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Assessment of a New ROS1 Immunohistochemistry Clone (SP384) for the Identification of *ROS1* Rearrangements in Non-Small Cell Lung Carcinoma Patients: the ROSING Study

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Running title: ROS1 Immunohistochemistry with clone SP384

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Conflict of interest statement

Grupo HM Hospitales has received honoraria from Roche, Pfizer, Thermo Fisher Scientific, Bristol-Myers Squibb and Abbvie.

E. Conde has received honoraria from Pfizer and Roche, and travel expenses from Roche, Merck Sharp & Dohme and Pfizer.

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E. Felip has received honoraria from Roche, Abbvie, AstraZeneca, Bergenbio, Blueprint Medicines, Boehringer Ingelheim, Bristol-Myers Squibb, Celgene, Eli

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Introduction

The *ROS1* gene rearrangement has become an important biomarker in non-small cell lung carcinomas (NSCLCs). The CAP/IASLC/AMP testing guidelines support the use of *ROS1* immunohistochemistry (IHC) as a screening test, followed by confirmation with fluorescence *in situ* hybridization (FISH) or a molecular test in all positive results. We have evaluated a novel anti-*ROS1* IHC antibody (SP384) in a large multicenter series to obtain real-world data.

Methods

Forty-three *ROS1* FISH-positive and 193 *ROS1* FISH-negative NSCLC samples were studied. All specimens were screened by two antibodies (clone D4D6 from Cell Signaling Technology and clone SP384 from Ventana) and the different interpretation criteria were compared with break-apart FISH (Vysis). FISH-positive samples were also analyzed with next-generation sequencing (OncoPrint™ Dx, Thermo Fisher Scientific).

Results

An H-score of ≥ 150 or the presence of $\geq 70\%$ of $\geq 2+$ stained tumor cells by SP384 clone were the optimal cut-off value (both with 93% sensitivity and 100% specificity). The D4D6 clone showed similar results with an H-score of ≥ 100 (91% sensitivity and 100% specificity). *ROS1* expression in normal lung was more frequent using the SP384 clone ($P < 0.0001$). *EZR-ROS1* variant was associated with membranous staining and an isolated green signal FISH pattern ($P = 0.001$ and $P = 0.017$, respectively).

Conclusions

The new SP384 ROS1 IHC clone showed excellent sensitivity without compromising specificity, so it is another excellent analytical option for the proposed testing algorithm.

Keywords: ROS1, immunohistochemistry, FISH, next-generation sequencing, lung carcinoma

ACCEPTED MANUSCRIPT

Introduction

The *c-ros* oncogene 1 (*ROS1*) gene rearrangement has now become an important predictive biomarker for targeted tyrosine kinase inhibitors (TKIs) in non-small cell lung carcinomas (NSCLCs). In March 2016, crizotinib was approved by the U.S. Food and Drug Administration (FDA) for the treatment of patients with advanced *ROS1*-rearranged NSCLCs without the requirement of the use of an FDA-approved companion diagnostic test.¹ Soon afterwards, the drug was approved by the European Medicines Agency (EMA), with the statement that “an accurate and validated *ROS1* assay is necessary for the selection of patients”.² Based on the excellent results of the crizotinib clinical trials and the development of other *ROS1* inhibitors with consistent efficacy results in this patient population, the importance of accurately identifying *ROS1*-positive lung cancer has never been greater.³⁻⁸

Regarding the detection of *ROS1* rearrangements, the recently updated CAP/IASLC/AMP molecular testing guidelines for the selection of lung cancer patients support the use of *ROS1* immunohistochemistry (IHC) as a screening test, followed by fluorescence *in situ* hybridization (FISH) (traditionally considered as the “gold standard” method)⁹ or a molecular test (i.e. reverse transcription PCR [RT-PCR] or next-generation sequencing [NGS]) in all cases with positive IHC results.¹⁰ To date, only one anti-*ROS1* IHC clone has been commercially available, and there is no universally accepted criterion for the interpretation of *ROS1* IHC.^{10,11}

This situation prompted us to evaluate a novel anti-*ROS1* IHC antibody in a large multicenter series to obtain real-world data for the proposed *ROS1* testing algorithm.

