

# Detection of *ROS1* rearrangement in non-small cell lung cancer: current and future perspectives

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**Abstract:** *ROS1* rearrangement characterizes a small subset (1%–2%) of non-small cell lung cancer and is associated with slight/never smoking patients and adenocarcinoma histology. Identification of *ROS1* rearrangement is mandatory to permit targeted therapy with specific inhibitors, demonstrating a significantly better survival when compared with conventional chemotherapy. Detection of *ROS1* rearrangement is based on in situ (immunohistochemistry, fluorescence in situ hybridization) and extractive non-in situ assays. While fluorescence in situ hybridization still represents the gold standard in clinical trials, this technique may fail to recognize rearrangements of *ROS1* with some gene fusion partner. On the other hand, immunohistochemistry is the most cost-effective screening technique, but it seems to be characterized by low specificity. Extractive molecular assays are expensive and laborious methods, but they specifically recognize almost all *ROS1* fusions using a limited amount of mRNA even from formalin-fixed, paraffin-embedded tumor tissues. This review is a discussion on the present and futuristic diagnostic scenario of *ROS1* identification in lung cancer.

**Keywords:** lung, adenocarcinoma, ROS1, FISH, immunohistochemistry, NGS, rearrangement

## Introduction

*ROS1* is a gene encoding a receptor tyrosine kinase; it is closely related to *ALK* and *LTK* and identified in several human tumors, including non-small cell lung cancer (NSCLC).<sup>1–8</sup>

Recently, the US Food and Drug Administration approved the use of crizotinib, (Xalkori<sup>®</sup>, Pfizer Inc., New York, NY, USA) a specific small molecule inhibitor, in the therapy of *ROS1* rearranged NSCLC.<sup>9–11</sup>

Detection of *ROS1* rearrangement is then a critical step in the treatment of NSCLC and may be performed using different techniques, including immunohistochemistry (IHC), fluorescence in situ hybridization (FISH) and molecular extractive methods (e.g., reverse transcription-polymerase chain reaction [RT-PCR]).

Since ROS1 protein is absent in normal lung tissue and the prevalence of *ROS1* rearrangement in NSCLC ranges from 0.5% to 2%, IHC appears a cost-effective screening assay, thus permitting rapid results with less cost.<sup>12–17</sup>

All the different methodologies adopted to identify *ROS1* rearrangement have some advantages as well as limitations when compared to each other. In the current clinical practice, FISH represents the gold standard in light of its use in determining ROS1 positivity in clinical trials. However, several studies comparing the sensitivity and specificity of other techniques with FISH results have been published.<sup>18–36</sup>

While coordinated use of IHC and FISH testing does represent the routine practice in real-life laboratories, emerging molecular assays, including mRNA expression of

the 3' region over 5' region of *ROS1* gene (NanoString assay) and next-generation sequencing (NGS), could become an appealing and futuristic standard, permitting simultaneous tests for several “druggable” drivers using limited amount of tumor tissue or liquid biopsies.<sup>37–40</sup>

