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Comparison of Different *ROS1* Immunohistochemistry Clones and Consistency with Fluorescence In Situ Hybridization Results in Non-Small Cell Lung Carcinoma

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Background: The study of *ROS1* rearrangement in non-small cell lung carcinoma (NSCLC) has gained importance as it enables personalized treatment of NSCLC with tyrosine kinase inhibitors. Therefore, it is important that the ROS1 assessment tests become more standardized. In this study, we compared the two immunohistochemistry (IHC) antibodies (D4D6 and SP384 clones) and consistency with the fluorescence in situ hybridization (FISH) results in NSCLC.

Aims: To investigate the effectiveness of the commonly used two IHC antibodies (SP384 and D4D6 clones) to detect ROS1 rearrangement in NSCLC.

Study Design: A retrospective cohort study.

Methods: The study included 103 samples diagnosed with NSCLC, confirmed using IHC and FISH ROS1 results (14 positives, four discordant, and 85 consecutive negatives), with sufficient tissue samples (≥ 50 tumor cells). All samples were initially tested with ROS1-IHC antibodies (D4D6 and SP384 clones); their ROS1 status was then analyzed using the FISH method. Finally, samples with discordant IHC and FISH results were confirmed using the reverse transcription polymerase chain reaction method.

Results: The sensitivity of SP384 and D4D6 clones of ROS1 antibody was 100% with a ≥ 1 + cut-off. When the ≥ 2 + cut-off was used, the sensitivity rate for the SP384 clone was 100%, whereas the sensitivity for the D4D6 clone was 42.86%. *ROS1* FISH rearranged samples were positive for both clones, but SP384 had generally higher intensity than D4D6. The mean IHC score was + 2 for SP384 and + 1.17 for D4D6. SP384 mostly tended to have a higher IHC score intensity, which made the evaluation easier than D4D6. SP384 has a higher sensitivity than D4D6. However, false positives were found in both clones. There was no significant correlation between ROS1 FISH-positivity percentage with SP384 (p = 0.713, ρ = 0.108) and D4D6 (p = 0.26, ρ = -0.323) IHC staining intensity. The staining patterns of both clones were similar (homogeneity/heterogeneity).

Conclusion: Our findings show that the SP384 clone is more sensitive than D4D6. However, SP384 can also cause false positive results like D4D6. Knowing the variable diagnostic performance of different ROS1 antibodies before using them in clinical applications is necessary. IHC-positive results should be confirmed using FISH.

INTRODUCTION

Recently, genomic or immune-based biomarkers associated with non-small cell lung carcinoma (NSCLC) have been increasingly used.[1-3] These biomarkers, which are predictive for molecularly targeted therapies, and the demonstration of new oncogene roles have led to significant changes in lung cancer treatment.[4,5] These developments improve survival and quality of life in patients with advanced lung cancer.[6]

Targeted therapies have become part of the standard care for the treatment of NSCLC.[7] Targeted therapies need biomarkers to identify patients in the subset where this treatment can be used. Patients with NSCLC are commonly evaluated for anaplastic lymphoma kinase (*ALK*) rearrangements, epidermal growth factor receptor (*EGFR*) mutations, and programmed death ligand-1 expression. The c-ros oncogene 1 (*ROS1*) rearrangement analysis was later added to the test paradigm. *ROS1* is currently an important predictive biomarker for lung adenocarcinomas.[8]

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The *ROS1* gene is found on the long arm of chromosome 6 at 6q22.[9] Although the physiological function of *ROS1* is unknown, it is believed to be similar to the oncogenic function of *ALK* and *EGFR*.[10] *ROS1* rearrangement is therapeutically actionable in 1-2% of patients with advanced NSCLC. *ROS1* rearrangement in NSCLC patients has received attention as a recently targetable genetic alteration for treatment.[11,12]

