ORIGINAL ARTICLE

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

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**ABSTRACT**

**Introduction:** The *ROS1* gene rearrangement has become an important biomarker in NSCLC. The College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology 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:** A total of 43 *ROS1* FISH–positive and 193 *ROS1* FISH–negative NSCLC samples were studied. All specimens were screened by using two antibodies (clone D4D6 from Cell Signaling Technology and clone SP384 from Ventana Medical Systems), and the different interpretation criteria were compared with break-apart FISH (Vysis). FISH-positive samples were also analyzed with next-generation sequencing (Oncomine Dx Target Test Panel, Thermo Fisher Scientific).

**Results:** An H-score of 150 or higher or the presence of at least 70% of tumor cells with an intensity of staining of 2+ or higher by the SP384 clone was the optimal cutoff value (both with 93% sensitivity and 100% specificity). The D4D6 clone showed similar results, with an H-score of at least 100 (91% sensitivity and 100% specificity). ROS1 expression in normal lung was more frequent with use of the SP384 clone ($p < 0.0001$). The ezrin gene (*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

## Introduction

The *ROS1* gene rearrangement has now become an important predictive biomarker for targeted tyrosine kinase inhibitors in NSCLC. 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 use of an

FDA-approved companion diagnostic test.[1] Soon afterward, the drug was approved by the European Medicines Agency, with the statement that "an accurate and validated *ROS1* assay is necessary for the selection of patients."[2] On the basis of 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 College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology (CAP/IASLC/AMP) molecular testing guidelines for the selection of patients with lung cancer support the use of ROS1 immunohistochemistry (IHC) as a screening test, followed by fluorescence in situ hybridization (FISH) (traditionally considered to be the criterion standard method)[9] or a molecular test (i.e., reverse-transcriptase polymerase chain reaction 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 results.[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. A total of 55 *ROS1*-positive samples from patients with NSCLCs that had been initially tested as part of routine clinical care in 23 different institutions were used for this study (also known as ROSING [ROS IHC and NGS]). To confirm the *ROS1*-positive status, FISH analysis (the criterion standard method) was performed at the referral institution (i.e., University Hospital HM Sanchinarro). Only cases with enough tissue available (i.e., $\geq$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 being

