ORIGINAL ARTICLE

Routine Clinically Detected Increased *ROS1* Transcripts Are Related With *ROS1* Expression by Immunohistochemistry and Associated With *EGFR* Mutations in Lung Adenocarcinoma

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ABSTRACT

Introduction: Translocations of the *ROS1* gene were found to drive tumorigenesis in 1% to 2% of lung adenocarcinoma. In clinical practice, *ROS1* rearrangements are often screened by immunohistochemistry (IHC) before confirmation with either fluorescence in situ hybridization or molecular techniques. This screening test leads to a non-negligible number of cases that have equivocal or positive ROS1 IHC, without *ROS1* translocation.

Methods: In this study, we have analyzed retrospectively 1021 cases of nonsquamous NSCLC having both ROS1 IHC and molecular analysis using next-generation sequencing.

Results: ROS1 IHC was negative in 938 cases (91.9%), equivocal in 65 cases (6.4%), and positive in 18 cases (1.7%). Among these 83 equivocal or positive cases, only two were ROS1 rearranged, leading to a low predictive positive value of the IHC test (2%). ROS1-positive IHC was correlated with an increased mRNA ROS1 transcripts. Moreover, we have found a mean statistically significant relationship between *ROS1* expression and *EGFR* gene mutations, suggesting a crosstalk mechanism between these oncogenic driver molecules.

Conclusion: This study demonstrates that ROS1 IHC represents true ROS1 mRNA expression, and raises the question of a potential benefit of combined targeted therapy in *EGFR*-mutated NSCLC.

Keywords: ROS1; NSCLC; Lung adenocarcinoma; EGFR; Biomarker; Immunohistochemistry

Introduction

Advances in precision oncology in the past few decades have led to the development of highly effective targeted therapies.[1] This is best exemplified in NSCLC, where mutations or gene rearrangement of several RTKs including *EGFR, ALK, MET, RET, NTRK,* and *ROS1* have been found to drive tumorigenesis.[2,3] Tyrosine kinase inhibitors (TKIs) have been subsequently developed to inhibit cancer-associated RTK and their therapy-

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resistant–mutated forms. Activating mutations in *EGFR* are of particular importance, being some of the most frequent targetable genetic alterations, present in nearly 15% of lung adenocarcinomas in the western population and up to 50% of East Asian descent.[4–7] In the past few years, the development of TKIs has extended to less common drivers such as *ROS1*, *MET*, *RET*, and *NTRK*. Although they have individually low prevalence, they may account together for 10% to 15% of all lung cancer cases, and dedicated drugs could benefit, worldwide, a large number of patients.

Among these low-prevalence oncogenic drivers, *ROS1* gene rearrangements were reported to drive tumorigenesis in approximately 1% to 2% of lung adenocarcinomas, mainly in nonsmokers.[8] ROS1, was initially identified as a homolog of the transforming sequence of the avian sarcoma RNA virus UR2.[9,10] Wild-type ROS1 consists of a 263.9-kDa, 2347 amino acid-long protein with an N-terminal extracellular domain, a single transmembrane domain, and a C-terminal intracellular kinase domain. Interestingly, ROS1 is an orphan receptor, whose ligand and normal function remain unknown. The first *ROS1* gene rearrangement was found in a glioblastoma cell line with the FIG protein.[11] Since then, a number of rearranged and mutated ROS1 variants have been found in a variety of human cancers, including glioblastoma,[12] cholangiocarcinoma,[13] inflammatory myofibroblastic tumor,[14,15] gastric cancer,[16] colorectal cancer,[17] ovarian cancer,[18] angiosarcoma,[19] epithelioid hemangioendothelioma,[19] spitz melanoma,[20] and NSCLC.[2] The kinase domain of the *ROS1* gene is fused to the aminoterminal region of various partners to form a *ROS1* fusion gene, leading to an increased expression of the fusion protein product which has an oncogenic activity. In lung adenocarcinomas, the most frequent partners are *CD74*, *EZR*, *SDC4*, and *TPM3*, which all have similar susceptibility to the targeted therapy crizotinib.[21]

In 2018, both the CAP/IASLC/AMP and the European Society for Medical Oncology guidelines were updated to add *ROS1* in the list of genes (*EGFR*, *ALK*) to be routinely tested in NSCLC.[22] In most laboratories, *ROS1* fusions are initially screened with immunohistochemistry (IHC), and equivocal and positive cases are then confirmed by fluorescence in situ hybridization (FISH) or molecular analysis, including next-generation sequencing (NGS). The antibodies recognize regular epitopes on ROS1 protein, suggesting that IHC identifies expression of ROS1 protein, independent of the cause of its activation (i.e., rearrangement). The clinical value of ROS1 IHC for ROS1 rearrangement screening is considered satisfactory in most published studies, with similar results between the two main antibody clones used in clinical practice: D4D6 (Cell Signaling) and SP384 (Ventana, Roche). Most studies report a sensitivity and specificity at approximately 90% to 100%, with an increased specificity when using H-score more than 100 and an increased sensitivity when using a low threshold of cytoplasm staining intensity (Supplementary Table 1).