Oncogene activation plays a vital role in the development of NSCLC. Oncogenic *ROS1* rearrangement in NSCLC was first described in 2007.[13] The *ROS1* oncogene was added to the list of NSCLC oncogenes in 2012.[14] Subsequently, *ROS1* rearrangement has become a therapeutic target.[15] Crizotinib was the first tyrosine kinase inhibitor (TKI) recommended as first-line therapy for ROS1 targeting. Clinical trials for new-generation TKIs are now underway.[16] Crizotinib was initially approved for the treatment of *ALK*-rearranged advanced NSCLC. Subsequent preclinical studies demonstrated that *ROS1* rearrangement was highly sensitive to crizotinib.[10] The use of crizotinib in patients with advanced NSCLC with *ROS1* rearrangement was approved by the American Food and Drug Administration and the European Medicines Agency in 2016.[17,18] Subsequent clinical studies reported objective response rates ranging from 69% to 83%.[16,19] The study of *ROS1* rearrangement in NSCLC has gained importance as it enables personalized treatment of NSCLC with tyrosine kinase inhibitors.[20]

The immunohistochemistry (IHC) method is recommended for screening *ROS1* fusions. It provides fast results and relatively low-cost advantages.[21] Although the pattern of ROS1 staining varies depending on the type of fusion and antibody used, diffuse staining of moderate-to-strong intensity indicates the presence of *ROS1* fusion.[22] Some tumors with EGFR mutations may exhibit nonspecific weak (1+) or strong (3+) ROS1 IHC expression, which is typically heterogeneous.[23] Most mucinous adenocarcinomas without *ROS1* rearrangement respond to IHC with a different granular pattern.[23,24] ROS1 IHC may exhibit nonspecific positive staining in nonneoplastic tissues, such as reactive pneumocytes, alveolar macrophages, and osteoclast-type giant cells.[24,25]

Fluorescence in situ hybridization (FISH) is a reliable technique for detecting *ROS1* rearrangement. Although it is quite reliable, it has limitations because the correct interpretation of results requires experience and depends on adherence to guidelines.[21] The FISH method is believed to detect all the rearrangements of the theoretically known *ROS1* breakpoints. *ROS1* fusions are typically caused by interchromosomal rearrangements, such as translocation or insertion, but rare intrachromosomal rearrangements, such as deletions, duplications, and inversions, have been reported.[21,26] However, false-negative results in the ROS1 FISH test are uncommon.[12,15] When *ROS1* fusions involve intrachromosomal rearrangements on the same chromosome, some break-apart patterns with the FISH technique may not identify them.[26] *ROS1* fusion partners are found on different chromosomes from the native *ROS1* gene, except for five genes. Fused in glioblastoma (FIG, also known as *GOPC*), *EZR*, *HLA-A*, *CEP85L*, and *TPD52L1* are intrachromosomal fusion partner genes located in the same chromosome with the native *ROS1* gene on chromosome 6.[21] Due to the close location, the FISH technique may make it challenging to identify intrachromosomal separated signals, such as FIG-ROS1 and EZR-ROS1.[12,26] Furthermore, rearrangements occurring at the transcriptional level and other genetic or epigenetic mechanisms, such as alternative transcript initiation, may lead to *ROS1* overexpression that FISH cannot detect. In this case, confirmation using alternative tests is required.[23,25]

Updated molecular testing guidelines (The College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology) suggest that the ROS1 IHC method can be used as an assay algorithm by confirming each IHC-positive cases with molecular tests, such as FISH, reverse transcription polymerase chain reaction (RT-PCR), or next-generation sequencing (NGS).[20,21,27]

D4D6 and SP384 are the most commonly used in the clinical practice for ROS1 IHC studies.[24] The SP384 clone is more recently developed and commercially available. This study aimed to compare the D4D6 and SP384 ROS1 IHC clones and examine their consistency with the FISH results in samples with NSCLC.

MATERIALS AND METHODS

Sample collection and study design

Approved with IHC and FISH results, ROS1 positive and IHC and FISH results in discordant samples, diagnosed with NSCLC, with sufficient tissue sample (≥ 50 tumor cells), were selected for the study. For this purpose, archival samples were scanned at the University Hospital. The study included 14 samples with sufficient tissue from IHC- and FISH-positive samples. Four samples with sufficient tissue were included in the study from IHC and FISH discordant samples. Eighty-five consecutive samples with IHC- and FISH-negative sufficient tissue were included in the study as negative controls. A total of 103 formalin-fixed, paraffin-embedded (FFPE) tissue block samples were sectioned at 4 μm thickness.