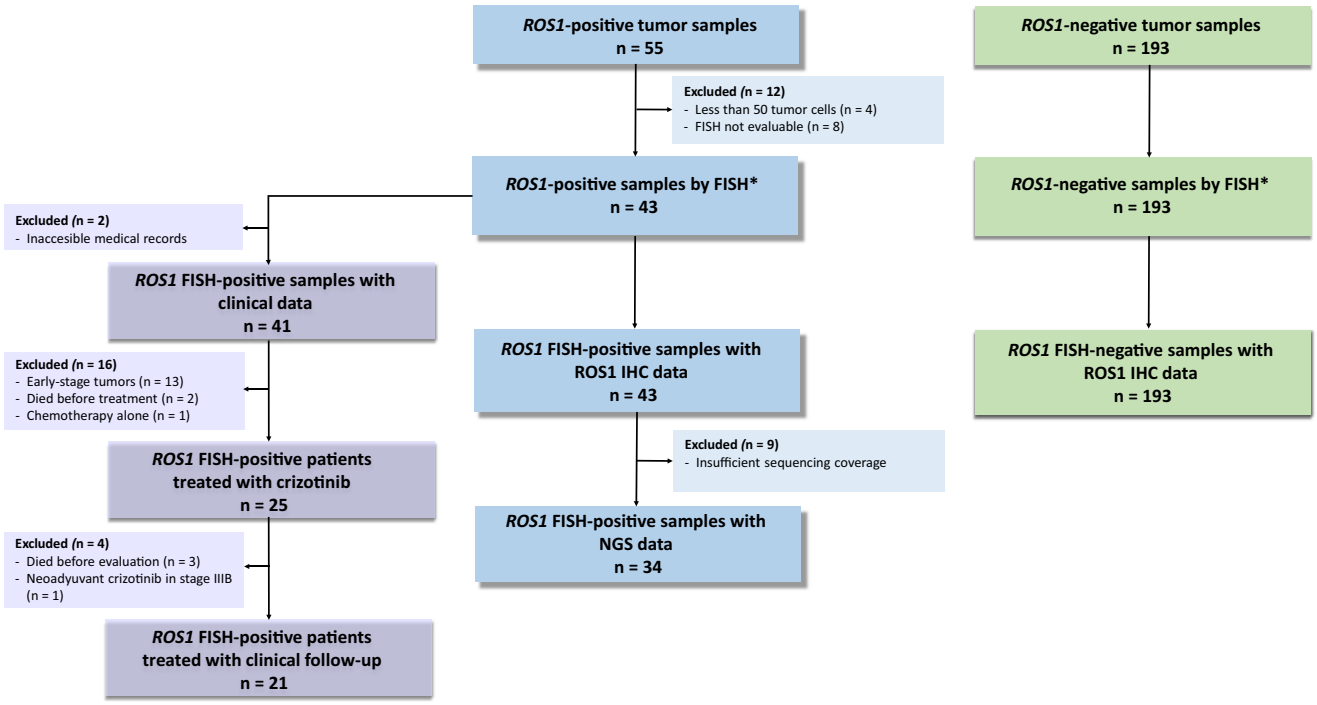


**Figure 1.** Flowchart of patients in the ROSING study. **ROS1* fluorescence in situ hybridization (FISH)-positive samples were defined as those with more than 25 (50%) break-apart (BA) signals or isolated green signals (IGS) in tumor cells. ROS1 FISH-negative samples were defined as those with fewer than five (10%) BA signals or IGS in tumor cells. *ROS1* FISH-positive samples were defined as those with more than 25 (50%) break-apart (BA) signals or isolated green signals (IGS) in tumor cells. IHC, immunohistochemistry; NGS, next-generation sequencing.

investigated by FISH, all specimens (negative and positive) were independently screened for ROS1 expression by using 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 Hospitals 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 4-$\mu$m-thick FFPE tumor tissue sections from all positive and negative cases. The Vysis 6q22 *ROS1* Break Apart FISH Probe Kit (Abbott Molecular, Des Plaines, IL) 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 highly recommended criteria.[11] A minimum of 50 tumor nuclei were counted. *ROS1* FISH–positive cases were defined as those with more than 25 break-apart (BA) signals (50%) (separated by at least one signal diameter) or an isolated green signal (IGS) in tumor cells. *ROS1* FISH–negative samples were defined as those with fewer than five (10%) BA signals or IGS in tumor cells. *ROS1* FISH cases were considered borderline if five to 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 calculated the percentage out of 100 cells. If the percentage of positive cells was lower than 15%, the sample was considered negative. If the percentage of positive cells was higher than 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). FFPE tumor tissues were sectioned at a thickness of 4 $\mu$m and stained with two different anti-ROS1 clones: SP384 (Ventana Medical Systems) and D4D6 (Cell Signaling Technology, Danvers, MA). 