Material and methods

Study design and tumor samples

The flow diagram is depicted in Figure 1. Fifty-five *ROS1*-positive samples from patients with NSCLCs, initially tested as part of routine clinical care in 23 different institutions, were used for this study (also known as ROSING, ROS Immunohistochemistry & Next-Generation sequencing). To confirm the *ROS1*-positive status, FISH analysis (the “gold standard” method) was performed at the referral institution (i.e. University Hospital HM Sanchinarro). Only cases with enough tissue available (i.e. a minimum of 50 tumor cells, as per the FISH test requirements) and *ROS1* FISH-confirmed positivity were included. In addition, 193 consecutive *ROS1* FISH-negative samples from NSCLCs tested at 14 of the participating institutions as part of routine clinical care were included as negative controls. The material available for all tumors was formalin-fixed and paraffin-embedded (FFPE). The specifics of formalin-fixation were unknown. All cases were reviewed by two pathologists (E.C. and F.L-R.). In addition to FISH, all specimens (negative and positive) were independently screened for *ROS1* expression by two IHC antibodies. *ROS1* FISH-positive cases were also tested by NGS. Clinical data from patients with *ROS1* FISH-positive tumors were collected. The Institutional Ethics Committee at Grupo HM Hospitales reviewed and approved this study. Each referring institution regulated the need for additional specific consent, as *ROS1* testing is part of routine clinical care. Clinical data were retrieved from the patient clinical records.

FISH for *ROS1* rearrangements

FISH was repeated centrally on unstained four μm -thick FFPE tumor tissue sections from all positive and negative cases. The Vysis 6q22 *ROS1* Break Apart FISH Probe Kit (Abbott Molecular, IL, USA) was used, following the manufacturer's instructions as previously described.¹² The *ROS1* FISH assay was independently captured and scored with the automated BioView Duet scanning system (BioView, Rehovot, Israel) by an experienced lung pathologist (E.C.) and molecular biologist (S.H.). The interpretation criteria strictly followed very recommended criteria.¹¹ A minimum of 50 tumor nuclei were counted. *ROS1* FISH-positive cases were defined as more than 25 (50%) break-apart (BA) signals (separated by ≥ 1 signal diameter) or an isolated green signal (IGS) in tumor cells. *ROS1* FISH-negative samples were defined as less than 5 (10%) BA or IGS cells. *ROS1* FISH cases were considered borderline if 5-25 (10-50%) cells were positive. In the case of borderline results, a second reader evaluated the slide, added cell count readings from the already automatically captured images, and a percentage was calculated out of 100 cells. If the positive cells percentage was lower than 15%, the sample was considered negative. If the positive cells percentage was higher or equal to 15%, the sample was considered positive.¹¹

IHC for *ROS1* expression

Automated IHC for *ROS1* expression was performed for all cases on a BenchMark ULTRA staining instrument (Ventana Medical Systems, Tucson, AZ, USA). FFPE tumor tissues were sectioned at a thickness of four μm and stained with two different anti-*ROS1* clones: SP384 (Ventana Medical Systems) and D4D6 (Cell Signaling Technology, Danvers, MA, USA). Briefly, the VENTANA

ROS1 (SP384) ready-to-use Rabbit Monoclonal Primary Antibody was applied with the OptiView DAB IHC Detection Kit and OptiView Amplification Kit, following the manufacturer's instructions. The D4D6 clone was used at a 1:50 dilution. Detection was performed with the same OptiView detection-amplification kit. FISH-validated *ROS1*-positive external controls were included in all the slides.

The slides were reviewed by two pathologists (E.C. and F.L-R.) blinded to the FISH results. When a discrepancy was observed, the final result was consensuated. Staining intensity was defined as follows: strong cytoplasmic staining (3+), clearly visible using a X2 or X4 objective; moderate staining (2+), requiring a X10 or X20 objective; weak staining (1+), involving a X40 objective; and negative staining (0), absence of expression.¹² The percentages of tumor cells with each staining intensity were also evaluated. Membrane staining was recorded when observed. ROS1 IHC staining results with both clones were finally interpreted using four previously described criteria: 1) an H-score with a threshold for ROS1 positivity defined as ≥ 100 ^{11,13}; 2) an H-score cut-off of ≥ 150 ^{11,14}; 3) an intensity criterion with cut-off of positivity defined as $\geq 2+$ in any tumor cells^{11,15,16}; and 4) a positive status based on $\geq 2+$ intensity in $\geq 30\%$ of total tumor cells.¹⁷ Intratumoral staining heterogeneity was also evaluated. It was defined as the presence of 0 or 1+ staining areas in positive cases.¹⁶ The positivity of normal lung tissue was recorded when it was present on the sections.