All samples were initially tested with ROS1 IHC D4D6 and SP384 clones. The *ROS1* status was then confirmed using the FISH method.[21] Finally, samples with discordant IHC and FISH results were analyzed using RT-PCR as a confirmation method.

Immunohistochemistry analysis

For both primary antibodies, ROS1 IHC expression was performed on a BenchMark ULTRA staining automated IHC system (Ventana Medical Systems, Tucson, Arizona). IHC staining was performed on 4-μm-thickness FFPE tissue samples using D4D6 (Cell Signaling Technology, Danvers, Massachusetts) and SP384 (Ventana Medical Systems, Tucson, Arizona) clones. The protocols of both antibodies are briefly shown in Table 1. For both clones, the staining protocols of the antibodies were made according to the datasheet, and the positive control staining was obtained before the study.

IHC scores were semiquantitatively graded according to their staining intensities as follows: strong staining (3 +) visible using x 4 objective with > 5% tumor cells, moderate staining (2 +) visible using x 20 objective with > 5% tumor cells, weak staining (1 +)

visible using x 40 objective with > 5% tumor cells or any staining intensity with ≤ 5% tumor cells, and negative staining (0) or absence of expression.[28] For all samples, IHC was also interpreted as homogeneously or heterogeneously stained.

FISH analysis

FISH analysis was performed on 4-µm-thickness FFPE tissue samples. *ROS1* rearrangement was detected using the 6q22 ROS1 Break Apart FISH probe (Abbott Molecular, Des Plaines, Illinois). The FISH preparation steps, including deparaffinization, prehybridization, and hybridization, were performed by the Abbott pretreatment kit and the probe datasheet.

Each hybridization was analyzed using a fluorescence microscope with an oil-immersion x 60 objective, identifying an area of high proportional neoplastic core density and evaluating a minimum of 50 non-overlapping interphase nuclei for the number of signals in each core. Tumor cells were captured using a fluorescence microscope (Olympus BX61, Olympus Optical, Japan) and compatible software (Duet®, Bioview Ltd., Israel). The FISH evaluation was interpreted and scored on images from Duet® Bioview digital analysis system.

A minimum of 50 nonoverlapping interphase tumor nuclei were counted for interpretation. Negative for *ROS1* rearrangement was defined when the proportion of positive cells was < 10% (< 5 in 50 cells). If the rate was > 30% (rearrangements in > 15 of 50 cells), *ROS1* rearrangement was considered positive. If the rate was 10% to 30% (5-15 of 50 cells), 50 more tumor cells were added to the evaluation. In the last evaluation with 100 tumor cells, > 15% (> 15 cells of 100 cells) were defined as positive.[21]

RT-PCR analysis

In samples with discordant FISH and IHC results, the AmoyDx gene fusion detection kit was used as a confirmatory RT-PCR method. The AmoyDX ROS1 Gene Fusion Detection Kit (Amoy Diagnostics Co., Ltd, Xiamen, China) is a CE-IVD-labeled RT-PCR to detect the 14 most common ROS1 fusions.

Statistical analysis

Immunohistochemistry clones were compared, and the correlation of IHC and FISH results was analyzed separately for each clone. We used the Shapiro-Wilk test to control the normality assumption. Because the data did not normally distribute, we used non-parametric hypothesis tests. The Spearman's rank correlation test was used to calculate the correlation between the IHC scores and FISH results of clones. The IHC score was used to identify IHC-positive samples (IHC-positive cut-off is ≥ 1+ intensity, any percentage of tumor cells). In the FISH evaluation, each sample's positivity percentage was determined (FISH-positivity cut-off is 15% of tumor cells). The Mann-Whitney U test was used to compare the distributions of positive FISH patterns. IBM SPSS Statistics Windows, Version 24.0 (IBM Corp., Armonk, New York) was used for statistical calculations, which were two-sided, and $p \leq 0.05$ indicated statistical significance.

RESULTS

FISH results

All samples (103 samples) were tested with the FISH technique. FISH analysis revealed that 14 samples were FISH-positive and 89 samples were FISH-negative. Among the 14 FISH-positive samples analyzed, 4 samples (28.6%) showed a break-apart pattern, and 10 samples (71.4%) showed an isolated 3' (single green signal) pattern.