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.) who were blinded to the FISH results. When a discrepancy was observed, the final result was obtained by consensus. Staining intensity was defined as follows: strong cytoplasmic staining (3+), which was clearly visible with use of a 2× or 4× objective; moderate staining (2+), which required use of a 10× or 20× objective; weak staining (1+), which involved use of a 40× objective; and negative staining (0), which was defined as 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 by using four previously described criteria: (1) an H-score with a threshold for ROS1 positivity defined as at least 100,[11,13] (2) an H-score cutoff of at least 150,[11,14] (3) an intensity criterion with the cutoff of positivity defined as 2+ or higher in any tumor cells,[11,15,16] and (4) a positive status based on an intensity of 2+ or higher in at least 30% of the total tumor cells.[17] Intratumoral staining heterogeneity was also evaluated. It was defined as the presence of areas of staining with an intensity of 0 or 1+ 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, freshly cut 5-$\mu$m-thick sections were collected for nucleic acid extraction: five sections for surgical specimens and 12 sections for small biopsy specimens. The first and last sections were stained with hematoxylin and eosin and reviewed by two pathologists (E. C. and F. L.-R.) to assess the percentage of tumor cells. RNA extraction was performed with the RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's instructions. The RNA was then purified and concentrated by using the 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 granted only for the RNA part of the procedure.