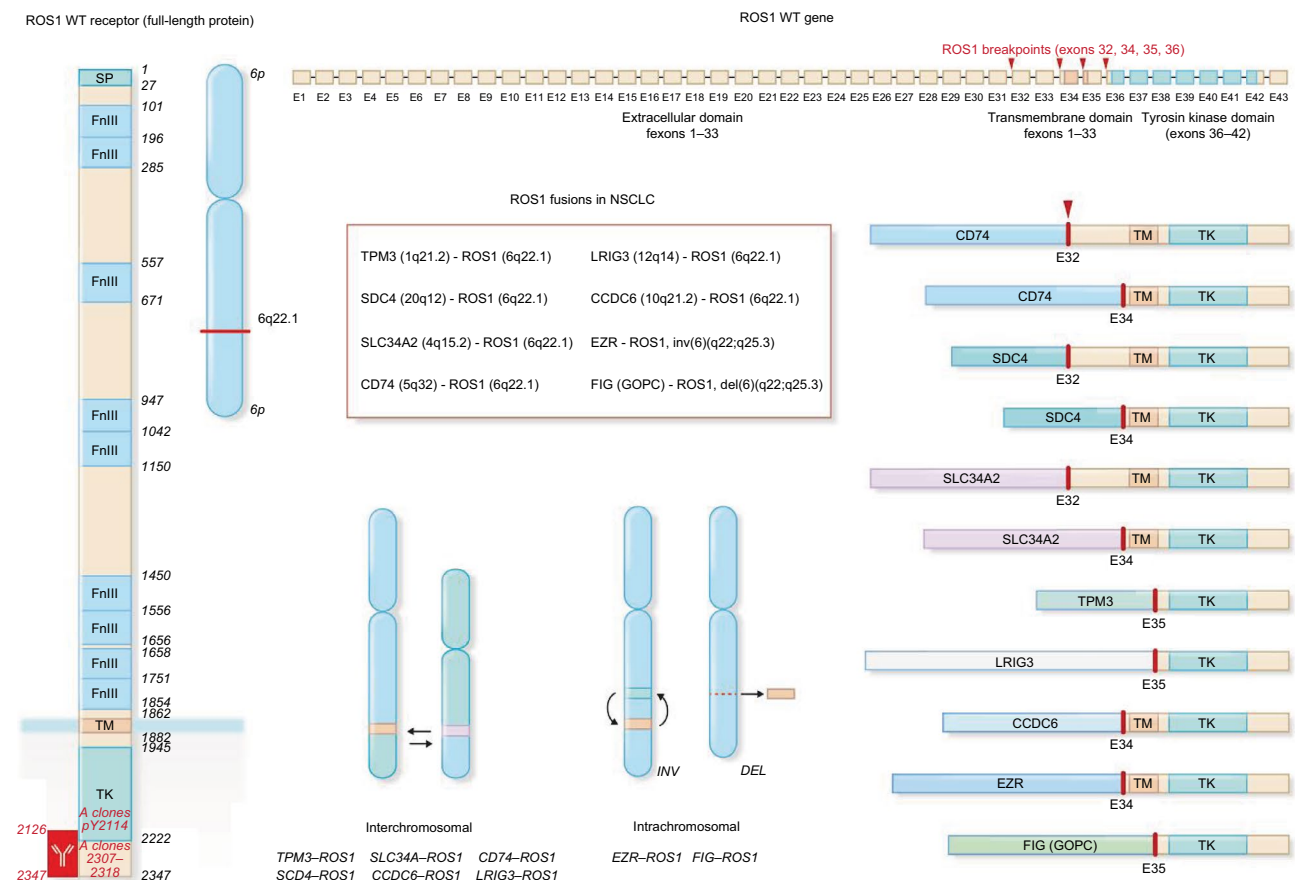
### The *ROS1* oncogene

*ROS1* gene is located at chromosome 6q22 and encodes for a receptor tyrosine kinase belonging to the insulin receptor family (Figure 1). The rearrangement of *ROS1* gene leads to a constitutively activated downstream signaling with oncogenic properties. *ROS1* rearrangement was firstly detected in a glioblastoma cell line,<sup>41</sup> but was also reported in cholangiocarcinoma, gastric adenocarcinoma, ovarian serous carcinoma, colonic adenocarcinoma, inflammatory myofibroblastic tumor, angiosarcoma, epithelioid hemangioendothelioma and spitzoid melanocytic tumors.<sup>2–7,34</sup> The fusion gene partners of *ROS1* comprise several genes, including *CD74*, *EZR*, *FIG1*, *CCD6*, *KDELRL2*, *LRI3*, *SDC4*, *SLC34A2*, *TPM3* and *TPD52L1* (Figure 1). All rearrangements involve the 3' region of the kinase domain of *ROS1* to the 5' region of the partner gene. *ROS1* rearrangements characterize about 0.5%–2% of unselected NSCLCs.

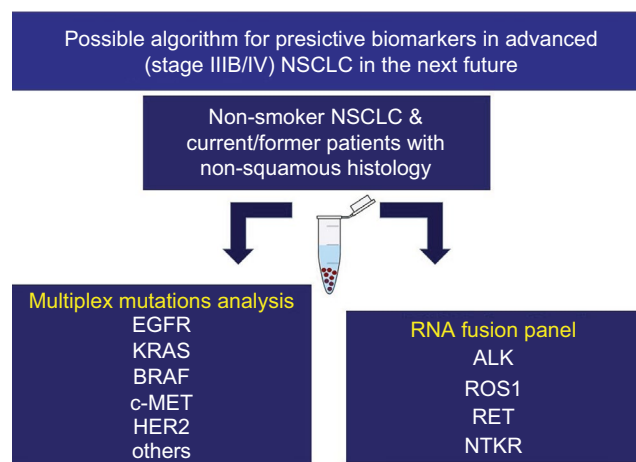
The seminal work by Rikova et al<sup>42</sup> first identified *ROS1* and *ALK* fusion genes in NSCLC using a phosphoproteomic approach, characterizing tyrosine kinase signaling in tumor cell lines and samples.

Although not every single fusion transcript has been evaluated for its oncogenic potential thus far, the preservation of the entire *ROS1* kinase domain, whatever the partner gene may be, should be sufficient to drive carcinogenesis.<sup>42</sup> Once constitutively activated, *ROS1* signaling mainly rests on extracellular regulated MAP kinase (ERK), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3K)/mechanistic target of rapamycin (mTOR) and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) intracellular pathways (Figure 2).<sup>1</sup>

Of note, *ROS1* and *ALK* are evolutionarily conserved and share >80% sequence identity within their ATP-binding sites.<sup>9,12</sup> These latter observations are strongly sustained by the clinical activity of crizotinib in both *ROS1*- and *ALK*-driven NSCLC and allow to approach them, with special regards to their inhibition by novel compounds, in a parallel way. Nevertheless, biologic differences between the two oncogenes, still not objectified at the cellular level, ostensibly



**Figure 1** *ROS1* gene is located at 6q22 and its rearrangement involves several fusion gene partners. **Abbreviations:** NSCLC, non-small cell lung cancer; TK, tyrosin kinase; TM; transmembrane; WT, wild type.



**Figure 2** A possible futuristic algorithm for detecting “druggable” oncogenic drivers in NSCLC using non-in situ methods.

**Abbreviation:** NSCLC, non-small cell lung cancer.

account for diversities observed in preclinical models and in the clinics for *ROS1*- and *ALK*-rearranged NSCLC.

The well-defined oncogenic role that *ROS1* plays in lung cancer carcinogenesis, as well as the major clinical improvement generated by the *ALK*/*MET*/*ROS1* inhibitor crizotinib (which, indeed, recently received the US Food and Drug Administration approval for *ROS1*-rearranged cases too) make the detection of *ROS1* rearrangement a crucial task in the field of molecular medicine.<sup>11</sup> Moreover, the relatively long survival of *ROS1*-positive NSCLC patients stimulates researchers and clinicians to find new therapeutic strategies to maintain an active *ROS1* deaddiction. As evidenced in *EGFR*-mutated and *ALK*-rearranged NSCLC, overcoming resistance to first-generation targeted treatments with novel molecules is achievable and dramatically fruitful, at least in significant quotes of patients.

This review is focused on the current status of the methodologies used to detect *ROS1* oncogene rearrangement in NSCLC. Presently, in situ (IHC and FISH) and extractive, non-in situ assays are available worldwide with important differences concerning advantages and limitations. In particular, a summary of the studies comparing IHC and FISH results is reported and the future scenario of *ROS1* detection in routine practice along with the advent of promising non-in situ techniques is reported. A present and futurist algorithmic approach to *ROS1* rearrangement in NSCLC is briefly discussed.