The mean percentage of positive cells in *ROS1* FISH-positive samples was 80.2% (median, 82%; range, 42-100%), and the mean percentage of positive cells in *ROS1* FISH-negative samples was 0.88% (median, 0%; range, 0-4%). Two positive FISH patterns (break-apart and isolated 3′) had similar numbers of rearranged cells, with mean of 82.75% and 79.2%, respectively ($p = 0.94$). A summary of FISH-positive results is shown in Table 2.

TABLE 1. Comparison of ROS1 Immunohistochemistry Antibodies Protocols.

	SP384 (Ventana Medical systems)	D4D6 (Cell Signaling technology)
Source/isotype	Rabbit	Rabbit
Platform	Ultra Benchmark Autostainer (Ventana)	Ultra Benchmark Autostainer (Ventana)
Section thickness	4 µm	4 µm
Dilution	Ready to use	1/250
Antigen retrieval	64-min CC1	64 min CC1
Antibody (primary) Incubating conditions	16 min, 37 °C	64 min, 37 °C
Counterstain	16-min hematoxylin II	16-min hematoxylin
Post counterstain	4-min bluing reagent	4-min bluing reagent
Detection system	Optiview	Optiview
OptiView HQ Linker	8 min	8 min
OptiView HRP multimer	8 min	8 min
Optiview amplification	Unselected	8 min

Immunohistochemistry results

The slides were interpreted by a pathologist blinded to the FISH results. All samples (103 samples) were tested with SP384 and D4D6 anti-ROS1 IHC antibodies.

SP384 IHC test results revealed that 86 samples (16.5%) were negative and 17 samples (83.5%) were positive. Positive samples were distributed as follows: 1 (5.88%) had a weak [1 +] intensity, 3 (17.65%) had a moderate [2 +] intensity, and 13 (76.47%) had a strong [3 +] intensity.

D4D6 IHC test results revealed that 86 samples (16.5%) were negative and 17 samples (83.5%) were positive. Positive samples were distributed as follows: 8 (47.06%) had a weak [1 +] intensity, 8 (47.06%) had a moderate [2 +] intensity, and 1 (5.88%) had a strong [3 +] intensity.

Comparison between D4D6 and SP384 clones in ROS1 FISH-positive samples

In FISH-positive samples, the SP384 clone showed 3 + strong staining in 11 of 14 samples (78.57%), 2 + moderate staining in three of 14 samples (21.43%), and 1 + weak staining in 0 of 14 samples (0%). IHC D4D6 clone showed 3+ strong staining in 0 of 14 samples (0%), 2 + moderate staining in six of 14 samples (42.86%), and 1 + weak staining in eight of 14 samples (57.14%; Table 3).

All *ROS1* FISH rearranged samples (14 samples) were positive for both clones; however, SP384 was mostly higher intensity than D4D6. The average IHC score of SP384 was 2, and the average IHC score of D4D6 was 1.17. Both clones were 100% sensitive for ≥ 1 + IHC score. However, for ≥ 2 + IHC score evaluation, the SP384 clone was 100% sensitive, whereas the D4D6 clone was 42.86% sensitive for the ROS1 rearranged samples (Table 3). Representative comparative images of ROS1 IHC using the D4D6 and the SP384 clones and the *ROS1* FISH analysis images are shown in Figure 1.

The IHC results of the two samples showed discordance in both clones and FISH results. Sample 20 was positive [3 +] with SP384 and negative with D4D6. Sample 21 was positive [2 +] with D4D6 and negative with SP384. These two discordant samples showed negative *ROS1* rearrangement FISH results. Two nonrearranged samples (17 and 18) stained either SP384 or D4D6 clones (Table 2). There was no significant correlation between ROS1 FISH-positivity percentage with SP384 ($p = 0.713$, $\rho = 0.108$) and D4D6 ($p = 0.26$, $\rho = -0.323$) IHC intensity (Table 3). Furthermore, when the samples were compared with the staining pattern (homogeneously/heterogeneously), mostly similar results were obtained from both clones.