### *Statistical Analysis*

On the basis of all the valid data obtained, we performed a descriptive analysis of all the variables of

**Table 1.** Performance of ROS1 IHC Using the Previously Published Criteria to Predict *ROS1* Rearrangements by FISH

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

CI, confidence interval; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; LR, likelihood ratio.

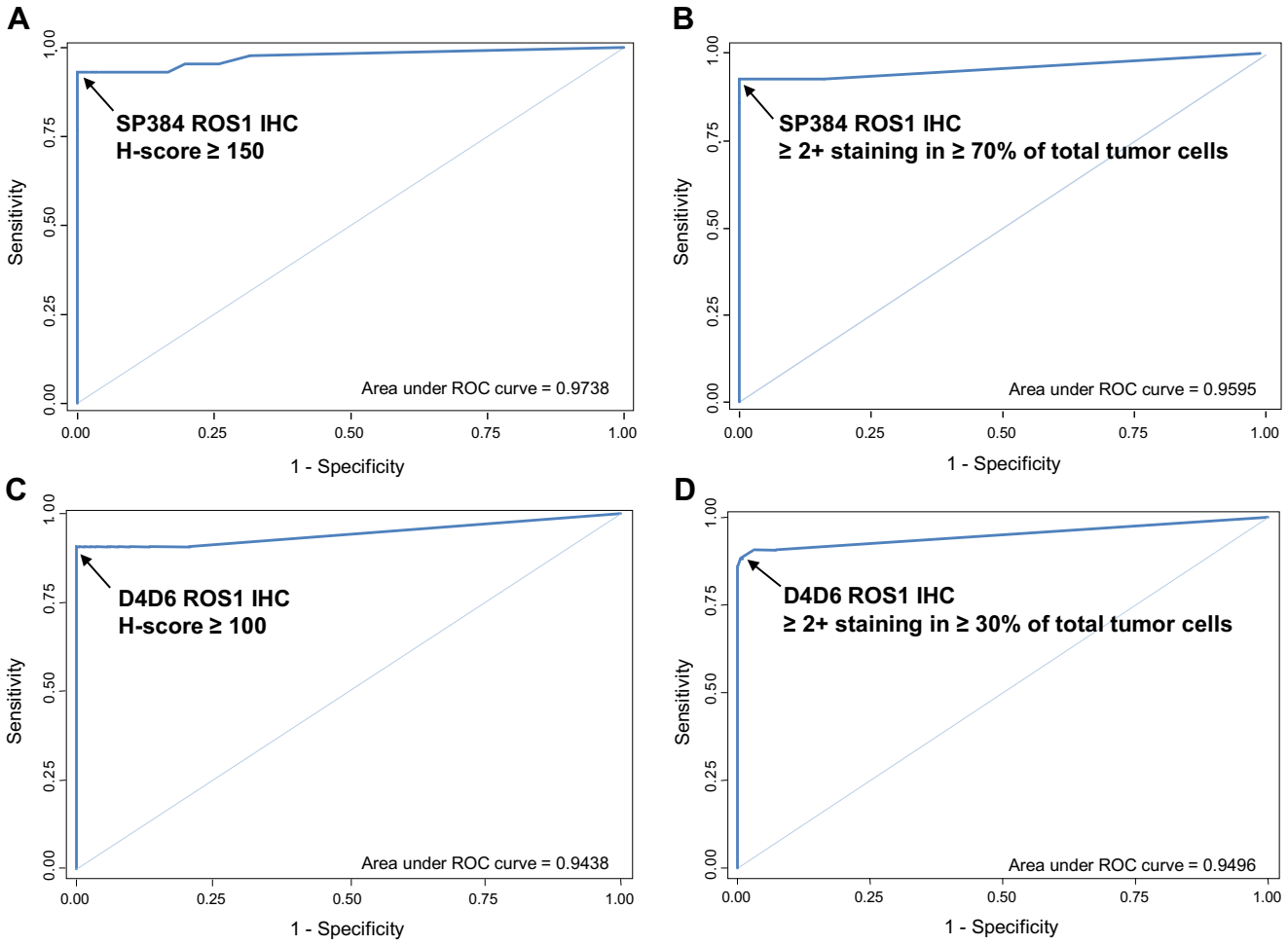


**Figure 2.** Receiver operating characteristic (ROC) curve analyses identified an H-score of 150 or higher (*A*) or the presence of at least 70% of cells with a staining intensity of 2+ or higher (*B*) with use of the SP384 clone as the optimal cutoff value for identifying *ROS1* translocations by fluorescence in situ hybridization (both with 93% sensitivity and 100% specificity). Regarding the D4D6 clone, the optimal cutoff value was an H-score of at least 100 (*C*) (with 91% sensitivity and 100% specificity), followed by the presence of at least 30% of cells with a staining intensity of 2+ or higher (*D*) (with 86% sensitivity and 100% specificity). IHC, immunohistochemistry.

interest. The test used for comparison of categorical variables was the Pearson chi-square test (frequency <5 [Fisher exact test]). For comparison of means we used the Mann-Whitney test. The sensitivity and specificity of both ROS1 IHC clones versus those of FISH were obtained. Receiver operating characteristic (ROC) curves were used to determine the optimal cutoff value that discriminates between patients with *ROS1*-rearranged and *ROS1*-non-rearranged tumors. We also analyzed the correlation between the different *ROS1* fusion variants and clinicopathologic features. Survival analysis was performed by using the Kaplan-Meier method with the log-rank test and Cox regression. All analyses were done with Stata 15.1 software (StataCorp, College Station, TX) and were two sided; *p* values less than 0.05 indicated statistical significance.

## Results

### ROS1 *Rearrangements Assessed by FISH*

Of the 55 *ROS1*-positive lung carcinoma specimens, four were excluded for lack of sufficient tumor tissue and eight were excluded because the FISH results were not evaluable (i.e., no or weak hybridization signals). Of the 193 *ROS1*-negative NSCLC specimens, all were included in the study (see Fig. 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%–10%). In the *ROS1* FISH–positive tumors, the