In clinical practice, ROS1-overexpressing NSCLC without *ROS1* gene rearrangement are not considered for targeted therapy, and interpreting ROS1 IHC has two caveats. If the laboratory chooses a sequential testing algorithm, using IHC first as a screening tool to select the cases to be tested by the accepted standard test (i.e., FISH or molecular analysis), a very high sensitivity (almost 100%) is needed to avoid missing patients who could benefit from targeted therapy. In this objective, it is safer to select a low threshold including equivocal and positive cases. Nevertheless, high sensitivity of the IHC test will lead to a non-negligible proportion of "false positive" cases (i.e., immunopositive but non-rearranged), especially when considering the low percentage of *ROS1*-rearranged carcinoma (1%–2%). Moreover, the meaning of these "false positive" is still unclear. More specifically, it is unclear whether the positive immunostain in those cases is related to a true expression of the ROS1 protein or reflects a cross-reaction.

In this real-world study, we analyzed retrospectively the relationships between ROS1 protein expression, ROS1 mRNA transcripts, and molecular characteristics obtained from NGS data to clarify the impact of ROS1 IHC positive but not rearranged cases in lung adenocarcinomas.

Materials and Methods

Case Selection

All consecutive cases of nonsquamous, non-neuroendocrine lung carcinomas (biopsies, cytology, or resections) where ROS1 IHC and NGS were performed at the McGill University Health Center Pathology Department between October 15, 2018, and December 31, 2020, were selected for this study. Given that this study was performed to optimize clinical laboratory tests, the use of anonymized data from these cases without consent is permitted by our research ethics board.

ROS1 IHC

IHC for ROS1 was performed using the D4D6 antibody (Cell Signaling Technologies, Danvers, MA) and the EnVision FLEX, High pH DAB detection kit (Agilent-Dako, Santa Clara, CA) on the Dako platform. The protocol was a result of a pan-Canadian optimization and validation project.[23] Starting December 2019, testing was switched to the Ventana SP384 antibody (Roche-Ventana, Oro Valley, AZ) on the Dako Omnis platform to improve staining quality. On each tested slide, a positive control