## Clinical implications of *ROS1* detection

The identification of *ROS1* rearrangement is of crucial interest in NSCLC patients due to the therapeutic consequences it generates.

Cytotoxic treatment with pemetrexed–platin doublets shows special activity in *ROS1*-positive patients,<sup>43</sup> as seen for lung tumors driven by either *ALK* or *RET* rearrangements.<sup>44,45</sup> Nevertheless, *ROS1* positivity appears to be the best biomarker of pemetrexed activity in terms of both response rate and long-term outcomes, with estimations of median progression-free survivals of around 7 months.<sup>46–48</sup>

After having demonstrated significant activity in *ALK*-driven diseases,<sup>49</sup> the tyrosine kinase inhibitor crizotinib, firstly developed as an anti-*MET* molecule, was shown to harbor relevant activity in *ROS1*-rearranged NSCLC too.<sup>9</sup> As seen for the first- and second-generation *EGFR* inhibitors gefitinib, erlotinib and afatinib, the biologic and clinical behavior of crizotinib in *ROS1*- (and *ALK*-) positive patients configures the scenario of the disruption of oncogene addiction. Being strictly dependent on *ROS1* signaling for survival, growth and progression, its pharmacologic inhibition engenders apoptosis of tumor cells, therefore translating in clinical benefit. Similar to the other oncogene-addicted models in NSCLC, tumor responses are observed in the vast majority of patients, making disease progressions rare events being often explained by diagnostic, pharmacokinetics or molecular caveats.<sup>9,10</sup> Albeit *ROS1* rearrangement does not seem to be an intrinsic prognostic factor, long-term disease control exerted by crizotinib is mostly relevant, almost doubling the one obtained in *EGFR*- and *ALK*-driven tumors undergoing specific treatment. According to the most recent updates, more than 19 months of progression-free survival are achievable with crizotinib in *ROS1*-positive cancers,<sup>50</sup> compared to the 9–12 months observed with the cited oncogenes.<sup>9,51,52</sup>

Nevertheless, as the intrinsic nature of advanced NSCLC implies, almost unequivocally, every *ROS1*-positive patient will undergo disease progression while receiving crizotinib. Already assimilated for *EGFR* and *ALK* models, beyond-progression strategies, together with local treatments in terms of oligo-progressive diseases involving or not the central nervous system,<sup>53–55</sup> are ostensibly applicable in *ROS1*-rearranged tumors too. Sooner or later anyway, the molecular escapes that cancer cells find out to slip away crizotinib inhibition need to face novel-generation inhibitors. As seen for the mentioned oncogene-addicted NSCLC, mutations occurring in *ROS1* kinase domains preclude crizotinib activity. Since their first report,<sup>56</sup> a few of them have been reported to be clinically meaningful.<sup>57</sup> Given the homology in their respective kinase domains, *ALK* and *ROS1* share a spectrum of active inhibitors beyond crizotinib (e.g., ceritinib, lorlatinib, entrectinib), developed in order to overcome the resistance to the first-generation molecule. Several clinical

trials, series and single cases reported the activity of these molecules, sometimes with regard to the precise reversion of the molecular event leading to crizotinib resistance.<sup>57</sup> Administration of such drugs after crizotinib exhaustion appears to be of major importance, engendering new responses and positive long-term outcomes, similar to what is observed in *ALK*-rearranged tumors<sup>57</sup> and in *EGFR*-mutated ones harboring the resistance mutation T790M, undergoing osimertinib treatment.<sup>58</sup>

Such evidence witnesses the dramatic relevance of the correct detection of *ROS1* rearrangements in patients whose cancers lack other genetic abnormalities. The clinical benefit originating from *ROS1* inhibition entails that not even a single *ROS1*-positive patient should be undiagnosed.

### Molecular diagnostics

The identification of *ROS1* gene rearrangement is mandatory to treat the patients with *ROS1*-positive NSCLC (Figure 3).

It is generally observed that *ROS1* gene rearrangements more often occur in younger and never/light smokers with adenocarcinoma. In a recent study on 727 lung adenocarci-