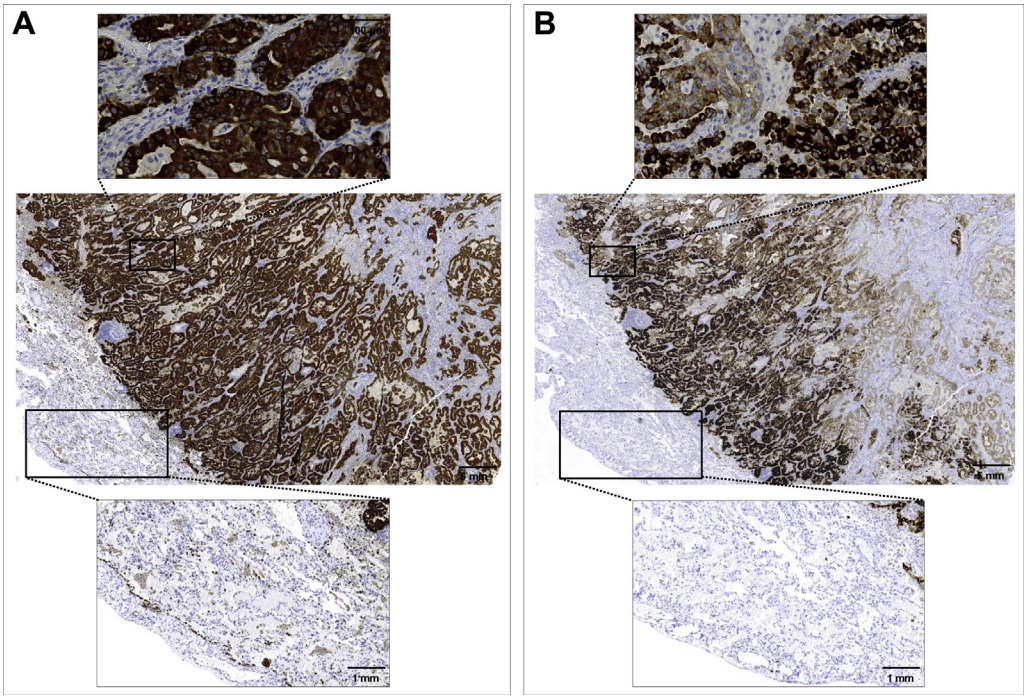


**Figure 3.** Most of the *ROS1*-positive tumors showed homogeneous staining with the SP384 clone (*A* [*top inset*]), whereas intratumoral heterogeneity was more frequently observed with the D4D6 antibody (*B* [*upper inset*]). Moreover, as shown in the lower insets, ROS1 expression was more frequent in nonneoplastic type II pneumocytes with use of the SP384 clone (*A*) than with use of the D4D6 antibody (*B*).

mean percentage of positive cells was 82.3% (median 86%, range 49%–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 or higher

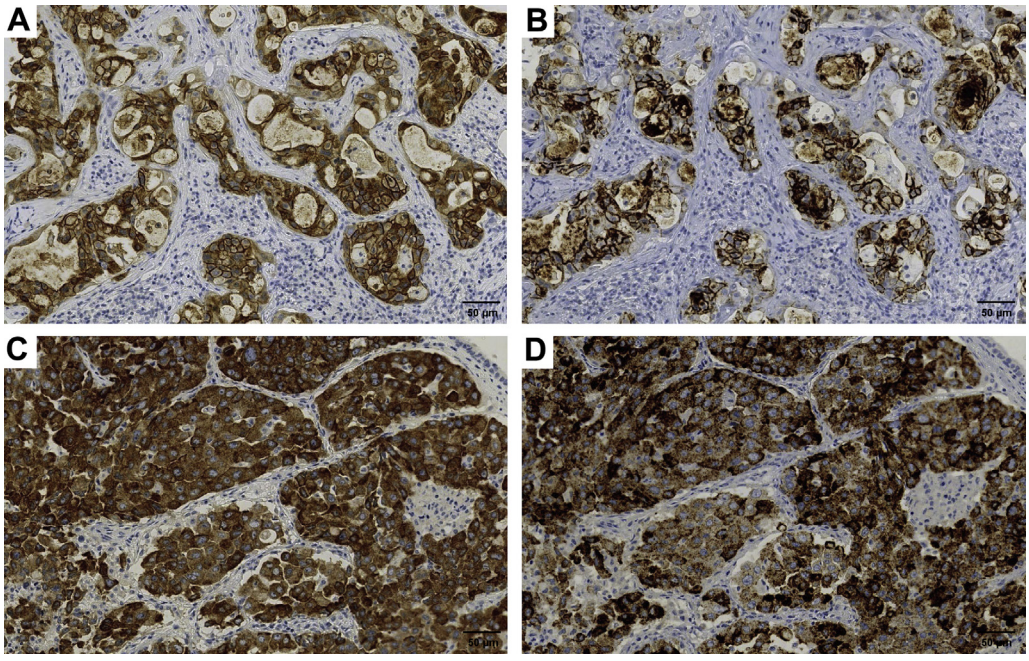


**Figure 4.** Representative images of the different topographic immunohistochemistry patterns. A tumor with a linear membranous accentuation staining with the SP384 (*A*) and D4D6 (*B*) clones, respectively. Another case with a diffuse and granular cytoplasmic staining using the SP384 clone (*C*) and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

(criterion 2) or the presence of at least 70% of cells with at least 2+ staining by the SP384 clone was the optimal cutoff value for identifying *ROS1* translocations by FISH (both with 93% sensitivity and 100% specificity). Regarding the D4D6 clone, the optimal cutoff value was criterion 1 (with 91% sensitivity and 100% specificity), followed by criterion 4 (Fig. 2). The IHC concordance between observers was almost perfect (data not shown).

