS1* point mutation occurring in crizotinib-treated patients, accounting for 30–40% of resistance mechanisms. This mutation was initially reported in 2013 in a NSCLC patient bearing a CD74-ROS1 fusion protein [46]. The G2032R mutation is a glycine-to-arginine substitution at the level of codon 2032 in the solvent front, causing resistance to crizotinib through steric interference with the drug binding site at the level of ROS1-kinase residues exposed to solvent. The G2032R mutation, in addition to causing crizotinib resistance, induces also two remarkable biologic effects: increased TWIST1 expression [47]; and epithelial to mesenchymal transition and increased tumor cell invasiveness and migration [48].

The G2032R *ROS1* mutation induces resistance to several TKIs, such as crizotinib, ceritinib, entrectinib and lorlatinib; the new-generation ROS1-TKIs repoprectinib, topotrectinib and SV-520 are active against this *ROS1* mutant.

Other *ROS1* point mutations observed in crizotinib-resistant patients are D2033N, S1986Y/F, L2026M, L2155S and L1951R. The *ROS1* D2033N mutation induces a modification of the ATP-binding site pocket and modified electrostatic interactions required for binding to ROS1-TKIs; this mutation confers resistance to crizotinib, entrectinib and ceritinib, but not to lorlatinib [49].

Gainor et al. reported on 16 patients developing resistance to crizotinib, 53% of the cases with *ROS1* resistance point mutations: G2032R in 41% of cases, D2033N in 6% of cases and S1986F in 6% of cases [50]. Lin and coworkers explored 41 *ROS1*-positive NSCLC patients developing resistance to crizotinib; 38% of these patients displayed *ROS1* point mutations: 34% G2032R, 2% D2033N and 2% S1986F [51].

Zhang et al. explored 49 *ROS1*-rearranged NSCLC patients developing resistance to crizotinib; 61% of these patients displayed *ROS1* point mutations: 28.5% were G2032R, 8.3% G2032K, 6.1% L2086F, 4.1% S1986Y, 2% S1986F, 2% L1174F and 2% L2155S [51]. Interestingly, a comparative analysis showed that patients with extracranial-only progression had a significantly higher frequency of *ROS1* point mutations compared to those with intracranial-only progression (72.7% vs. 15.2%, respectively) [52]. The fact that no point mutations were detected in a large proportion of patients with intracranial-only mutations may in large part reflect a pharmacokinetic failure related to the scarce capacity of crizotinib to penetrate the blood–brain barrier [52].

1.3.2. Resistance to Lorlatinib

A more limited number of studies have explored the molecular mechanisms responsible for resistance to lorlatinib. Liu et al. analyzed 28 *ROS1*-positive patients developing resistance to lorlatinib treatment; *ROS1* point mutations were observed in 46% of these patients: G2032R in 32% of cases, as well as L2086F (3.6%), G2032R/L2086F (3.6%), G2032R/S1986F/L2086F (3.6%) and S1986F/L2000V (3.6%) [53]. Analysis of lorlatinib-resistant cases, where matched post-crizotinib/pre-lorlatinib samples were available, showed that in about 55% of cases no *ROS1* mutations were detected in either post-crizotinib or post-lorlatinib specimens; 27% of cases acquired new *ROS1* mutations on lorlatinib and the remaining 18% maintained the same mutations in both post-crizotinib and post-lorlatinib biopsies [52].

Wang et al. reported the retrospective analysis of 101 *ROS1*-positive NSCLC patients; all these patients were treated with crizotinib and 21 with lorlatinib after crizotinib progression [53]. The PFS was 12.9 months for crizotinib and 6.4 months for lorlatinib; patients with *CD74-ROS1* and *SLC34A2-ROS1* fusions had significantly longer PFS than those with other *ROS1* fusions [52]. An accumulation of both on-target (baseline vs. post-crizotinib vs. post-lorlatinib: 0% vs. 43% vs. 62%, respectively) and off-target (baseline vs. post-crizotinib vs. post-lorlatinib: 22% vs. 26% vs. 43%) mechanisms of resistance was observed [53].

1.3.3. ROS1-Extrinsic Mechanisms

Several extrinsic mechanisms of resistance have been reported.