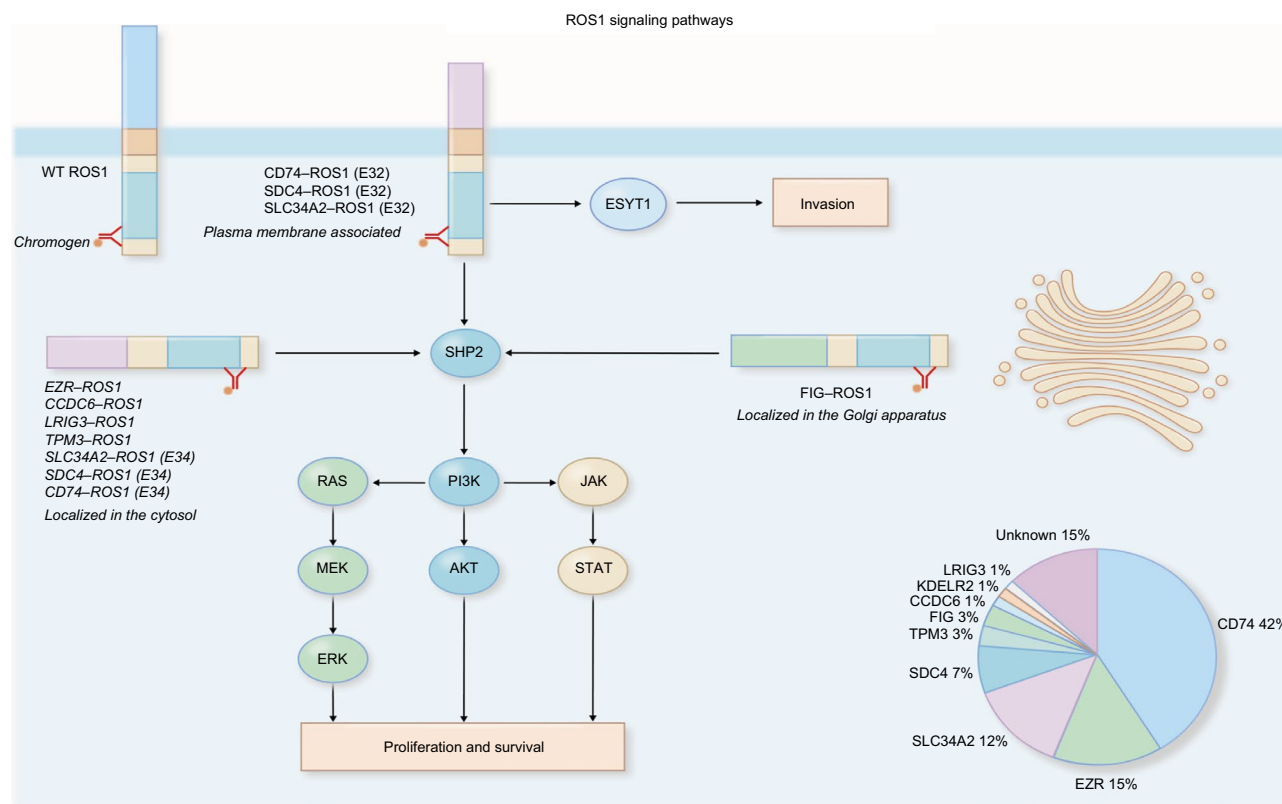
nomas from patients with stage IV disease, *ROS1* fusions were independently associated with female sex, younger age at diagnosis and absence of smoking history.<sup>59</sup> Compared with *ALK*-positive adenocarcinoma, the *ROS1* positive counterpart is more significantly associated with a peripheral location.<sup>60–62</sup>

Despite all these data, the clinicopathologic features cannot be robustly used to recognize *ROS1*-positive patients. In addition, since this genetic alteration occurs in about 0.5%–2% of all NSCLCs, a screening test is necessary in terms of cost-effectiveness.<sup>19</sup>

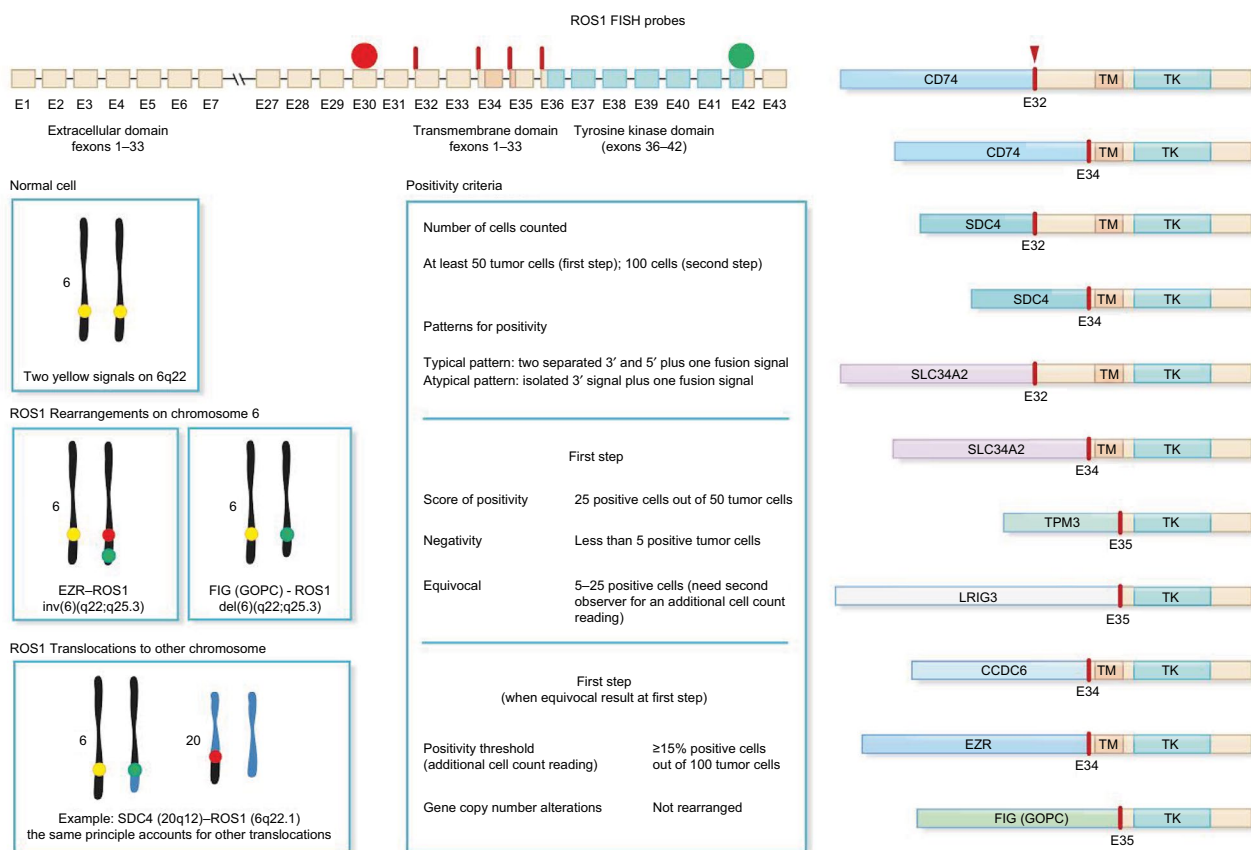
*ROS1* rearrangement is generally detected using in situ methods, namely FISH and IHC (Figure 4).