In accordance with the defined optimal criteria, when the SP384 clone was used, 40 cases (16.9%) were positive and 196 cases (83.1%) were negative. The mean H-score of the SP384 ROS1–positive cases was 291 (median 300, range 160–300), and the mean of cells with staining scored as 2+ or higher was 98.9% (median 100, range 70–100). Interestingly, in 37 out of 40 SP384 ROS1–positive cases (92.5%), immunoreactivity was evident as diffuse staining with an intensity of 2+ or higher. Heterogeneity was present in 7.5% of cases (Fig. 3*A*). With the D4D6 clone, we observed 39 positive cases (16.5%) and 197 negative tumors (83.5%). The mean H-score of the D4D6 ROS1–positive cases was 243 (median 260, range 100–300), and the mean percentage of tumor cells with staining graded 2+ or higher was 82.3% (median 90, range 10–100). Of the 39 D4D6 ROS1–positive cases, 22 (56.4%) showed intratumoral heterogeneity (Fig. 3*B*). Interestingly, in the positive cases the difference in intratumoral heterogeneity between the two clones was statistically significant ($p < 0.0001$).

Regarding the SP384 ROS1–negative tumors, immunoreactivity ranged from absent (133 of 196 [67.9%]) to focal and weak (1+) or moderate (2+) staining (63 of 196 [32.1%]), with a mean H-score of 10.6 (median 0, range 0–130) and a mean percentage of cells with staining graded 2 or higher of 1.9% (median 0, range 0–40). With the D4D6 clone, 157 out of 197 ROS1 IHC–negative cases (79.7%) showed absence of immunoreactivity, whereas the remaining cases (40 of 197 [20.3%]) exhibited a focal staining pattern graded 1+ to 2+. The mean H-score was 3.8 (median 0, range 0–75) and the mean percentage of cells with staining graded 2+ or higher 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 ROS1-negative cases), whereas a linear membranous accentuation was observed only in ROS1-positive tumors (14 of 40 examined by SP384 IHC [35%] and 14 of 39 examined by D4D6 IHC [35.9%]) (Fig. 4). There was no significant association between the topographic IHC patterns and the FISH patterns.

Finally, ROS1 expression in nonneoplastic type II pneumocytes (especially in the periphery of the tumor nodule or in a subpleural location) was statistically more frequent with use of the SP384 clone (104 of 107 [97.2%]) than with use of the D4D6 antibody (63 of 107 [58.9%]) ($p < 0.0001$) (see Fig. 3).

**Table 2.** Clinicopathologic Features of Patients with *ROS1* Rearrangements

| Feature | Patients, n (%)[a] (n = 43) |
|---|---|
| Tumor histologic type | |
| 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, y [a] | |
| Mean | 59 |
| Median | 60 |
| Range | 32–83 |
| Sex[a] | |
| Male | 24 (58.5) |
| Female | 17 (41.5) |
| Smoking status[a] | |
| Nonsmoker | 26 (63.4) |
| Smoker | 15 (36.6) |
| Stage at initial diagnosis[a] | |
| I | 8 (19.5) |
| II | 5 (12.2) |
| III | 10 (24.4) |
| IV | 18 (43.9) |
| Metastasis sites for stage IV disease[a] | 26 |
| 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[b] | |
| First | 12 (48) |
| Second | 8 (32) |
| ≥Third | 5 (20) |
| Response rate of crizotinib[c] | |
| PD | 3 (14.3) |
| Stable disease | 1 (4.8) |
| PR | 16 (76.2) |
| CR | 1 (4.8) |

[a]Clinical information was available for 41 out of 43 patients.
[b]Patients with stage IV disease treated with crizotinib (n = 25).
[c]Patients who were treated with crizotinib and had clinical follow-up information available (n = 21).
AC, adenocarcinoma; CR, complete response; NOS, not otherwise specified; PR, partial response; PD, progressive disease; SCC, squamous cell carcinoma.