NGS for *ROS1* rearrangements

For each FFPE tumor sample, five μm thickness freshly cut sections were collected for nucleic acid extraction: five sections for surgical specimens and 12 sections for small biopsies. The first and last sections were stained with H&E and reviewed by two pathologists (E.C. and F.L.-R.) to assess the percentage of tumor cells. RNA extraction was performed with RecoverAll™ Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's instructions. RNA was then purified and concentrated using GeneJET RNA cleanup and concentration micro kit (Thermo Fisher Scientific).

The Oncomine™ Dx Target Test panel (Thermo Fisher Scientific) was the selected approach because it requires very little input RNA and it was the first FDA-approved NGS test. The protocol for the NGS analysis followed the manufacturer's instructions, and a minimum of 5000 mapped fusion panel reads was required for *ROS1* fusion analysis. Consent was only granted for the RNA part of the procedure.

Statistical analysis

Based on all the valid data obtained, we performed a descriptive analysis of all the variables of interest. The test used for comparison of categorical variables was Pearson's χ^2 test (frequency < 5 , Fisher). For comparison of means we used the Mann-Whitney test. The sensitivity and specificity of both *ROS1* IHC clones *versus* FISH were obtained. Receiver Operating Characteristics (ROC) curves were used to determine the optimal cut-off value that discriminates between patients with *ROS1*-rearranged and -non-rearranged tumors. We also analyzed the correlation between the different *ROS1* fusion

variants and clinicopathologic features. Survival analysis was performed using the Kaplan-Meier method via the log-rank test and Cox Regression. All analyses were done in Stata 15.1, were two-sided, and P-values < 0.05 indicated statistical significance.

Results

ROS1 rearrangements assessed by FISH

Of the 55 *ROS1*-positive lung carcinoma specimens, four cases were excluded for lack of sufficient tumor tissue and eight samples due to FISH results being not evaluable (i.e. no or weak hybridization signals). Of the 193 *ROS1*-negative NSCLCs, all specimens were included in the study (Figure 1). Among the 43 *ROS1* FISH-positive cases analyzed, 27 tumors (62.8%) had a BA pattern, and 16 (37.2%) showed an IGS pattern. The total number of tumor cells analyzed was 50 in all cases (97.7%), except in one specimen (2.3%) (a case with initial borderline results in which 100 nuclei had to be scored). In *ROS1* FISH-negative cases, the mean percentage of positive tumor cells was 0.4% (median 0%; range 0 to 10%). In *ROS1* FISH-positive tumors, the mean percentage of positive cells was 82.3% (median 86%; range 49 to 98%). There were no significant differences in the percentages of positive cells between the two patterns of positivity.

ROS1 immunoreactivity by IHC

The IHC results using the previously published criteria are summarized in Table 1. In addition, the ROC analyses showed that an H-score of ≥ 150 (criterion 2) or the presence of $\geq 70\%$ of $\geq 2+$ stained cells by SP384 clone were

the optimal cut-off value for identifying *ROS1* translocations by FISH (both with 93% sensitivity and 100% specificity). Regarding the D4D6 clone, the optimal cut-off value was criterion 1 (with 91% sensitivity and 100% specificity), followed by criterion 4 (Figure 2). The IHC concordance between observers was almost perfect (data not shown).

Following the optimal criteria defined, 40 cases (16.9%) were positive with the SP384 clone, whereas 196 (83.1%) cases were negative. The mean H-score of SP384 *ROS1*-positive cases was 291 (median: 300; range: 160-300) and the mean of $\geq 2+$ stained cells was 98.9% (median: 100; range: 70-100). Interestingly, 37 out of 40 SP384 *ROS1*-positive cases (92.5%) showed an immunoreactivity in a diffuse and $\geq 2+$ staining manner. Heterogeneity was present in 7.5% of cases (Figure 3A). With the D4D6 clone, we observed 39 (16.5%) positive cases, whereas 197 (83.5%) tumors were negative. The mean H-score of D4D6 *ROS1*-positive cases was 243 (median: 260; range: 100-300) and the mean of $\geq 2+$ stained tumor cells was 82.3% (median: 90; range: 10-100). Twenty-two out of 39 D4D6 *ROS1*-positive cases (56.4%) showed intratumoral heterogeneity (Figure 3B). Interestingly, in positive cases the difference in intratumoral heterogeneity between both clones was statistically significant ($P < 0.0001$).