Since the clinical trials demonstrating crizotinib efficacy in *ROS1*-positive patients have substantially adopted FISH testing, this method is considered the “gold standard” for determining *ROS1* positivity. Nevertheless, several experiences have evidenced a fair-to-perfect agreement between FISH and IHC tests using the *ROS1* rabbit primary antibody D4D6.<sup>19–36</sup>

At the same time, validated extractive technologies (RT-PCR, NGS, nCounter platform) represent new promising



**Figure 3** The oncogenic activation of *ROS1* gene determines an intracytoplasmic cascade of signaling comprising ERK, PI3K, JAK/STAT. **Abbreviations:** FISH, fluorescence in situ hybridization; WT, wild type.



**Figure 4** FISH testing is still considered the gold standard technique to identify ROS1, although this method cannot discriminate the exact gene fusion involved.

**Abbreviations:** FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

tools in detecting multiple actionable fusions (*ALK*, *ROS1*, *RET*, *NTRK*) using small amount of tumor RNA.<sup>33,63–65</sup>

## FISH and IHC

FISH using a dual color “break-apart” probes approach is considered the “gold standard” in detecting *ROS1* gene rearrangement. If tumor cells are rearranged, the two ends of *ROS1* are separated and the portion containing the tyrosine kinase domain is fused with another partner to create an *ROS1* fusion gene.

The probes label the 3' (centromeric) part of the fusion breakpoint with green fluorochrome and the 5' (telomeric) part with orange fluorochrome. The criteria for *ROS1* FISH identification in NSCLC are identical to those proposed for *ALK* rearrangement, with two main patterns as follows: 1) the break-apart pattern (“conventional” pattern) with one fusion signal and two separated 3' and 5' signals and 2) an atypical pattern showing an isolated 3' signal (usually one fusion signal and one isolated 3' green signal without the corresponding 5' signal; Figure 5).<sup>19</sup> The green fluorochrome

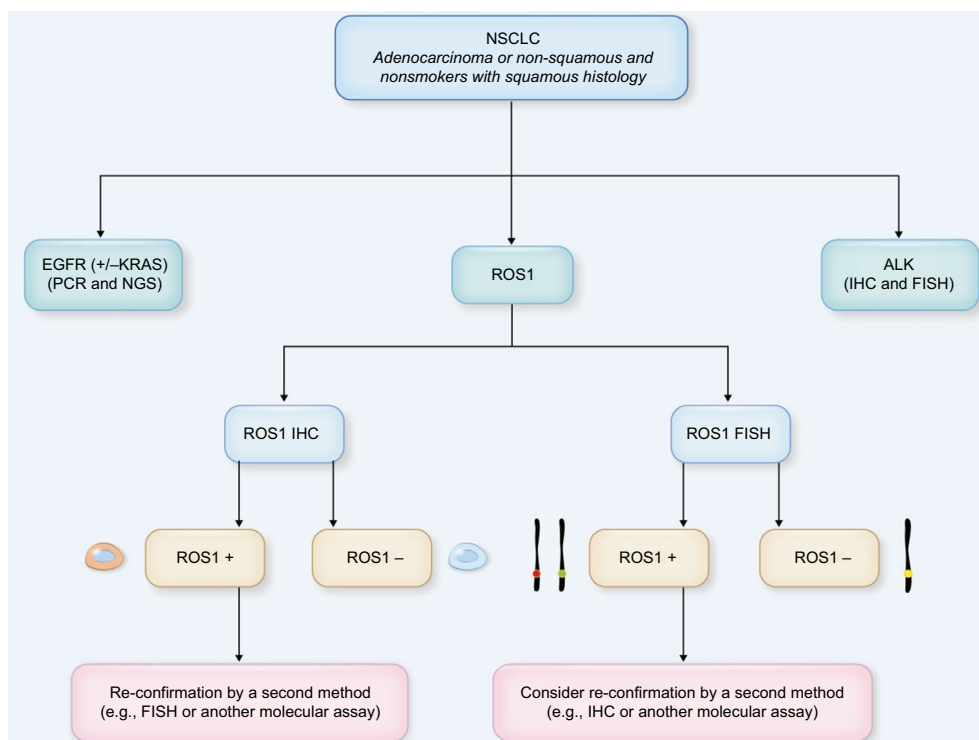
is the part containing the kinase domain of *ROS1* gene. FISH testing for *ROS1* is applicable either on biopsy or in cytologic specimens, and the cut-off of rearranged signals to quote *ROS1* positivity is based on detection of 15% or more among 50 neoplastic nuclei.<sup>66,67</sup>

Different probes have been used in literature to detect *ROS1* rearrangement (Table 1), and it is important to note that some break-apart FISH assays for *ROS1* fusion cannot detect intrachromosomal deletion, as *GOPC-ROS1* rearrangement.<sup>68</sup>

IHC is an effective screening tool to detect *ROS1*-positive NSCLC, since either sensitivity or specificity may reach over 90% when compared with FISH results (Table 1).<sup>18–36</sup> Indeed, screening of NSCLC (all nonsmokers and smokers with nonsquamous NSCLC) by IHC may prevent unnecessary FISH analysis in *ROS1*-negative cases and, thus, dramatically reduce the costs of testing.<sup>69</sup>

Data obtained from IHC are based on the use of the rabbit primary monoclonal antibody D4D6 (Cell Signaling Technology, Danvers, MA, USA) applied at different dilutions





**Figure 5** The combination of FISH and protein expression by IHC is the main methodology used in routine practice to detect ROS1 rearrangement. In this, even in the presence of IHC positivity, it is necessary to confirm ROS1 positivity by FISH.