## ROS1 *Rearrangements Assessed by NGS*

Analysis by NGS was successful in 34 tumors (79%). Results could not be assessed in nine cases owing to

insufficient sequencing coverage: four cases had a very limited tumor cell content (i.e., 5%–10%), and in five cases results could not be obtained because of RNA degradation (for example, one of the biopsy specimens was a decalcified bone sample). Fourteen cases (41.2%) had a CD74 molecule gene (*CD74*)-*ROS1* fusion (11 cases corresponding to *CD74* exon 6–*ROS1* exon 34 fusion and three corresponding to *CD74* exon 6–*ROS1* exon 32 fusion), nine cases (26.5%) showed an ezrin gene (*EZR*) exon 10–*ROS1* exon 34 fusion, six cases (17.6%) had a syndecan 4 gene (*SDC4*) exon 2–*ROS1* exon 32 fusion, four cases (11.8%) presented a solute carrier family 34 member 2 gene (*SLC34A2*)-*ROS1* fusion (three corresponding to *SLC34A2* exon 13–*ROS1* exon 32 fusion and one corresponding to *SLC34A2* exon 13–*ROS1* exon 34 fusion), and finally one sample (2.9%) contained a tropomyosin 3 gene (*TPM3*) exon 7–*ROS1* exon 35 fusion. 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 more frequently exhibited 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 the FISH-positive cases are detailed in Supplementary Table 1.

### *Discordances between* ROS1 *Assays*

Of the 43 *ROS1* FISH–positive tumors, three showed absent (0) or focal (1+) cytoplasmic staining with both antibodies and were therefore considered ROS1 IHC–negative with use of all the criteria. Unfortunately, NGS results were not available for these cases. Clinically, all three patients were males with a history of smoking. Interestingly, one patient had metastatic poorly differentiated squamous cell carcinoma diagnosed by a bronchial biopsy (i.e., p40 positive by IHC), with a predominantly BA FISH pattern (78% of positive cells); the patient received crizotinib treatment but had progressive disease. The remaining two patients had adenocarcinomas (ACs) that were diagnosed by 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 with use of the SP384 clone (with an H-score of 160 and staining in 70% of tumor cells graded 2+ or higher) and was considered ROS1 IHC–positive with use of all the criteria. Conversely, immunoreactivity was absent with use of the D4D6 ROS1 antibody. Clinically, the patient was a 67-year-old smoking male with a stage IV lung AC that had been diagnosed in a cell block; he received crizotinib with a partial response. An NGS result was not available.

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

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

### *Correlation between* ROS1 *Rearrangements and Clinicopathologic Data*

The clinicopathologic features of the 43 *ROS1* FISH–positive tumors are detailed in Table 2. Briefly, 31 cases (72.1%) were diagnosed as primary lung cancer (cancer originating in the lung), whereas 12 (27.9%) were metastases from different sites. Thirty-nine tumors (90.7%) were ACs, one (2.3%) was a squamous cell carcinoma, and the remaining three (7%) were NSCLCs not otherwise specified. Of the 39 ACs, 20 (51.3%) were observed to have a predominant acinar pattern, 14 (35.9%) presented a solid architecture, two (5.1%) had a predominant lepidic pattern, one (2.6%) showed a papillary growth, and one (2.6%) had a predominant micropapillary pattern. Mucinous and/or signet ring cells were observed in six out of 39 ACs (15.4%). 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 (see Fig. 1 and Table 2). Briefly, the overall response rate was 81% and the disease control rate was 85.7%. At the time of report, the median progression-free survival (PFS) and overall survival times were 10.8 and 16.6 months, respectively. There were no relevant associations between *ROS1* fusion variants and clinicopathologic characteristics, except for a nonsignificant trend toward better PFS in patients with the *EZR-ROS1* variant ($p = 0.199$).