RT-PCR results

The RT-PCR method was used to assess *ROS1* rearrangement in six samples, including FISH- and IHC-positive control[9] and negative control samples[19] and four discordant samples.[15-18] RT-PCR results were negative, except for the FISH- and IHC-positive control samples.[9] Furthermore, the RT-PCR results were consistent with the FISH results. The results of all methods used to compare the ROS1 status are shown in Table 4.

TABLE 2. Immunohistochemistry and Fluorescence In Situ Hybridization Results of Positive and Discordant Samples.

Sample no.	SP384 intensity	Staining pattern	D4D6 intensity	Staining pattern	FISH results
1	2	Heterogeneous	1	Heterogeneous	Rearranged (isolated 3′) 100%
2	3	Heterogeneous	1	Heterogeneous	Rearranged (isolated 3′) 88%
3	2	Heterogeneous	1	Heterogeneous	Rearranged (isolated 3′) 78%
4	3	Homogeneous	2	Homogeneous	Rearranged (break apart) 80%
5	3	Heterogeneous	1	Homogeneous	Rearranged (break apart) 90%
6	2	Heterogeneous	1	Heterogeneous	Rearranged (isolated 3′) 42%
7	3	Homogeneous	2	Homogeneous	Rearranged (break apart) 67%
8	3	Homogeneous	2	Heterogeneous	Rearranged (isolated 3′) 86%
9	3	Homogeneous	2	Homogeneous	Rearranged (isolated 3′) 70%
10	3	Homogeneous	1	Homogeneous	Rearranged (isolated 3′) 82%
11	3	Homogeneous	1	Homogeneous	Rearranged (isolated 3′) 82%
12	3	Heterogeneous	1	Heterogeneous	Rearranged (isolated 3′) 96%
13	3	Homogeneous	2	Homogeneous	Rearranged (isolated 3′) 68%
14	3	Homogeneous	2	Homogeneous	Rearranged (break apart) 94%
15	3	Homogeneous	0	-	Non-rearranged
16	0	-	2	Homogeneous	Non-rearranged
17	1	Heterogeneous	2	Heterogeneous	Non-rearranged
18	3	Homogeneous	3	Homogeneous	Non-rearranged

DISCUSSION

It has been defined that there is a concordance between the IHC and FISH methods for the ALK test.[29] Recently, it has been reported that immunohistochemical studies are sufficient for the ALK test and can be used instead of the FISH test.[30] Recent research has suggested that the close correlation between ALK IHC and FISH may not be equally present for ROS1 IHC and FISH using currently available reagents.[10] However, a similar screening approach used for ALK testing in many centers has been maintained in the hope

TABLE 3. Distribution of D4D6 and SP384 Immunohistochemistry Scores and Correlation of Immunohistochemistry Scores with Fluorescence In Situ Hybridization Positivity Percentage in ROS1 Fluorescence In Situ Hybridization Positive Samples.

		SP384		p/ρ
IHC scores	Weak 1 +	Moderate 2 +	Strong 3 +	
FISH percentage range	-	42-100%	67-96%	
Samples (n = 14)	0 (0%)	3 (21.43%)	11 (78.57%)	0.713/0.108
		D4D6		p/ρ
IHC scores	Weak 1 +	Moderate 2 +	Strong 3 +	
FISH percentage range	42-100%	67-94%	-	
Samples (n = 14)	8 (57.14%)	6 (42.86%)	0 (0%)	0.26/-0.323