**Abbreviations:** FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction.

(usually from 1:50 to 1:500) with different amplification kits and detection systems in various automated immune stainers (Table 1).

ROS1 protein is basically absent in normal human lung tissue, but in IHC, its expression may be observed in reactive alveolar type II pneumocytes and macrophages (Figure 6). Therefore, it is mandatory to insert a positive external control or a cell block obtained from *ROS1*-rearranged cell line, preferentially on the same slide of the tumor to be tested or on a separate slide to be included in the same batch.

Pathologists should also be aware that in bone biopsies performed in metastatic NSCLC, it is possible to observe osteoclasts revealing a moderate-to-strong granular cytoplasmic staining.

Among the different scoring systems to detect *ROS1* rearrangement by IHC, a semi-quantitative method using the expression intensity (negative [score 0], weak [1+], moderate [2+] or strong staining [3+]) was considered. Some authors adopted an h-score (multiplying the percentage of positive tumor cells and the intensity of staining from 0 to 3+, ranging from 0 to 300), with optimal threshold for ROS1 positivity defined as >100.<sup>20</sup> ROS1-rearranged tumors usually show diffuse immunohistochemical expression (>75% of tumor

cells) with a 2+/3+ staining intensity, basically corresponding to an h-score >100.

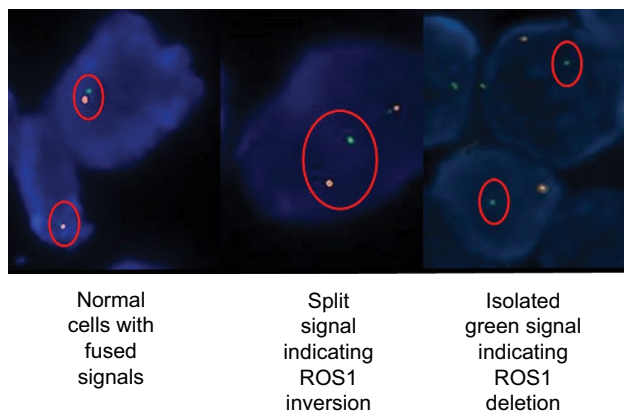
ROS1 expression at IHC level typically reveals finely granular cytoplasmic and/or membranous staining (Figure 7), although the staining pattern may depend on the different gene fusion partners.<sup>19</sup> The same IHC protocols used in formalin-fixed, paraffin-embedded (FFPE) tissue specimens may be employed on cell blocks obtained from fine-needle aspirates and pleural/pericardial effusions. Although ROS1 IHC works on conventional alcohol-fixed cytology, FISH technique is superior to IHC on nonbloody cytologic smears and cytospin slides.<sup>65,66</sup> Conversely, IHC should be preferred in FFPE small biopsy and cell block containing few tumor cells (Figure 8). Some authors suggested to consider IHC ROS1 expression only when at least 20 tumor cells are clearly identifiable in the samples to be analyzed,<sup>22</sup> but, in the absence of a clear-cut IHC cut-off, even a positive result on a few neoplastic cells should be considered appropriate and sufficient to quote a tumor as positive/rearranged.

When using clone D4D6 together with highly sensitive amplification kits, the correlation between IHC and FISH is good, although presently, IHC seems to be less specific than FISH when compared with the more robust similar diagnostic

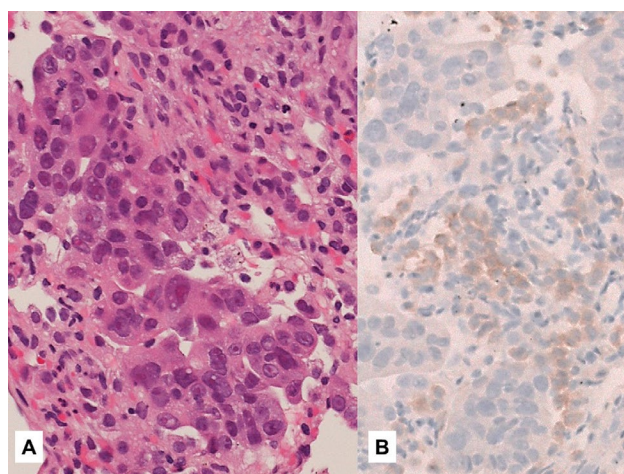
**Table 1** Summary of studies comparing identification of ROS1 rearrangement with FISH and IHC