1.3.4. Intracranial Failure

Brain metastases are a major cause of morbidity and mortality in patients with NSCLC. Central nervous system (CNS) metastases were observed in 36% of stage IV *ROS1*-positive NSCLC patients and the CNS was the first and the sole site of progression in 47% of these patients [54]. The incidence of brain metastases was similar in LUADs with *ROS1, ALK, EGFR, BRAF* or other mutations [54]. At variance with that study, Gainor et al. reported a lower frequency of brain metastases in *ROS1*-rearranged than in *ALK*-rearranged NSCLCs, at initial diagnosis [49]. This lower frequency of brain metastases was observed also following crizotinib treatment: 34% in *ROS1*-rearranged LUADs vs. 73% in *ALK*-rearranged LUADs [50].

It was estimated that the frequency of CNS metastases ranged from 20 to 35% at diagnosis in *ROS1*-positive NSCLC patients and can be as high as 50% or more post-crizotinib treatment [55]. This phenomenon is due to the low capacity of crizotinib to penetrate across the blood–brain barrier since this compound is a substrate of P-glycoprotein and of human ATP-binding cassette subfamily efflux transporters. In fact, cerebrospinal fluid concentrations of crizotinib are low and not sufficient to exert adequate antitumor effects [56].

Entrectinib, lorlatinib and, particularly, repotrectinib are more active than crizotinib in exerting intracranial activity in *ROS1*-positive NSCLC patients.

1.3.5. Off-Target Activation of Signaling Pathways

Resistance to ROS1-TKIs may be mediated by activation of other signaling pathways. Acquisition of these activation pathways may be related to two different mechanisms: (i) presence at diagnosis or acquisition of mutations or gene amplifications at the level of some oncogenic pathways; (ii) stimulation by ROS1 of signaling pathways resistant to inhibition mediated by ROS1-TKIs.

The presence of some co-mutations negatively affects the response of *ROS1*-rearranged NSCLCs.

TP53 mutations are observed in about 20–30% of *ROS1*-positive NSCLC patients. A large retrospective study on 86 ROS1-positive NSCLCs showed the presence of *TP53* co-mutations in 13% of patients with *CD74-ROS1* fusions and in 18.8% of those with non-*CD74-ROS1* fusions [32]. Patients with concomitant *TP53* mutations had significantly shorter PFS than those with wild-type *TP53* (6.5 months vs. 21.0 months, respectively) [32].

Gen et al. explored 39 *ROS1*-positive patients undergoing treatment with crizotinib; the presence of *TP53* mutations (observed in 33% of patients), as well as the presence of brain metastases, were associated with shorter PFS compared to patients without these mutations or without brain metastases [57]. Wang et al. explored 101 patients with *ROS1*-positive NSCLC treated with crizotinib and observed in those bearing baseline *TP53* mutations a worse PFS compared to those *TP53*-WT [53]. Frost et al. explored a group of 52 patients with *ROS1*- or *ALK*-rearranged NSCLCs treated with lorlatinib; 28% of these patients displayed *TP53* mutations, which were associated with a substantially reduced PFS (3.7 months vs. 10.8 months) [58].

Mc Coach et al. observed in 5/12 *ROS1*-positive NSCLC patients undergoing crizotinib treatment the presence of off-target mechanisms that could cause ROS1-TKI persistence; there were Kit and β-catenin mutations in a single patient each and copy number variation in proto-oncogenes in three other patients [59]. Other studies have reported the acquisition of $BRAF^{V600E}$ mutation [59,60] or *ALK* mutation [61] or *MET* point mutation [62] or *MET* gene amplification [63] in crizotinib-treated *ROS1*-rearranged NSCLC patients.

In lorlatinib-resistant *ROS1*-positive lung cancers, Lin et al. identified *MET* amplification (4%), $KLRAS^{G12C}$ mutation (4%), *KRAS* amplification (4%), *NRAS* mutation (4%) and *MAP2K1* mutation (4%) [51].

MYC amplification was observed in about 19% of lorlatinib-resistant *ROS1*-driven NSCLCs [64]. In vitro and in vivo studies showed that inhibition of *MYC*-amplified *ROS1*-positive tumors can be obtained through the combination of ROS1-TKI and CDK4/6 inhibition [65].