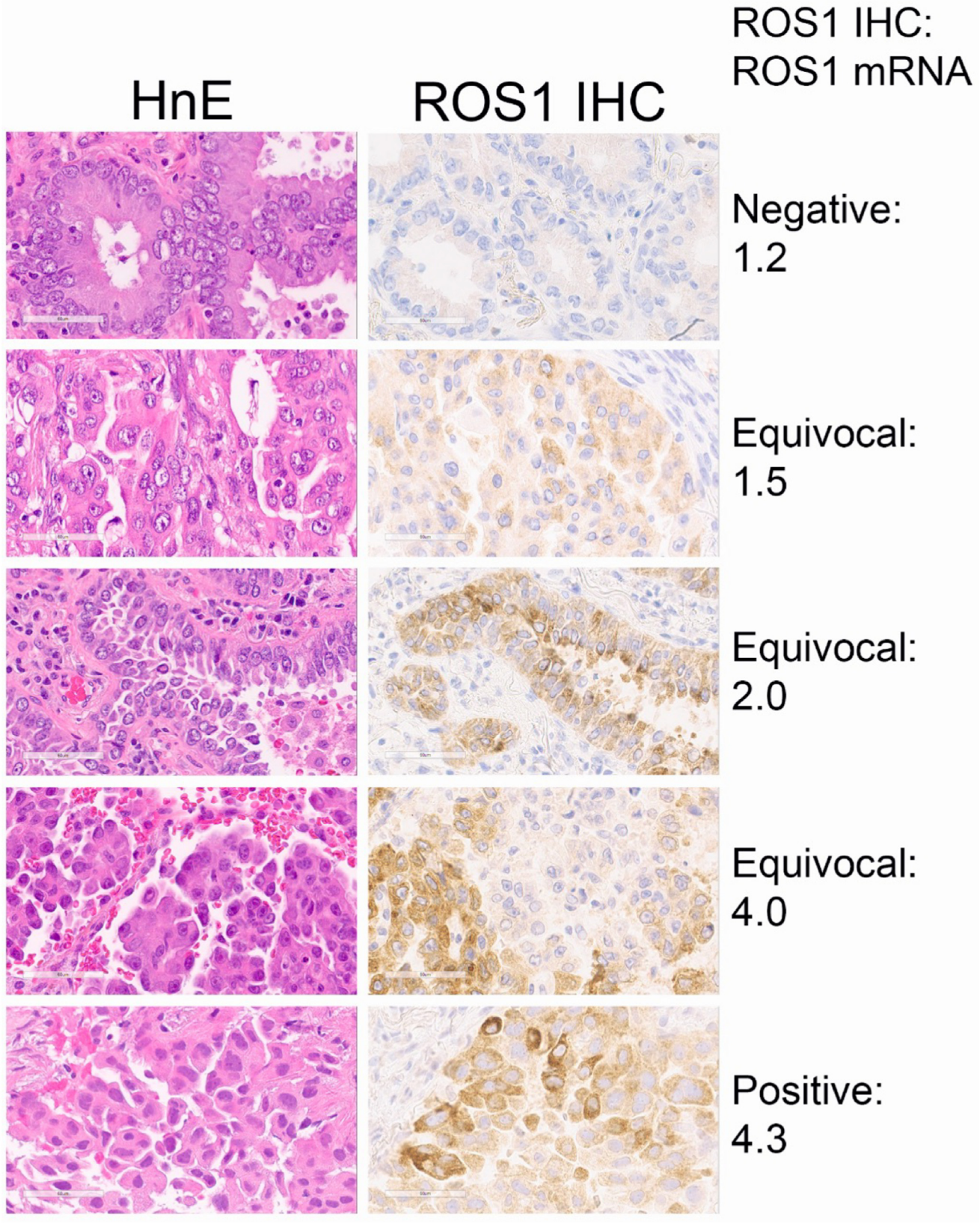

Figure 1. Representative ROS1 immunohistochemistry and mRNA levels (total ROS1 transcripts/total housekeeping gene transcripts). HnE, hematoxylin and eosin; IHC, immunohistochemistry.

(known *ROS1*-rearranged case or a pellet of the HCC78 ROS1-rearranged NSCLC cell line) was present. IHC was scored as negative (score of 0), equivocal (1+ to 2+), or positive (3+) with a semiquantitative scale (Fig. 1).

RNA and DNA Extraction

DNA and RNA were extracted from NSCLC tissues using QIAmp Allprep FFPE Tissue Kit (Qiagen, Toronto, Canada) following manufacturer's protocol (excluding deparaffinization). DNA and RNA concentrations were determined by fluorometric quantitation using Qubit 2.0 Fluorimeter with Qubit DNA dsDNA BR Assay Kit and Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Montreal, Canada) as appropriate or by qPCR.

Next-Generation Sequencing

Complementary DNA synthesis before library preparation for RNA was carried out with the AmpliSeq for Illumina Focus Panel (Illumina; San Diego, CA) by following the manufacturer's protocol. Library preparation was carried out using a total of 21 ng input DNA and RNA per sample. A maximum of 30 RNA samples were prepared per run (60 samples if both DNA and RNA analyses were required) and sequenced on MiSeq platform (2× 150). The DNA panel can identify hotspot mutations and a selected number of copy number variants. The RNA panel can identify rearrangements in the following genes: ABL1, AKT3, ALK, AXL, BRAF, EGFR, ERBB2, ERG, ETV1, ETV4, ETV5, FGFR1, FGFR2, FGFR3, MET, NTRK1, NTRK2, NTRK3, PDGFRA, PPARG, RAF1, RET, and ROS1.

All cases with positive IHC (for ROS1 or ALK) and negative NGS for translocation were countertested with Nanostring sequencing. The limit of detection of the Ampliseq panel is 0.28 ng RNA for translocations. Our coverage of ROS1 includes CD74-ROS1 (exon 6 for CD74, exons 32 and 34 for ROS1), EZR-ROS1 (exon 10 for EZR, exon 34 for ROS1), LRIG3 (exon 16 for LRIG3, exon 35 for ROS1), SDC4-ROS1 (SDC4 exons 2 and 4, ROS1 exons 32 and 34), SLC34A2-ROS1 (exons 4 and 13 for SLC34A2, exons 32, 34, and 36 for ROS1), ERC1-ROS1 (exon 11 for ERC1, exon 36 for ROS1), CLTC-ROS1 (exon 31 for CLTC, exon 35 for ROS1), HLA_A-ROS1 (exons 6 and 7 for HLA_A, exon 34 for ROS1), KIAA1598-ROS1 (exon 11 for KIAA1598, exon 36 for ROS1), MYO5A-ROS1 (exon 23 for MYO5A, exon 35 for ROS1), PPFIBP1-ROS1 (exon 9 for PPFIBP1, exon 35 for ROS1), PWWP2A-ROS1 (exon 1 for PWWP2A, exon 35 for ROS1), TPM3-ROS1 (exons 3 and 7 for TPM3, exons 35 and 36 for ROS1), ZCCHC8-ROS1 (exons 1 and 2 for ZCCHC8, exon 36 for ROS1), TFG-ROS1 (exon 4 for TFG, exon 35 for ROS1), CD74-ROS1 (exons 3 and 6 for CD74, exons 33 and 35 for ROS1), EZR-ROS1 (exon 10 for EZR, exon 35 for ROS1), MSN-ROS1 (exon 9 for MSN, exon 34 for ROS1), CCDC6-ROS1 (exon 5 for CCDC6, exon 35 for ROS1), KDELR2-ROS1 (exon 5 for KDELR2, exon 35 for ROS1), and CLIP1-ROS1 (exon 19 for CLIP1, exon 36 for ROS1). To test for cryptic ROS1 translocations, 5′/3′ imbalances for ROS1 were evaluated with exons 18 and 19 for N′ and exons 38 and 39 for 3′.