Reference	Number of analyzed cases	Histology	Positive cases	ROSI-positive histology	Clone	Scoring method	Platform	Amplification kit	FISH	Sensitivity, %	Specificity, %
Rimkunas et al <sup>24</sup>	556	NSCLC	9	8 adeno 1 LCC	D4D6 I:100	Any positivity	NA	EnVision Flex	Abbott Molecular	100	87.5
Sholl et al <sup>22</sup>	218	Adeno	9	Adeno	D4D6 I/1000	≥2+	NA	EDTA, pH 8.0	BACPAC Resource	100	92
Mescam-Mancini et al <sup>23</sup>	121	Adeno	9	Adeno	D4D6 I:50	≥2	Ventana Benchmark	Ultrascan	Abbott Molecular	100	97
Yoshida et al <sup>21</sup>	270	Adeno	17	Adeno	D4D6 I:100	h-score ≥150	NA	EnVision Flex	Chromosome Science Lab	94	98
Cha et al <sup>31</sup>	330	NSCLC	13	Adeno	D4D6 I:50	h-score ≥100	Ventana Benchmark	Optiview	Abbott molecular	100	73
Rogers et al <sup>28</sup>	322	NSCLC	3	Adeno	D4D6 I:50	≥1+	Ventana Benchmark	Optiview	Abbott molecular	33	99
Boyle et al <sup>26</sup>	33	Adeno	6	Adeno	D4D6 I:100	h.score ≥100	Ventana Benchmark	Ultrascan	Abbott molecular	100	100
Shan et al <sup>25</sup>	60	Adeno	16	Adeno	D4D6 I:150	>10% at least 2+	NA	EDTA pH 9.0	ZytoVision	100	94
Cao et al <sup>27</sup>	183	Adeno	3	Adeno	D4D6 I:50	≥1+	Dako	EnVision Flex	Abbott Molecular	100	96.7
Viola et al <sup>20</sup>	103	Lung cancer	2	Adeno	D4D6 I:200	h-score ≥100	Dako	EnVision Flex	Cytocell	100	83
Warth et al <sup>36</sup>	1478	NSCLC	9	8 adeno 1 LCC	D4D6 I:100	Any positivity	Dako	Pretreatment at pH 8	ZytoVision	100	15
Zhou et al <sup>35</sup>	27	Adeno	27	Adeno	D4D6 I:200	3+/2+	Ventana Benchmark	Optiview	Abbott Molecular	100	94.4
Fu et al <sup>30</sup>	204	NSCLC	4	Adeno	D4D6 I:50	2+/3+ >75%	Benchmark Ventana	NA	Abbott Molecular	NA	NA
Reguart et al <sup>33</sup>	108	NSCLC	21	Adeno	D4D6 I:100	3+ or 2+ in ≥50%	Ventana Ultra	Optiview	ZytoVision	55.6	96
Su et al <sup>34</sup>	57	Mixed (lung, colon, others)	10	Adeno	D4D6 I:200	≥1+	Bond 3 Leica	NA	Abbott Molecular	100	85
Selinger et al <sup>32</sup>	278 retro 104 pros	Adeno	88 19	Adeno	D4D6 I:50	h-score	Ventana Ultra	Ultrascan	ZytoVision Abbott Molecular	100	76
Wu et al <sup>37</sup>	238	NSCLC	10	Adeno	D4D6 I:200	h-score >150 60% 2+/3+	Ventana Benchmark	Ultrascan	Abbott Molecular	100	100

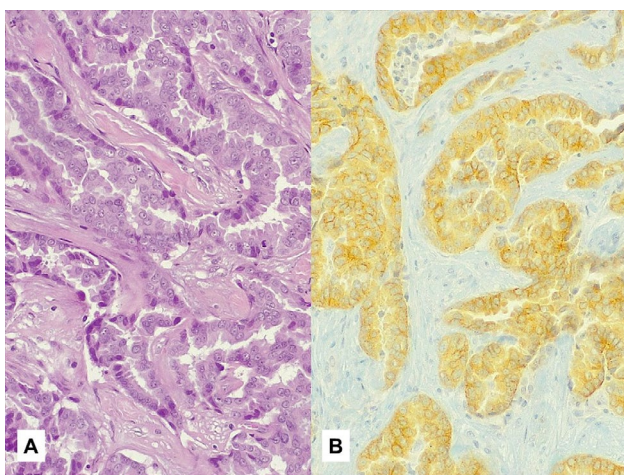
**Abbreviations:** NSCLC, non-small cell lung cancer; adeno, adenocarcinoma; LCC, large cell carcinoma; retro, retrospective; pros, prospective; NA, not available.



**Figure 6** FISH testing may lead to normal setup with fused signals (on the left) or alterations of signals with the classic split signal (in the middle) or deletion signal (on the right).



**Figure 7** A transbronchial biopsy with a lung adenocarcinoma. **Notes:** (A) Hematoxylin–eosin stain, lacking ROS1 expression, but showing positive reactive pneumocytes. (B) Immunohistochemistry, clone D4D6.



**Figure 8** A surgically resected lung adenocarcinoma. **Notes:** (A) Hematoxylin–eosin stain, strongly expressing ROS1. (B) Immunohistochemistry, clone D4D6, score 3+. Magnification:  $\times 200$ .

scenario already established for *ALK* rearrangement. Several other promising anti-ROS1 primary monoclonal and polyclonal antibodies are commercially available, although no overt data have been published so far. Another promising primary rabbit monoclonal antibody (EPMGHR2; Abcam, Cambridge, UK) is now commercially available.

### Extractive technologies

Extractive assays are based on RT-PCR and massive parallel NGS with kits including several fusion genes (*ROS1*, *ALK*, *RET*, *NTRK*).<sup>70</sup> While the sensitivity and specificity of RT-PCR are quite good, the presence of numerous *ROS1* fusion partners identified (e.g., *CD74*, *FIG*, *SLC34A2*, *TPM3*, *SDC4*, *EZR*, *LRIG3*, *GOPC*, *MSN*, *KDEL2* and *CCDC6*)<sup>1</sup> or still unknown in the absence of a clear predictive value and the availability of good-quality RNA obtained from FFPE small specimens could limit the use of RT-PCR in routine practice.

nCounter platform (NanoString assay) is a multiplex mRNA-based promising test showing high specificity and sensitivity with referenced tests in detecting *ROS1*.<sup>70</sup> This method may detect several gene fusions by direct profiling using a limited amount of RNA.

Reguart et al<sup>33</sup> recently demonstrated the identification of *ALK*, *RET* and *ROS1* fusions using nCounter technology in FFPE samples of advanced stage NSCLC. Agreement in detecting *ROS1* was 87.2% and 86% with IHC and FISH, respectively.

A combined assay involving a pan-receptor tyrosine kinase immunohistochemical cocktail of antibodies (*NTRK1*, *NTRK2*, *NTRK3*, *ROS1* and *ALK*) followed by an RNA-based anchored multiplex PCR NGS assay has been recently proposed.<sup>71</sup>

The promising results of extractive methodologies applied in clinical practice to determine *ROS1* rearrangement seem to candidate non-in situ methods as the near-standalone assays, thus limiting the role of IHC and FISH.