Regarding SP384 *ROS1*-negative tumors, the immunoreactivity ranged from absent (133/196, 67.9%) to focal and weak (1+) or moderate (2+) staining (63/196, 32.1%), with a mean H-score of 10.6 (median: 0; range: 0-130) and with a mean of $\geq 2+$ stained cells of 1.9% (median: 0; range: 0-40). With the D4D6 clone, 157 out of 197 *ROS1* IHC-negative cases (79.7%) showed absent of immunoreactivity, whereas the remaining cases (40/197, 20.3%) exhibited a

focal and 1+ to 2+ staining pattern. The mean H-score was 3.8 (median: 0; range: 0-75) and the mean of $\geq 2+$ stained cells was 0.6% (median: 0; range: 0-20).

We observed the same topographic staining pattern with both ROS1 IHC antibodies. A granular or diffuse cytoplasmic staining was present in all cases with immunoreactivity (ROS1-positive and -negative cases), whereas a linear membranous accentuation was observed only in ROS1-positive tumors (14/40, 35% by SP384 and 14/39, 35.9% by D4D6) (Figure 4). There was no significant association between the topographic IHC pattern and the FISH patterns.

Finally, ROS1 expression in non-neoplastic type II pneumocytes (especially in the periphery of the tumor nodule or in a subpleural location) was statistically more frequent when using the SP384 clone (104/107, 97.2%) than with the D4D6 antibody (63/107, 58.9%) ($P < 0.0001$) (Figure 3).

ROS1 rearrangements assessed by NGS

Analysis by NGS was successful in 34 (79%) tumors. Results could not be assessed in nine cases due to insufficient sequencing coverage (four of them had very limited tumor cell content [i.e. 5-10%], and in five cases results could not be obtained due to RNA degradation [for example, one of the biopsies was a decalcified bone sample]). Fourteen (41.2%) cases had a *CD74-ROS1* fusion (eleven corresponding to *CD74(6)-ROS1(34)* and three to *CD74(6)-ROS1(32)*), nine (26.5%) showed an *EZR(10)-ROS1(34)*, six (17.6%) had a *SDC4(2)-ROS1(32)*, four (11.8%) presented a *SLC34A2-ROS1* (three corresponding to *SLC34A2(13)-ROS1(32)* and one to *SLC34A2(13)-ROS1(34)*), and finally one (2.9%) sample contained a *TMP3(7)-ROS1(35)*. Interestingly,

among the nine *EZR-ROS1* positive tumors, eight (88.9%) showed membranous accentuation staining with both ROS1 IHC antibodies and six (66.7%) presented an IGS FISH pattern. Both associations were statistically significant ($P = 0.001$ and $P = 0.017$, respectively). *CD74-ROS1* positive tumors exhibited more frequently a cytoplasmic staining with both ROS1 IHC clones (12 *versus* two; $P = 0.009$) and a BA FISH pattern (10 *versus* four; $P = 0.495$). The results of all three assays in FISH-positive cases are detailed in Supplementary Table S1.

Discordances between *ROS1* assays

Out of the 43 *ROS1* FISH-positive, three tumors showed absent (0) or focal 1+ cytoplasmic staining with both antibodies and were therefore considered *ROS1* IHC-negative using all criteria. Unfortunately, NGS results were not available for these cases. Clinically, all three patients were males with a smoking history. Interestingly, one patient was a metastatic poorly differentiated squamous cell carcinoma (SCC) diagnosed in a bronchial biopsy (i.e. p40 positive by IHC), with a predominantly BA FISH pattern (78% of positive cells), that received crizotinib treatment but had progressive disease. The remaining two patients were adenocarcinomas (ACs) diagnosed in surgical specimens (i.e. lung and bone resections) with an IGS FISH pattern (90% and 52% of positive cells, respectively). Only one of these two patients received crizotinib and had progressive disease.