Among the ROS1-independent mechanisms of acquired TKI resistance, a relevant role is played by bypass signaling through the MAPK pathway. Acquired *KRAS* mutations or amplifications have been reported in a subset of patients with *ROS1*-rearranged NSCLCs [50]. Particularly, *KRAS* mutations were observed in 8.6% of *ROS1*-positive NSCLC patients developing resistance to crizotinib or lorlatinib, with acquisition of *KRAS* mutations such as *KRSG12C* and *KRASQ61H* not present in the treatment-naïve lung cancer tissue [51]. Some *KRAS* mutations involved variants of unknown function [50]. A case report on an *ROS1*-positive patient exhibiting the evolution of acquired resistance at the diagnosis showed an *ROS1*-rearranged NSCLC with concomitant $KRAS^{G12C}$ co-mutation, with a variant allele frequency of 3.2%; the patient was initially treated with entrectinib and showed only a short duration of disease control; at progression, the patient continued treatment with entrectinib, associated with sotorasib, a small molecule inhibitor of $KRAS^{G12C}$; unfortunately, the patient did not receive the expected clinical benefit, seemingly due to an evolving $KRAS^{G12C}$ amplification [66]. Furthermore, other mutations activating MAPK pathways, such as *NF1* mutations, were observed in 7% of crizotinib-resistant *ROS1*-resistant patients, the majority being represented by mutations associated with loss of function and, in some instances, with *MET* amplification or mutation [51].

Alterations of the *MET* gene, such as amplifications (2.9%) or *MET* mutations (4.3%), were also observed in crizotinib- or lorlatinib-resistant *ROS1*-positive patients [51]. A recent study showed that *MET* amplification and overexpression were frequently observed in samples from NSCLC patients who relapsed on ROS1, ALK or RET TKIs [67]. This MET-mediated resistance may be overcome by a drug combination based on the association of targeted therapy with MET or SHP2 inhibitors [66]. These observations suggest a therapeutic strategy for *ROS1*-rearranged NSCLC patients developing TKI resistance associated with *MET* amplification/overexpression based on the combined administration of an MET or SHP2 inhibitor and an ROS1-TKI [66].

1.3.6. Histological Transformation into Small Cell Lung Cancer (SCLC) of ROS1-Rearranged NSCLC

Tumor lineage changes, such as histological transformation into SCLC or epithelial-to-mesenchymal transition, represent a mechanism of TKI resistance, not related to a specific target. SCLC transformation is an event observed in about 3–10% of TKI-resistant, EGFR-mutant NSCLCs, and is associated with an aggressive clinical evolution and poor response to therapy. There are also a few case reports of SCLC transformation in TKI-resistant, ROS1-rearranged NSCLCs. Lin et al. reported the SCLC transformation in an ROS1-rearranged NSCLC patient receiving sequential treatment with ROS1 inhibitors; in this patient, evidence of SCLC transformation was observed in all metastatic sites at autopsy, with loss of ROS1 fusion expression and inactivation of TP53 and RB1 [68]. These authors estimated a frequency of SCLC transformation in 2% of ROS1-positive NSCLCs [68]. A second study reported the SCLC transformation in an ROS1-positive NSCLC patient developing crizotinib resistance after 8 months of treatment; SCLC transformation was observed at the level of a mediastinal lymph node and was associated with retention of ROS1 rearrangement [69].

These observations support the need for tissue biopsy for patients who acquire resistance to ROS1-TKIs.

Gou et al. used models of NSCLC cell lines engineered to express *CD74-ROS1* fusion, bearing or not bearing the G2032R mutation; the expression of either *CD74-ROS1* or *CD74-ROS1-G2032R* in these cells (A549 cells) induced EMT, markedly increased the ability of invasion and migration, and clearly increased the expression of matrix metalloproteinase (MMP)-9 and of the transcription factor Twist1 [47]. The inhibition of Twist1 expression using a specific siRNA reversed the EMT induced by *CD74-ROS1 G2032R;* the concomitant

addition of crizotinib and Twist1 siRNA markedly reduced the vitality of these cells [47]. These observations suggest a role for Twist1 upregulation as a mechanism through which CD71-ROS1 *G2032R* induces EMT and drug resistance.