Data Processing

ROS1/control ratios were determined by adding both the 5′ and 3′ high-quality ROS1 reads and dividing them by the total high-quality control RNA reads, which includes the following five housekeeping genes: TBP, LRP1, MYC, HMBS, and ITGB7. These are the same housekeeping genes routinely used as controls for our clinical NGS panel. Only cases with adequate DNA quality, defined as having at least 95% of all targets covered by more than 250 reads, and adequate RNA quality, defined as more than 20,000 high-quality reads, were used for further analysis.

Statistical Analysis

To analyze whether ROS1-positive IHC was correlated to a true expression of the ROS1 gene, we launched a nested case-control study gathering cases and controls with at least 20,000 high-quality reads for RNA expression gene controls. The cases consisted in all tumors equivocal or positive for ROS1 by IHC (2+ or 3+, called in the following "combined positive" cases) and an approximately equal number of negative controls (79 cases, ROS1 IHC staining 0 or 1+), randomly selected from the same NGS batches and having adequate RNA quality. Chi-square and Fisher's exact tests when appropriate were used to compare categorical variables. Two-way analysis of variance test was used to compare distributions with two variables. Unpaired t test with Welch correction was used to assess relative ROS1 RNA to ROS1 IHC results.

Results

Case Characteristics

A total number of 1116 nonsquamous NSCLC cases where ROS1 IHC and NGS were performed were collected between October 15, 2018 and December 31, 2020 (Table 1). Of those, ROS1 IHC had negative staining in 1012 cases (90.7%), equivocal staining in 83 cases (7.4%), and strong positive staining in 21 cases (1.9%). In the following, the term of "combined positive" cases will represent the combination of all equivocal and positive cases (104, 9.3%). No significant difference in the proportion of positive cases was found when comparing results obtained from the two different

Table 1. ROS1 IHC Results With the Two Antibody Clones D4D6 and SP384

	Total	D4D6 Clone[a]	SP384 Clone[a]
Nonsquamous NSCLC cases tested for ROS1 IHC, n	1116	608	508
Negative score, n (%)	1012 (90.7)	562 (92.4)	450 (88.6)
Equivocal score, n (%)	83 (7.4)	38 (6.3)	45 (8.9)
Positive score, n (%)	21 (1.9)	8 (1.3)	13 (2.5)

[a]Chi-square test: $p = 0.109874$.
IHC, immunohistochemistry.

clones, D4D6 and SP384 ($p = 0.109874$; Table 1). Therefore, we combined results from both clones in further analyses.

NGS was performed with adequate DNA and RNA quality in a total of 1021 cases, including 938 ROS1 IHC-negative cases (91.9%), 65 ROS1 IHC equivocal cases (6.4%), and 18 ROS1 IHC-positive cases (1.7%). Of these cases, only two of them (0.2%) were found to harbor a *ROS1* gene translocation detected by NGS. Both were positive for ROS1 IHC (Table 2).

ROS1 IHC test had a sensitivity of 100% and a specificity of 92.1%, similarly to previously published studies (Supplementary Table 1). Within the limit of our limited series, the negative predictive value of ROS1 IHC test was 100%, whereas its positive predictive value was low, estimated at 2.4% (Table 2). In total, 81 of 1021 (7.9%) tested cases had positivity for ROS1 immunostaining (2+ equivocal or 3+ positive), without ROS1 rearrangement.

ROS1 Immunohistochemical Results Are Correlated With ROS1 mRNA Levels

To further characterize the ROS1 IHC "combined positive" cases, we launched a nested case-control study investigating the ROS1 mRNA transcript level in the group of 83 ROS1 IHC combined positive cases and in 79 control cases (or negative cases) with negative ROS1 IHC and adequate RNA quality (Supplementary Table 2). To determine whether ROS1 IHC positivity is correlated with an increased *ROS1* relative transcript expression, we compared the ROS1/control ratios of the combined positive cases with the control (i.e., negative) cases. We found a significant 2.2-fold increase in *ROS1* mRNA transcripts for the combined positive cases, as compared with negative cases ($p < 0.0001$) (Fig. 2*A*). This result was independent of the antibody clone ($p = 0.1411$), with D4D6 and SP384 having no significant difference (Fig. 2*B*). These results suggest that ROS1 positivity in IHC corresponds to a true *ROS1* gene expression, as found by an increase of its mRNA transcripts. The increase in *ROS1* mRNA was also present (×1.8 relative levels of *ROS1* mRNA compared with no rearrangement) in the two cases revealing ROS1 gene rearrangement, in concordance with an increased expression of the protein by translocation.