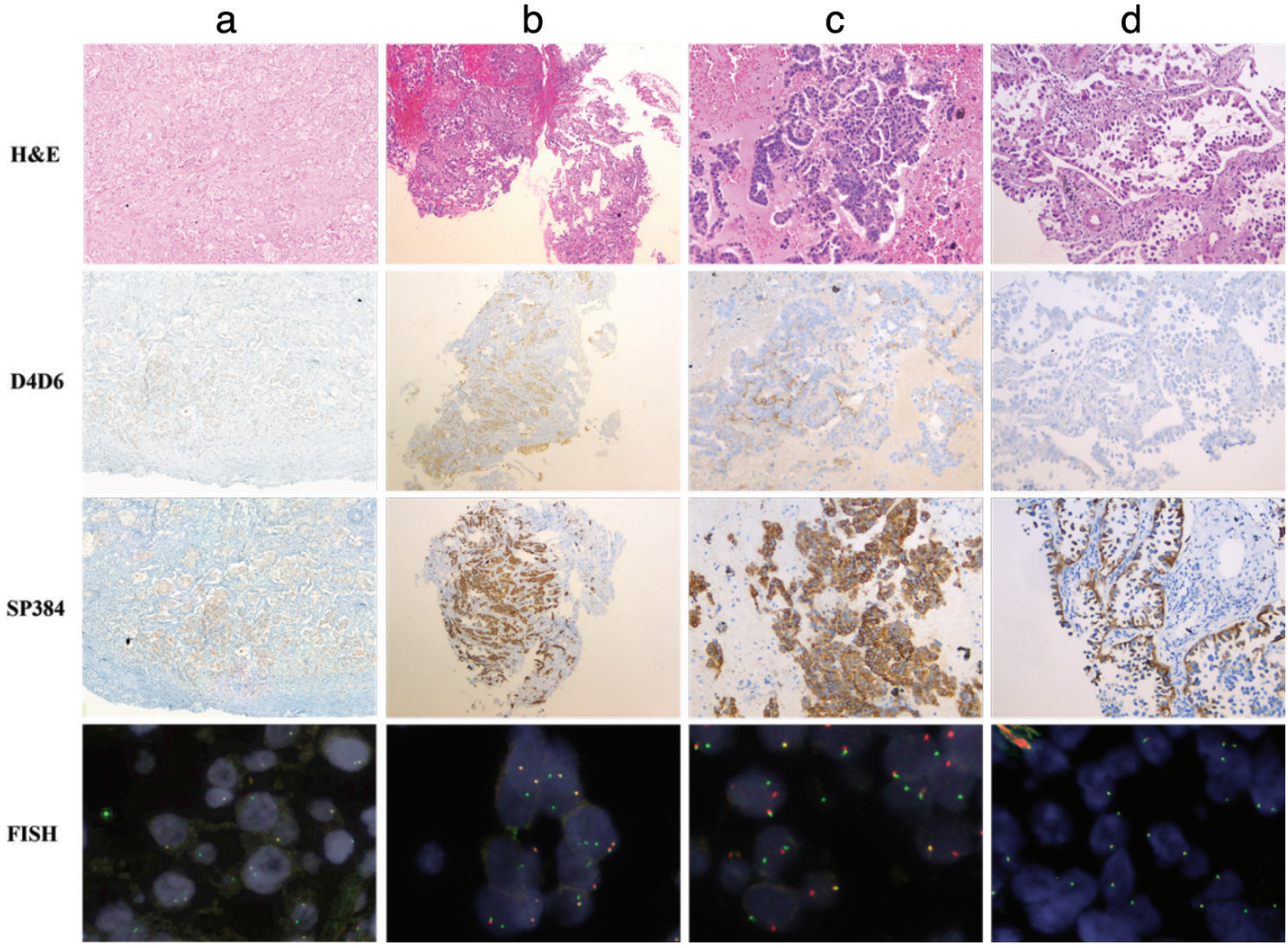

FIG. 1. Representative comparative samples of the hematoxylin and eosin (H&E), ROS1 IHC (SP384 and D4D6), and ROS1 FISH images. (a)-(d) represent samples 6, 7, 14, and 15, respectively. (a) [1 +] weak staining with D4D6 IHC and [2 +] moderate staining with SP384 IHC in isolated 3' (42%) pattern ROS1 FISH-positive. (b) [2 +] moderate staining with D4D6 IHC and [3 +] strong staining with SP384 IHC in isolated 3′ (67%) pattern ROS1 FISH-positive. (c) [2 +] moderate staining with D4D6 IHC and [3 +] strong staining with SP384 IHC in break apart (94%) pattern ROS1 FISH-positive. (d) [0] negative staining with D4D6 IHC and [3+] strong staining with SP384 IHC in ROS1 FISH-negative. H&E and IHC digital images are at 200x, and FISH digital images are at 600x magnification.

TABLE 4. Comparison of ROS1 Status in Discordant Samples in Terms of Immunohistochemistry, Fluorescence In Situ Hybridization, and RT-PCR Results.