## Discussion

This multicenter study provided real-world data on *ROS1* rearrangements in patients with NSCLC. To the best of our knowledge, this series represents one of the largest *ROS1*-positive lung cancer cohorts ever assembled. Considering that patients with *ROS1* rearrangement represent only 1% to 2% of the overall population with NSCLC, 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 it 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 those of 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 allow 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 in vitro diagnostic 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. First, there is the extremely frequent homogeneous staining (>92%) for ROS1. Considering the small size and limited number of fragments of most lung biopsy specimens, the sensitivity of some predictive IHC tests on small biopsy specimens has been challenged owing to heterogeneous expression.[29] Therefore, it is tempting to speculate that a less heterogeneous pattern of staining is an advantage in this setting. Second, there is the constant staining of nonneoplastic 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 preanalytical parameters.[12] No matter how much one monitors 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 that are usually more prone to preanalytical 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 afterward so as not to overinterpret the immunoreactivity in such normal or hyperplastic pneumocytes.[11,15] Along these lines, positivity with D4D6 has been described in *ROS1*-nonrearranged tumors having lepidic patterns of growth or containing *EGFR* mutations (see the next paragraph).[14,30] This potentially confounding situation could be used to our advantage when searching for external positive controls.

Although our findings in the *ROS1*-nonrearranged cohort should be interpreted with extreme caution to avoid sample size bias,[31,32] we truly believe that 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 were 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 cutoffs 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*-nonrearranged cases containing other druggable alterations (mainly *EGFR* mutations, but also *KRAS* mutations, *BRAF* mutations, ALK receptor tyrosine kinase gene [*ALK*] fusions, and erb-b2 receptor tyrosine kinase 2 gene [*HER2*] abnormalities), and we have had anecdotal analogous experience with SP384 (E. Conde, unpublished observation, 2019).[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% cutoff, a result similar to that of our ROC curve analyses.[35] Nonetheless, the interreader precision of SP384 has been reported as high even with use of a lower cutoff (30%), so higher cutoffs should not be an interpretation challenge in the real clinical world.[17] Accordingly, a recent study has also reported a high interpathologist agreement when interpreting both clones.[23] In the light of the aforementioned, extreme caution is advised in settings with a very high incidence of patients with *EGFR* mutation (or other druggable non-*ROS1* genomic drivers, for that matter) so as not to render useless the screening value of ROS1 IHC (see later in this discussion).

Although break-apart FISH has traditionally been the criterion standard test for 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 numbers of positive cells in positive tumors were very high (>80%, which is well above the threshold) and obviously contributed to the excellent correlation with FISH, but it must be emphasized that some rare fusion partners ([*GOPC*], which is also known as *FIG*, occurs in 3% of *ROS1* patients and is not represented in the present study) are a well-known source of false-negative FISH results.[14,39,41,42] Conversely, we and others have reported that bona fide *ROS1*-nonrearranged tumors can contain a number of positive nuclei (10%–12%), which is close to the 15% cutoff used in many studies.[9,15,16] At least some published reports with a 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 were similar to those described.[24,37,45] The percentage of cases in which the suboptimal RNA quality and/or 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, with the *EZR* variant usually being associated with a membranous accentuation and isolated 3′ signals, respectively.[13,14,25,45] This latter association could explain some of the cases with false-negative FISH results that 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 nonsignificant trend of better PFS for patients with the *EZR-ROS1* fusion might be in alignment with the results of 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 multicenter 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 suggests 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 cutoff, 3′ isolated pattern], and if the result is inconsistent, it is sensible to use a third methodology [i.e., NGS], which 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. 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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## Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at https://doi.org/10.1016/j.jtho.2019.07.005.

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