Moreover, one *ROS1* FISH-positive case (i.e. 98% of positive cells with an IGS FISH pattern) showed immunoreactivity by SP384 clone (with an H-score of 160 and with $\geq 2+$ stained in 70% of tumor cells) and was considered

ROS1 IHC-positive using all criteria. Conversely, the immunoreactivity by D4D6 ROS1 antibody was absent. Clinically, the patient was a 67-year-old smoking male diagnosed in a cell block with a stage IV lung AC, who received crizotinib with a partial response. The NGS result was not available.

In addition, if we consider criteria 2 and 4, two *ROS1* FISH-positive cases were clearly positive by SP384 antibody (i.e. H-score of 230 and 300, and with $\geq 2+$ staining in 95% and 100% of tumor cells, respectively), whereas they should be considered negative by D4D6 clone (i.e. H-score of 105 and 100, and with $\geq 2+$ in 20% and 10% of tumor cells, respectively). NGS confirmed the *ROS1* fusions (*EZR-ROS1* and *CD74-ROS1* variants, respectively). Clinically, both patients were non-smoking males with ACs that received crizotinib resulting in objective responses.

All discordant cases were independently reviewed (F.L-R.) and the results confirmed. Remarkably, all *ROS1* NGS-positive tumors were in agreement with FISH.

Correlation between *ROS1*-rearrangements and clinicopathologic data

The clinicopathologic features of the 43 *ROS1* FISH-positive tumors are detailed in Table 2. Briefly, thirty-one cases (72.1%) were diagnosed as primary lung origin whereas 12 (27.9%) were metastases from different sites. Thirty-nine tumors (90.7%) were ACs, one (2.3%) was a SCC and the remaining 3 cases (7%) were NSCLCs not otherwise specified (NSCLC-NOS). Among the ACs, a predominant acinar pattern was observed in 20 out of 39 (51.3%); 14 (35.9%) cases presented solid architecture; two (5.1%) a predominant lepidic pattern; one (2.6%) showed a papillary growth; and one (2.6%) a predominant

micropapillary pattern. Mucinous and/or signet ring cells were observed in six out of 39 (15.4%) ACs. Interestingly, psammomatous calcifications and pleomorphic features were frequently observed (in 18.6% and 30.2% of tumors, respectively).

Clinical data were available for 41 patients (Figure 1 and Table 2). Briefly, overall response rate was 81% and disease control rate was 85.7%. At the time of report, median progression-free survival (PFS) and overall survival were 10.8 and 16.6 months, respectively. There were no relevant associations between *ROS1* fusion variants and clinicopathologic characteristics, except for a non-significant trend with better PFS in patients with the *EZR-ROS1* variant ($P = 0.199$).

Discussion

This multicenter study provided real-world data of *ROS1* rearrangements in NSCLC patients. To the best of our knowledge, this series represents one of the largest *ROS1*-positive lung cancer cohorts ever assembled. Considering that *ROS1*-rearranged patients represent only 1-2% of the overall NSCLC population, few reports contain more than 50 patients.¹⁸⁻²³ Moreover, a careful review of published studies identified only two larger series in which positive tumors had been investigated with more than two methodologies.^{19,22} One potential caveat of our work is that this is a retrospective series and therefore conclusions regarding *ROS1* inhibition are limited. To partially overcome this shortcoming, it is relevant to emphasize that all samples were initially tested with intention-to-treat, so our findings represent the clinical reality. In fact, the clinical results are in complete agreement with other series.^{4,24} Moreover, we

used commercially available tools, so our findings could be replicated elsewhere.