Staining Heterogeneity and Reactive Pneumocyte Expression Contribute to Outliers

Although the average *ROS1* mRNA levels are significantly correlated with ROS1 IHC staining (Figs. 1 and 2*A*), there are some discrepancies in individual cases (Supplementary Fig. 1*A*). In particular, several cases had high *ROS1* mRNA levels and a negative ROS1 IHC pattern, raising the question of the cell type expressing ROS1 (e.g., tumor cells, reactive pneumocytes). To uncover variables underlying these discrepancies, 15 slides corresponding to borderline or outlier cases were selected and reviewed blindly by two staff pathologists (SCB, POF). The presence of ROS1-positive reactive type II pneumocytes was identified as the most common source of discrepancies and explained in some cases the presence of elevated *ROS1* mRNA transcripts (≥1.4× transcripts) in ROS1-negative cases by IHC (six of 10 negative ROS1 IHC discrepant cases, representative example in Supplementary Fig. 1*C*). Conversely, low tumor cell content (10% to 30%) and heterogeneous tumor staining (e.g., 30% of tumor cells with 2+ staining and 70% of tumor cells with 0 or 1+ staining) were

Table 2. ROS1 IHC and ROS1 Rearrangement in 1021 Cases

	ROS1 Gene Rearrangement		
ROS1 IHC	Presence	Absence	
Combined positive	2	81	PPV: 2.4%
Negative	0	938	NPV: 100%
	Sensitivity: 100%	Specificity: 92.1%	

Note: Only cases with high-quality RNA/DNA have been selected. ROS1 combined positive cases gather all cases with equivocal (2+) and positive (3+) cases on IHC.
IHC, immunohistochemistry; NPV, negative predictive value; PPV, positive predictive value.

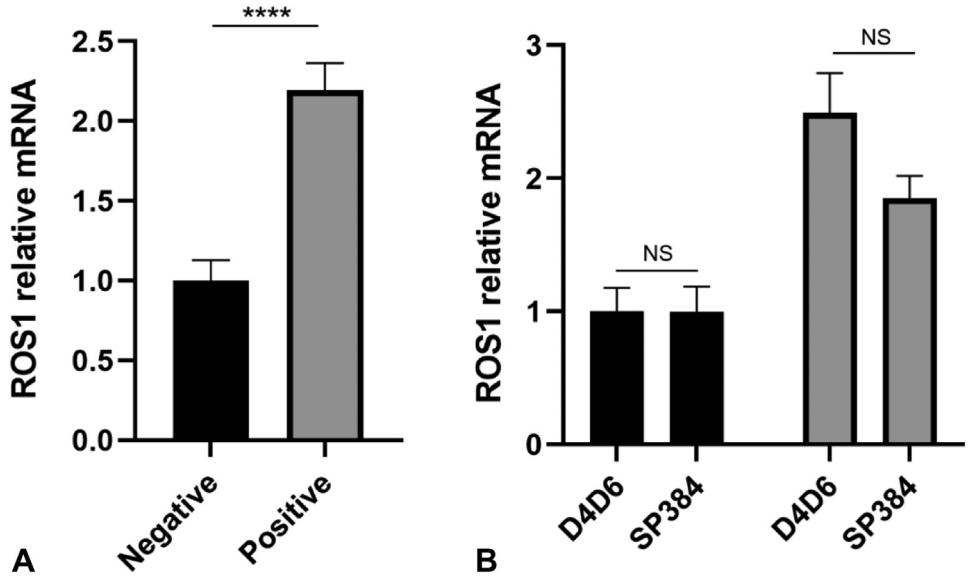

Figure 2. Relative ROS1 mRNA level correlation with ROS1 IHC results. (*A*) *ROS1* relative mRNA levels are increased in ROS1 IHC-positive cases. (*B*) There is no significant difference between ROS1 IHC antibodies clone D4D6 and SP384 (black set: negative ROS1 IHC; gray set: positive ROS1 IHC). IHC, immunohistochemistry; NS, not significant.

identified as the main variables leading to low *ROS1* mRNA transcripts in cases with ROS1 IHC interpreted as equivocal or positive (three of five equivocal/positive ROS1 IHC discrepant cases). Finally, high background led to discrepancies both in cases with elevated *ROS1* mRNA transcripts (four of 10 negative ROS1 IHC discrepant cases), where ROS1 tumor cell staining was obscured and interpreted as negative, and in cases with low *ROS1* mRNA transcripts (two of five equivocal/positive ROS1 IHC discrepant cases), where background cannot be distinguished from equivocal or positive ROS1 IHC. These discrepancies were found mainly in cytologic samples, which are particularly susceptible to having few tumor cells, numerous inflammatory cells, and more intense background. No association was found between ROS1 RNA transcripts and ROS1 IHC patterns (Supplementary Fig. 1*B*). This suggests that particular care should be taken when assessing ROS1 IHC in cytology cases.