*ROS1* rearrangement is commonly mutually exclusive with other alterations involving driver oncogenes in lung cancer. According to this view, a recent study on 62 patients with *ROS1*-rearranged NSCLC failed to evidence concurrent *ALK* and *EGFR* gene alterations, while concomitant *KRAS* mutations were detected in two cases (3.2%). No concurrent mutations in *BRAF*, *ERBB2*, *PIK3CA*, *AKT1* or *MAP2K1* were detected. While no concomitant *ALK* and *ROS1* rearrangements were identified, exceedingly rare mutations in *EGFR* and *KRAS* were observed.<sup>72</sup>



Of note, the occurrence of *KRAS* mutation in *ROS1*-rearranged lung adenocarcinoma seems to lead to crizotinib resistance in preclinical studies.<sup>73</sup>

By contrast, a recent work evidenced frequent (36%) concomitant oncogenic driver mutations involving *EGFR* (6 cases), *KRAS* (2 cases), *PIK3CA* and *BRAF* among 25 lung adenocarcinomas showing *ROS1* positivity by IHC.<sup>74</sup>

Similarly, among 15 cases of *ROS1*-positive lung adenocarcinomas, 2 patients had *TP53* mutations, 1 patient showed an R248L mutation co-occurring with a *MAP2K1* missense mutation (K57N) and 1 had an *EGFR* mutation in exon 21 (P848L). Two out of 17 patients analyzed with NGS or by Sanger sequencing had *BRAF* mutations and 1 patient showed *c-MET* mutation (R988C). Overall, 66.7% of patients with *ROS1*-positive adenocarcinoma analyzed by NGS had further genetic aberrations.<sup>75</sup>

## ROS1 testing into a practical algorithm

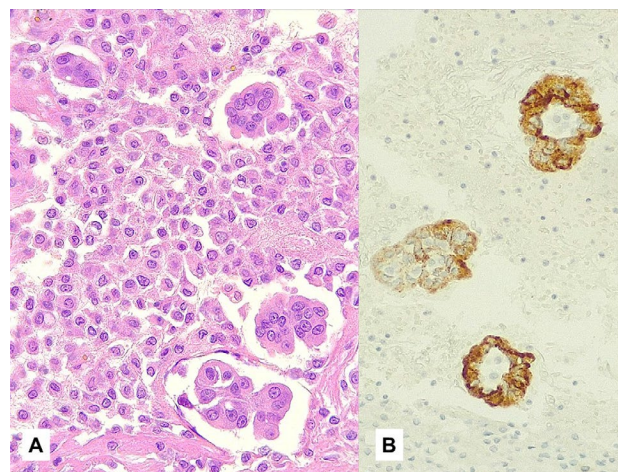
The availability of specific inhibitors of ROS1 should lead to test *ROS1* rearrangement simultaneously with *EGFR* mutation and *ALK* rearrangement in all advanced stage never/light smokers with squamous cell carcinoma and nonsquamous NSCLC.<sup>19,38,76</sup>

Optimization of tumor tissue with simultaneous preparation of extra blank sections in small biopsy and cell block is another possibility aimed at maximizing the yield of molecular testing, including *ROS1* rearrangement.<sup>37</sup>

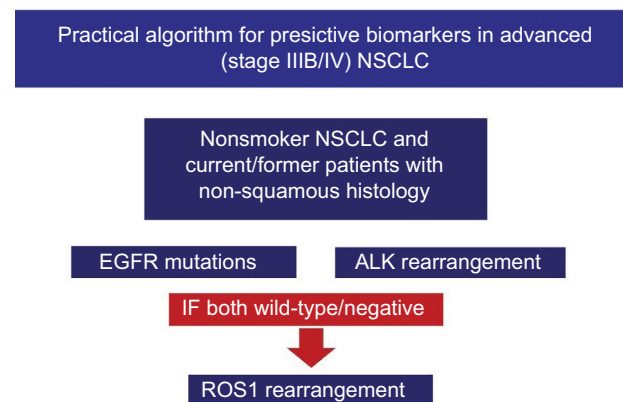
*ROS1* rearrangement is generally mutually exclusive with other genetic alterations in NSCLC, but a subset may concurrently harbor *EGFR* or *KRAS* mutations.

The role of *KRAS* mutations in an algorithm of predictive biomarkers in routine practice is still debatable. However, when considered as the most common oncogenic driver occurring in about one-third of adenocarcinomas in the Caucasian population, its value as a negative selecting biomarker precluding useless search of further rare targetable gene alterations seems at least reasonable.

According to the recent proposal by an European Board of Pathologists, a routine clinical practice algorithm based on IHC screening with further confirmation by *ROS1* break-apart FISH assay in IHC-positive and doubtful cases seems entirely appropriate (Figure 9).<sup>19</sup> In the near future, the advent of highly sensitive extractive methods using a small amount of tumor RNA in a single-tube assay to simultaneously detect several oncogenic fusions (*ALK*, *RET*, *ROS1* and *NTRK1* gene rearrangements; Figure 10) and mutations, coupled to a decrease in the cost of instruments will be implemented



**Figure 9** A cell block from a pleural effusion with a metastatic lung adenocarcinoma. **Notes:** (A) Hematoxylin-eosin stain, magnification  $\times 200$ . (B) Immunohistochemistry, clone D4D6, score 3+. Magnification  $\times 200$ .



**Figure 10** A practical algorithm commonly used in real-life routine practice. **Abbreviation:** NSCLC, non-small cell lung cancer.

even in routine practice, possibly limiting the use of IHC and FISH methods.<sup>33,77,78</sup>

## Disclosure

The authors report no conflicts of interest in this work.

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