Although the recently updated CAP/IASLC/AMP molecular testing guidelines allows the use of ROS1 IHC for screening purposes, there has been only one antibody available to date (D4D6).⁹⁻¹¹ The sensitivity for this clone was controversial, probably reflecting the different interpretation criteria and the small numbers that were tested in most studies (reviewed in^{9,10,25-28}). The recent release of a new clone (SP384), with only one published report available to date, provides an IVD alternative.²³

Several conclusions can be drawn from our study. SP384 is more sensitive than D4D6 when compared with FISH, regardless of the criterion used. There are two differential features of SP384 that can be extremely useful to reduce the risk of a false-negative result. Firstly, the extremely frequent homogeneous staining (>92%) for ROS1. Considering the small size and limited number of fragments of most lung biopsies, sensitivity in small biopsies of some predictive IHC tests has been challenged due to heterogeneous expression.²⁹ Therefore, it is tempting to speculate that a less heterogeneous pattern of staining is an advantage in this setting. Secondly, the constant staining of non-neoplastic type II pneumocytes (>95%), which can be used as an *in situ* performance control. External positive controls should not be used to rule out a false-negative result caused by suboptimal pre-analytical parameters.¹² No matter how much you monitor this phase of the procedure, samples will occasionally fail. Along these lines, all but one of the IHC false-negative samples in our series were precisely specimens which are usually more prone to pre-analytical artifacts: two surgical resections, a decalcified bone specimen,

and a cell block (the only true discordant positive sample between both clones). Accordingly, pathologists should try to select blocks for ROS1 IHC testing that contain normal lung and extreme caution must be taken afterwards not to overinterpret the immunoreactivity in such normal or hyperplastic pneumocytes.^{11,15} Along these lines, positivity with D4D6 has been described in *ROS1*-non-rearranged tumors with lepidic patterns of growth or containing *EGFR* mutations (see below).^{14,30} This potentially confounding situation could be used to our advantage when searching for external positive controls.

Although our findings in the *ROS1*-non-rearranged cohort should be interpreted with extreme caution to avoid sample size bias,^{31,32} we truly believe the results might represent the clinical reality (i.e., these were not referral cases and we chose not to use tissue microarrays). The specificity of the two clones could very well be 100% if very stringent interpretation criteria are used. The best option would be an H-score of at least 100 for D4D6, but the higher sensitivity of SP384 comes at a cost and higher cut-off are needed to avoid what could be considered an excessive number of orthogonal tests (98% *versus* 100% specificity). However, a broadly held consensus on the interpretation criteria required for a positive IHC score has yet to emerge.^{10,11} There are several lines of evidence that are worth considering when addressing this matter. Unquestionable ROS1 IHC expression (i.e., even strong but focal) with D4D6 has been described in *ROS1*-non-rearranged cases containing other druggable alterations (mainly *EGFR* mutations, but also *KRAS* mutations, *BRAF* mutations, *ALK* fusions and *HER2* abnormalities) and we have had anecdotal analogous experience with SP384 (E. Conde, *unpublished observation*).^{14-16,25,30,33,34} Therefore, it is not surprising that the analytical comparison data of

SP384 *versus* FISH released by the manufacturer achieves the best balance between negative and positive agreement at the 50% cut-off, a result that is like our ROC curve analyses.³⁵ Nonetheless, SP384 inter-reader precision has been reported as high even when using a lower cut-off (30%), so higher cut-offs should not be an interpretation challenge in the real clinical world.¹⁷ Accordingly, a recent study has also reported a high inter-pathologist agreement when interpreting both clones.²³ In the light of the above, extreme caution is sensible in settings with very high incidence of *EGFR*-mutated patients (or other druggable non-*ROS1* genomic drivers, for this matter) not to render useless the screening value of *ROS1* IHC (see below).

Although break-apart FISH has traditionally been the gold-standard test for the detection of *ROS1* rearrangements, the *ROS1* FISH is especially difficult to interpret and may be prone to both false-negatives and false-positives.^{9,11,19,36–40} To increase the robustness of the results, we decided to repeat all FISH tests *in-house* and score them with an outstanding automated FISH scanning system using a high threshold for positivity. The mean and median number of positive cells in positive tumors was very high (>80%, well above the threshold) and obviously contributed to the excellent correlation with FISH, but it must be emphasized that some rare fusion partners (*GOPC*, also known as *FIG*, is 3% of *ROS1* patients and not represented in the present study) are a well-known source of FISH false-negative results.^{14,39,41,42} Conversely, we and others have reported that *bona fide ROS1*-non-rearranged tumors can contain a number of positive nuclei (10-12%), close to the 15% cut-off used in many studies.^{9,15,16} At least some published reports with high prevalence of concomitant oncogene mutations may reflect problems with the