Relationship Between ROS1 Expression and Other Molecular Alterations

To investigate the relationship between ROS1 protein expression and molecular alterations, we retrospectively analyzed ROS1 IHC and molecular alterations in the series of 1021 cases of nonsquamous NSCLC (Table 3). The percentage of case with a specific oncogenic driver within the negative or combined positive ROS1 IHC categories was calculated (Table 3). Interestingly, we found that cases with EGFR, and to a lesser extent MET

Table 3. Relationship Between Molecular Alterations and ROS1 Protein Expression

Molecular Alteration	Frequency of Alteration (%)	ROS1 Negative Number (%)	ROS1 Combined Positive Number (%)	*p* Value
	Total Number of Cases	938	83	
KRAS	421 (41.2)	397 (42.3)	24 (28.9)	0.6143
None	298 (29.2)	284 (30.2)	14 (16.9)	N/A
EGFR	138 (13.5)	110 (11.7)	28 (33.7)	0.0001
BRAF	40 (3.9)	40 (4.3)	0 (0)	0.3876
Others	40 (3.9)	38 (4.0)	2 (2.4)	1.0000
MET	28 (2.7)	23 (2.4)	5 (6.0)	0.0162
PIK3CA	20 (2.0)	19 (2.4)	1 (1.2)	1.0000
ALK	17 (1.7)	14 (1.4)	3 (3.6)	0.0555
ERBB2	11 (1.1)	9 (0.9)	2 (2.4)	0.1052
RET	6 (0.6)	4 (0.4)	2 (2.4)	0.0345[a]
ROS1	2 (0.2)	0 (0)	2 (2.4)	0.0027[a]

Note: ROS1-positive expression gathered combined positive and ROS1 negative cases.
[a]These cases have significant *p* values, but the number of positive cases is limited and interpretation is uncertain.
N/A, not applicable.

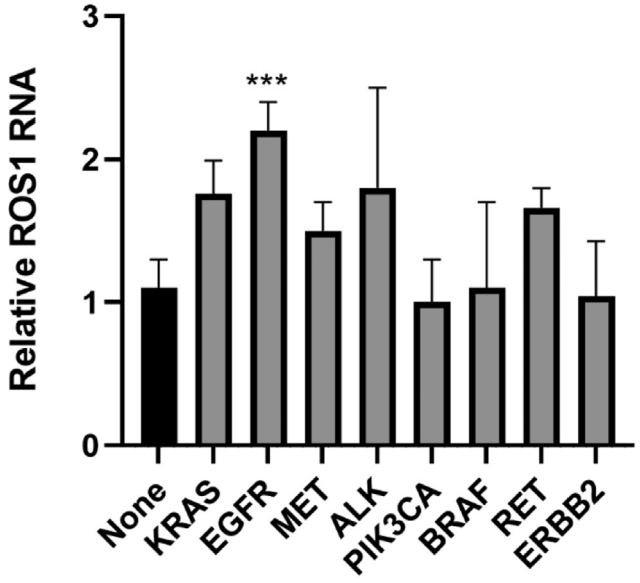

Figure 3. Relative ROS1 mRNA levels by oncogenic driver. $^{**}p < 01$. $^{***}p < 0.001$.

driver mutations, were significantly overrepresented in the ROS1 IHC-positive category compared with cases without an identified driver mutation ($p = 0.0001$ and $p = 0.0162$, respectively; Table 3). In contrast, cases with KRAS, BRAF, and PI3KCA mutations had distribution within ROS1 IHC–negative and –positive categories similar to cases with no driver mutation ($p = 0.6143$, $p = 0.3876$, $p = 1.0000$; Table 3). The remainder of the cases with an oncogenic driver, although not revealing any significant difference with cases without an oncogenic driver, is in insufficient numbers to be properly assessed. Interestingly, the association between EGFR-altered cases and ROS1 IHC positivity is reflected in the ROS1 relative RNA levels, which are significantly elevated in EGFR-altered cases compared with case with no major oncogenic drivers (2.2 ± 0.2 versus 1.1 ± 0.2, respectively, $p < 0.001$; Fig. 3). Conversely, MET-altered cases only revealed a trend toward elevated ROS1-relative RNA levels compared with cases with no major oncogenic drivers (1.5 ± 0.2 versus 1.1 ± 0.2, respectively; Fig. 3).