2. Limitations of the Clinical Studies Carried Out on ROS1-Rearranged LUADs

The clinical studies carried out in *ROS1*-positive LUADs had intrinsic limitations mainly related to the paucity of these patients. Because of this limitation, phase III clinical studies comparing ROS1-TKIs to chemotherapy or comparing two different ROS1-TKIs are lacking. In this context, the randomized open-label, multicenter, phase III trial NCT 04603807 was recently proposed, aiming to compare the safety and efficacy of entrectinib vs. crizotinib in TKI-naïve adult *ROS1*-positive patients with advanced/metastatic disease; the patients will be stratified according to the presence or not of CNS metastases and prior brain radiotherapy [70].

There are no head-to-head studies directly comparing the response of *ROS1*-positive NSCLC patients to different ROS1-TKIs. A recent study reported the results of an indirect comparison between the outcomes of *ROS1*-positive patients treated with crizotinib and those treated with entrectinib using a simulated treatment comparison [70]. The results of this analysis showed that crizotinib and entrectinib have comparable efficacy in *ROS1*-rearranged NSCLC patients [71].

Furthermore, most of the clinical studies on *ROS1*-positive NSCLC patients were based on a limited follow-up, thus rendering impossible an accurate evaluation of the impact of each of these drugs on event-free survival and on overall survival.

3. Conclusions

The ROS1 oncogene is involved in chromosome rearrangements that occur in 1–2% of NSCLCs and generate fusion proteins with various fusion partners, resulting in constitutive activation of ROS1 kinase activity. *ROS1*-rearranged NSCLCs can be specifically targeted using ROS1-TKIs.

Several pivotal trials have shown that the TKI crizotinib induces a durable response and extends the PFS of patients with *ROS1*-rearranged NSCLC, thus establishing ROS1 as a valid therapeutic target in *ROS1*-rearranged lung cancers. Although crizotinib remains the first-line reference therapy, this molecule displayed several therapeutic limitations related to (i) scarce capacity to penetrate the blood–brain barrier and (ii) the occurrence of some adverse events related to gastrointestinal disturbances and ocular toxicities.

To improve the therapeutic activity on brain metastases, new molecules better able to penetrate the blood–brain barrier, such as entrectinib and lorlatinib, have been introduced in the therapy of *ROS-1*-rearranged NSCLCs. To limit the problem of adverse events and to improve the bioavailability, some ongoing clinical trials are evaluating unecritinib, a novel derivative of crizotinib with a comparable anti-tumor activity.

A better understanding of the resistance mechanisms of *ROS1*-rearranged NSCLCs is fundamental for the rational development and evaluation of novel TKIs and of innovative therapeutic strategies. The mechanisms underlying the resistance of *ROS1*-positive NSCLCs to TKIs are heterogeneous and include the frequent development of *ROS1* point mutations under selective pressure exerted by ROS1-TKIs, or the occurrence of genetic alterations, such as co-mutations present in tumors at the moment of therapy with ROS1-TKIs. In this context, new ROS1-TKIs, such as repotrectinib and taletrectinib, have shown a potent anti-tumor activity, extended also to counter the $ROS1^{G2032R}$ fusion variant, frequently observed in patients treated with crizotinib or other TKIs. According to the results obtained in the TRIDENT-1 clinical trial, repotrectinib could represent a best-in-class option for TKI-naïve patients and a potential first-in-class option for patients with *ROS1*-positive NSCLC who have been previously treated with TKIs.

However, repotrectinib and taletrectinib are associated with gastrointestinal and neurological toxicities. NV-520, a new ROS1-TKI with selectively potent inhibition only of ROS1 and ALK, good bioavailability and an effective inhibitory activity on the $ROS1^{G2032R}$

variant, is under clinical evaluation, and in the first studies in ROS1-positive patients it did not display neurotoxic secondary effects.

It is important to note that the rarity of NSCLC patients with *ROS1* rearrangements represents a major limitation for devising randomized clinical trials and this problem is particularly relevant for rare *ROS1* fusion variants. However, in spite of this important limitation, the molecular characterization of individual *ROS1*-positive NSCLC patients may offer a unique tool for the development of therapies adapted to the molecular profile of each of these patients.

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