FISH interpretation.^{43,44} The use of imaging systems and/or a higher threshold for positivity are strategies that should ensure specificity.^{9,11,15,16}

In the last phase of the study, we performed an RNA-based NGS assay in FISH-positive cases to understand the molecular epidemiology of the different rearrangements and try to correlate them with the clinical and pathological features. It must be emphasized that this was not a formal comparison study between different methodologies. Overall, the variety and prevalence of *ROS1* partners identified was like those described.^{24,37,45} The percentage of cases in which the suboptimal RNA quality/quantity resulted in low sequencing coverage highlights the need for an evidence-based algorithmic approach.^{39,46,47} The fusion partner can influence both the IHC staining and the FISH pattern, the *EZR* variant being usually associated with a membranous accentuation and isolated 3' signals, respectively.^{13,14,25,45} This latter association could explain some FISH false-negative cases than were found to contain the *EZR-ROS1* transcript, as this atypical pattern is in fact the most difficult to score because the isolated 3' signals can sometimes be absent or barely visible.^{9,13,40} Finally, our non-significant trend of better PFS for patients with the *EZR-ROS1* fusion might be in alignment with series in which almost every patient with an IGS achieved a complete response and with the recently published differential efficacy of crizotinib in the non-*CD74-ROS1* group.^{24,48} Unfortunately, this is still a controversial topic that would need larger multicentre series with longer follow-up and standardized NGS to draw definitive conclusions.^{22,37}

A review of published studies in the light of our findings suggest that there are two scenarios that can have important clinical consequences when

ROS1 IHC is to be used as the primary screening method for *ROS1* therapy: (1) A *ROS1* FISH-false positive result in a patient with another druggable alteration that is causing the ROS1 IHC positivity. Awareness of the FISH potential pitfalls is essential (i.e., percentage of positive nuclei around the cut-off, 3' isolated pattern), and if the result is inconsistent it is sensible to use a third methodology (i.e., NGS) that will potentially discover the reason for the IHC positivity, and (2) a *ROS1* NGS-negative or failed report in a *ROS1*-rearranged sample that exhibited intense and homogeneous IHC staining.^{38,44} The choice of RNA-based NGS can reduce the risk of false negatives and using another sample or a third technology (i.e., FISH) when the initial NGS approach fails is mandatory to confirm those positive IHC results.^{39,47}

In conclusion, the new SP384 clone showed high sensitivity without compromising specificity, so it is another excellent analytical option for the proposed CAP/IASLC/AMP molecular testing algorithm. A consideration of the clinical problem of NSCLC highlights the need to be aware of how the methods that we use perform in the real-world setting.⁴⁶

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Figure legends

Figure 1. Flow chart of patients in the ROSING study. FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; NGS, next-generation sequencing. **ROS1* FISH-positive cases were defined as more than 50% break-apart (BA) signals or an isolated green signal (IGS) in tumor cells (i.e. more than 25 of 50 cells). *ROS1* FISH-negative samples were defined as less than 10% BA or IGS cells (i.e. fewer than five of 50 cells). *ROS1* FISH cases were considered borderline if 10-50% cells were positive. In this latter scenario, the final rate was calculated out of 100 cells, and the sample was considered rearranged if the positive cells percentage was higher or equal to 15%.

Figure 2. Receiver Operating Characteristics (ROC) curves analyses identified an H-score of ≥ 150 (A) or the presence of $\geq 70\%$ of $\geq 2+$ stained cells (B) by SP384 clone as the optimal cut-off value for identifying *ROS1* translocations by FISH (both with 93% sensitivity and 100% specificity). Regarding the D4D6 clone, the optimal cut-off value was an H-score of ≥ 100 (C) (with 91% sensitivity and 100% specificity), followed by the presence of $\geq 30\%$ of $\geq 2+$ stained cells (D) (with 86% sensitivity and 100% specificity). IHC, immunohistochemistry.

Figure 3. Most of the *ROS1*-positive tumors showed a homogenous staining with the SP384 clone (A, detail on the top inset), whereas intratumoral heterogeneity was more frequently observed with the D4D6 antibody (B, detail on the upper inset). Moreover, as shown in the lower insets, *ROS1* expression was more frequent in non-neoplastic type II pneumocytes when using the SP384 clone (A) than with the D4D6 antibody (B).