Discussion

Despite the great sensitivity (100%) and fair specificity (91%) of the ROS1 IHC test, the low prevalence of ROS1 rearrangement in our series leads to a high number of "false positive cases" (positive or equivocal immunostaining in ROS1 non-rearranged cases), resulting in a very low positive predictive value (approximately 2%). The surprisingly low prevalence of ROS1 rearrangement (0.2% versus 0.3% to 11.5%, Table 1; reviews by Bubendorf et al.[8]) may result, at least in part, from differences in our studied population. ROS1-rearranged cases have been reported to be particularly overrepresented in the female nonsmoker population[24] and associated with high-stage cancer.[25] Therefore, one of the hypotheses for our low proportion of ROS1-rearranged cases would be a difference in smoker demographics in our cohort. Although the current prevalence of smokers in Quebec (18%) is only slightly higher to that of the rest of Canada (16%) but lower than other countries, including United States (25%) and Japan (21%),[26,27] it was for a long time higher than elsewhere in Canada and United States, at 64.2% to 54.9% from 1965 to 1975 (closest being Atlantic provinces at 53.4%–46.8%),[28] and 33% to 36% from 1985 to 1999 (closer to Manitoba, 35.3%–30.1%).[29] Thus, the lower incidence of nonsmoker-associated genetic alterations in NSCLC is likely a consequence of the smoking demographics in the past 5 to 7 decades rather than a reflection of the current smoking demographics in Quebec. Indeed, already in 1998, the incidence of lung adenocarcinoma in Quebec was higher than for the other Canadian provinces[30], and remains to date highest in Canada with other Atlantic provinces.[31] Moreover, the hypothesis of different exposure in our population is consistent with a different oncogenic driver distribution in our cohort, revealing a higher proportion of *KRAS* mutations (40.7% versus 25%[1]) and a lower proportion of other alterations usually associated with nonsmokers, such as *ALK* (1.7% versus 2%–7%[1]).[32] Second, it has been described that *ROS1*-rearranged lung cancers are often diagnosed at an advanced stage (III or IV), with frequent central nervous system metastases.[24,25] Because our center is one of the major thoracic surgical centers in the Quebec province, our population is biased with a higher proportion of resectable disease, which may explain in part the low frequency of *ROS1* molecular alterations. Finally, our study relies on the NGS focus panel test, considered as the accepted standard, to determine *ROS1* rearrangements, after a screening by IHC. A small focused NGS panel has the great advantage to test simultaneously, using a small amount of formalin-fixed, paraffin-embedded material, the main known targetable molecular alterations in NSCLC, including rare oncogenic fusions involving the *ALK*, *RET*, *ROS1*, and *NTRK1* genes. A limitation is that the test may miss rare events or unpublished partners. In those cases, finding a 5′/3′ imbalance may suggest a possible fusion. We tested for and did not identify ROS1 5′/3′ imbalances and tested for a large number of ROS1 translocations (see the Methods section). Moreover, cases with positive ROS1 IHC and negative NGS for ROS1 translocation were counterscreened with Nanostring sequencing, which did not identify any ROS1 translocations. Altogether, it is unlikely that our molecular testing missed a significant number of ROS1

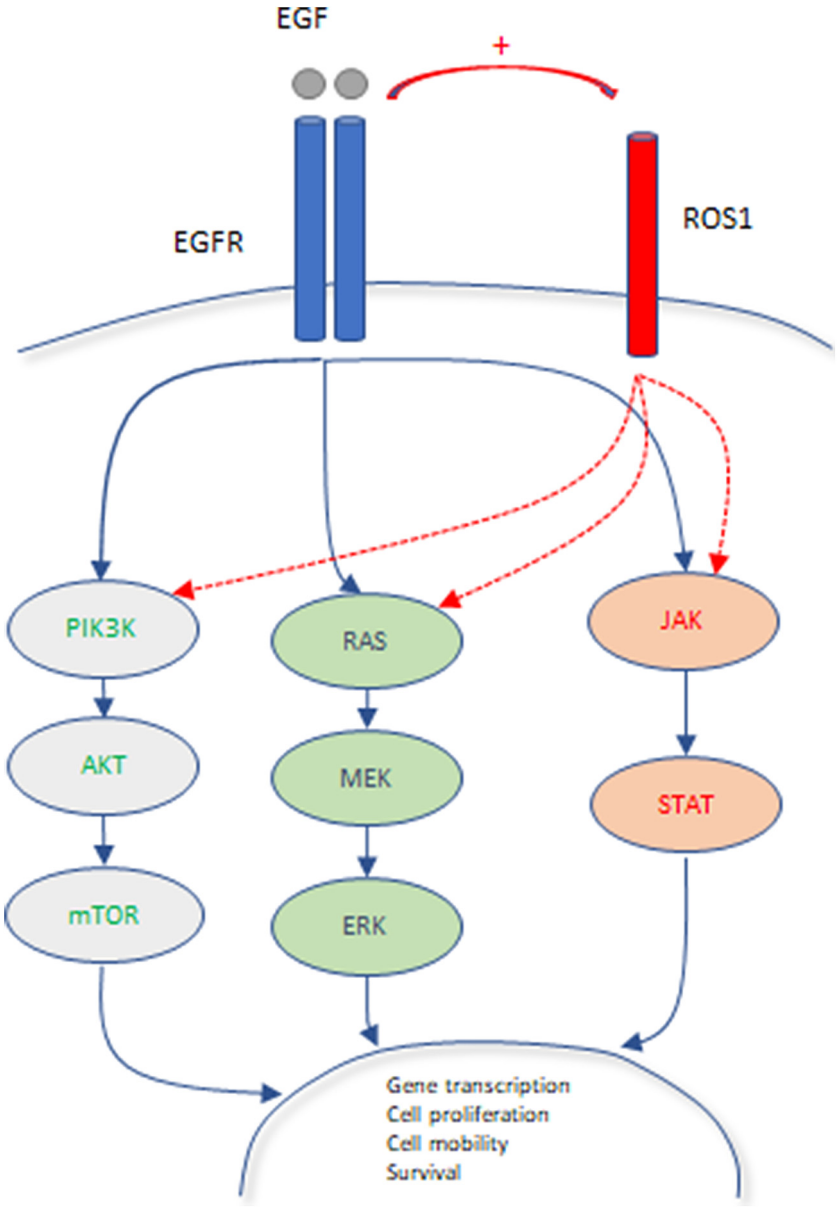

Figure 4. EGFR and ROS1 downstream pathways are not specific and interact with each other.

translocations. Nevertheless, most of the previously published series relied on reverse transcriptase-polymerase chain reaction or the FISH technique. The FISH technique has the advantage to rely on a Break-Apart Probe Kit, able to detect a break in the *ROS1* gene, independent of its involved partner gene (known or unknown). However, FISH is labor intensive and sometimes difficult to interpret. In addition, similar to other single-gene tests, its use will be limited in the future as the list of genes to be tested grows.

To our knowledge, this is the first study revealing in a "real world" clinical setting that ROS1 protein expression as detected by IHC correlates with ROS1 RNA transcripts. This result is also concordant with previous reports that *ROS1* transcripts are elevated in murine models[33–35] and in more than 30% of human lung adenocarcinoma cases, independent of ROS1 rearrangements.[36–40] Altogether, this ROS1 expression, independent of *ROS1* rearrangement, in part explains the lack of specificity and low positive predictive value of ROS1 IHC as a surrogate test for assessing *ROS1* gene rearrangement. It is well known that positive type II pneumocyte hyperplasia and alveolar macrophages should not be considered when interpreting ROS1 immunostaining.[41] Nonetheless, and especially in small samples, the distinction between pneumocyte hyperplasia and lepidic spread is not always straightforward and may in part explain the lack of correlation between ROS1 IHC and ROS1 mRNA in cytologic samples. In our

study, only tumor cells were considered, and our results are concordant with the hypothesis of a true expression of ROS1 gene, in a subset of non-rearranged lung adenocarcinomas.

Only cases having *ROS1* rearrangement are targetable by crizotinib, whereas ROS1 non-rearranged expression is not considered. It remains unclear, however, whether an increased *ROS1* expression may have, by itself, an oncogenic effect. Interrogating secondary messengers downstream of ROS1, such as STAT, MAPK, and AKT, in these cases could help resolve this question but is beyond the aim of this study. Within the limit of our small numbers, the level of *ROS1* mRNA expression of our two *ROS1*-rearranged cases was not different from other ROS1 IHC-positive cases. In addition, ROS1 is expressed in normal tissue, including the testis and lung.[42,43] The ROS1 increased expression in type II pneumocyte hyperplasia suggesting that ROS1 expression could be induced by various non-neoplastic situations, including regeneration of alveolar epithelium after injury. Nevertheless, apart from rearrangement, other biological mechanisms regulating ROS1 expression are still unknown.

We have also found a significant relationship between *EGFR* mutation and *ROS1* expression at both transcript and protein levels. EGFR and ROS1 are two tyrosine kinase receptors that act on the same major signaling pathways, which fuel cell proliferation and survival. The pathways include the PI3 kinase-mTOR pathway and the RAS pathway (Fig. 4). This crosstalk may explain the fact that driving molecular alterations are mutually exclusive, especially if the oncogenes participate in the same signal transduction pathway.[44] Furthermore, this redundancy may explain acquired resistance to TKIs, including additional mutation or copy gain of a downstream factor. It has been found previously that bypass signaling from EGFR plays a role in crizotinib resistance in ROS1-rearranged NSCLC malignancies,[45] suggesting a possible benefit of ROS1 and EGFR co-inhibition. Similarly, a mechanism of resistance to the EGFR inhibitor osimertinib is related to ROS1 alterations.[46,47] In the present study, we report that part of the EGFR-mutated NSCLC (81%) has an increased expression of ROS1 in the tumor cells (>1.5 ratio of ROS1 transcripts/housekeeping genes), raising the question of its role in anti-EGFR resistance or benefit of combined targeted therapies. Although cases with MET alterations also had a significant association with positive ROS1 IHC, we were not able to confirm a statistically meaningful association at the molecular level, possibly owing to a markedly lower number of MET-altered cases. Nevertheless, it would be interesting to explore this association in further studies with a larger number of MET-altered cases.

Finally, we have revealed in this study that approximately 8% of nonsquamous NSCLC has an overexpression of ROS1 protein, detected by IHC. This expression is present also at the mRNA level, independent of *ROS1* gene rearrangement. Compellingly, we report that ROS1 expression is associated with other driver mutations, especially with EGFR mutation. This suggests that ROS1 might be increased in response to specific oncogenic driver activation (including EGFR mutation) and might have a broader role in NSCLC, independent of ROS1 rearrangements. Although the exact molecular mechanism of this correlation is unclear, it supports a crosstalk between ROS1 and EGFR signaling in NSCLC and raises the question of a potential synergistic therapeutic benefit of combined targeted therapies, inhibiting ROS1 in addition to EGFR in EGFR-mutated NSCLC.

Credit Authorship Contribution Statement

Karl Grenier: Conceptualization, Data acquisition and curation, Methodology, Formal analysis, Writing—original draft, Writing—review and editing.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *JTO Clinical and Research Reports* at www.jtocrr.org and at https://doi.org/10.1016/j.jtocrr.2023.100530.

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