Figure 4. Representative images of the different topographic IHC patterns. A tumor with a linear membranous accentuation staining with the SP384 (A) and the D4D6 (B) clones, respectively. Other case with a diffuse and granular cytoplasmic staining using the SP384 clone (C) and the D4D6 antibody (D).

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Table 1. Performance of ROS1 IHC using the previously published criteria to predict ROS1 rearrangements by FISH

ROS1 IHC	ROS1 FISH							
		FISH+	FISH-	Total (%)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	LR+ (95% CI)	LR- (95% CI)
Clone SP384								
Criterion 1:	IHC+	40	1	41 (17.4)	93 (81-98)	99 (97-100)	180 (25.4-1270)	0.1 (0-0.2)
H-score \geq 100	IHC-	3	192	195 (82.6)				
Criterion 2:	IHC+	40	0	40 (16.9)	93 (81-98)	100 (98-100)	.	0.1 (0-0.2)
H-score \geq 150	IHC-	3	193	196 (83.1)				
Criterion 3:	IHC+	40	31	71 (30.1)	93 (81-98)	84 (78-89)	5.8 (4.1-8)	0.1 (0-0.2)
\geq 2+ staining	IHC-	3	162	165 (69.9)				
Criterion 4:	IHC+	40	1	41 (17.4)	93 (81-98)	99 (97-100)	180 (25.4-1270)	0.1 (0-0.2)
\geq 2+ staining in \geq 30% of total tumor cells	IHC-	3	192	195 (82.6)				
Clone D4D6								
Criterion 1:	IHC+	39	0	39 (16.5)	91 (78-97)	100 (98-100)	.	0.1 (0-0.2)
H-score \geq 100	IHC-	4	193	197 (83.5)				
Criterion 2:	IHC+	37	0	37 (15.7)	86 (72-95)	100 (98-100)	.	0.1 (0.1-0.3)
H-score \geq 150	IHC-	6	193	199 (84.3)				
Criterion 3:	IHC+	39	14	53 (22.5)	91 (78-97)	93 (88-96)	12.5 (7.5-20.9)	0.1 (0-0.2)
\geq 2+ staining	IHC-	4	179	183 (77.5)				
Criterion 4:	IHC+	37	0	37 (15.7)	86 (72-95)	100 (98-100)	.	0.1 (0.1-0.3)
\geq 2+ staining in \geq 30% of total tumor cells	IHC-	6	193	199 (84.3)				

CI, confidence interval; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; LR+, likelihood ratio positive; LR-, likelihood ratio negative

Table 2. Clinicopathologic features of patients with ROS1 rearrangements

	No. of Patients* N = 43 (%)
Tumour histology	
AC	39 (90.7)
SCC	1 (2.3)
NSCLC-NOS	3 (7)
Specimen type	
Surgical	28 (65.1)
Small biopsy	11 (25.6)
Cell block	4 (9.3)
Age at diagnosis, years*	
Mean	59
Median	60
Range	32-83
Sex*	
Male	24 (58.5)
Female	17 (41.5)
Smoking status*	
Non-smoker	26 (63.4)
Smoker	15 (36.6)
Stage at initial diagnosis*	
I	8 (19.5)
II	5 (12.2)
III	10 (24.4)
IV	18 (43.9)
Metastasis sites for stage IV disease*	
Lung	3 (11.5)
Brain	1 (3.8)
Bone	3 (11.5)
Lymph nodes	1 (3.8)
Pleural	3 (11.5)
Multiple organs	12 (46.2)
Other or unknown	3 (11.5)
Crizotinib treatment line†	
First	12 (48)
Second	8 (32)
≥Third	5 (20)
Response rate of crizotinib‡	
PD	3 (14.3)
SD	1 (4.8)
PR	16 (76.2)
CR	1 (4.8)

*Clinical information was available for 41 out of 43 patients

†Stage IV patients treated with crizotinib (n=25)

‡Patients treated with crizotinib and clinical follow-up available (n=21)

AC, adenocarcinoma; CR, complete response; NSCLC-NOS, non-small cell lung carcinoma, not otherwise specified; PR, partial response; PD, progressive disease; SCC, squamous cell carcinoma; SD, stable disease