Sample no.	SP384 results	D4D6 results	FISH results	RT-PCR results	Consensus status
9	Positive	Positive	Positive	Positive	Positive
19	Negative	Negative	Negative	Negative	Negative
15	Positive	Negative	Negative	Negative	Negative
16	Negative	Positive	Negative	Negative	Negative
17	Positive	Positive	Negative	Negative	Negative
18	Positive	Positive	Negative	Negative	Negative

Samples 9 and 19 are positive and negative controls, respectively.

that it may be an effective method to detect ROS1 abnormality. However, some tumors with EGFR mutations may exhibit 3 + ROS1 IHC, which is a nonspecific, weak expression, and heterogeneous.[23] In addition, many mucinous adenocarcinomas without ROS1 rearrangement may show reactivity with IHC with a different granular pattern.[23,24] Similarly, nonneoplastic tissues, including reactive pneumocytes, alveolar macrophages, and osteoclast-type giant cells, may show nonspecific positive staining by ROS1 IHC.[24,25] As a result, the ROS1 IHC remained only a preliminary screening test. Nevertheless, guidelines recommend knowing these pitfalls and confirming positive samples with FISH or another molecular technique (RT-PCR and NGS).[20,21,27] Therefore, we aimed to compare the commonly used two ROS1 IHC antibodies (D4D6 and SP484) and their consistency with FISH results in this study.

The *ROS1* rearrangement represents only 1-2% of the NSCLC samples.[12] The ROS1 protein in the cytoplasm of tumor cells is used as a potential analysis tool to detect rearrangement in the 6q22 *ROS1* gene.[20] The most important advantages of using IHC biomarkers are speed, cost, and easy assay interpretation. SP384 and D4D6 clones are used to determine ROS1 status with IHC. There are variable results in studies comparing two clones in the literature. In a study investigating the efficacy of both clones, SP384 was found to be more sensitive than D4D6.[24] In contrast, in another study, both clones were reported to have variable sensitivity and specificity, with D4D6 providing more specific and accurate results than SP384 in samples whose ROS1 rearrangement was confirmed by the FISH method.[31] In our study, we compared the efficacy of two clones on ROS1 FISH-positive samples and found that the SP384 clone was more sensitive and had a higher intensity of staining in positive samples. We observed the same sensitivity with a ≥ 1 + cut-off in ROS1 FISH-positive samples between the two clones (both with 100%). However, when the ≥ 2 + cut-off was used, the sensitivity rate for the SP384 clone was 100%, whereas the sensitivity decreased to 42.86% for the D4D6 clone. The specificity of the SP384 and D4D6 clones was the same when using the ≥ 1 + cut-off (both with 100%), whereas using the ≥ 2 + cut-off was similar (97.75% and 96.62%, respectively).

We found that the SP384 antibody was more sensitive and positive samples stained better. The average IHC score for SP384 was + 2, whereas the average IHC score for D4D6 was + 1.17. The higher average IHC score of SP384 than the D4D6 antibody led to easier assessment. In addition, the intensities of overall stained tumor cells were higher in the SP384 clone than in clone D4D6. In the samples compared in terms of the staining pattern (homogeneously/heterogeneously), mostly similar results were obtained from both clones, except for two samples (samples 5 and 8). No significant correlation was reported between the D4D6 ROS1 IHC intensity and FISH-positivity percentage in the literature.[32] We found no significant correlation between IHC staining intensity and FISH-positivity percentage (SP384, $p = 0.713$ and D4D6, $p = 0.26$).

Some reports revealed that the D4D6 clone may show false-positive results.[32] Our study similarly found false-positive results in the D4D6 clone. In addition, we determined that the SP384 clone may also show false positivity. All samples were initially tested with ROS1 IHC D4D6 and SP384 clones. It was then tested for ROS1 status using the FISH method, and the results were compared. IHC and FISH results in discordant cases were analyzed using RT-PCR to confirm. As a result of this comparison, we determined that some samples stained with SP384 clones were negative by FISH and RT-PCR. This situation was observed in the same sample with the D4D6 clone and in a different sample. As a result, we determined that although SP384 is more sensitive than D4D6, the SP384 clone may give false-positive results.

Our study has limitations, including its retrospective nature, selection bias, and sample size. Nevertheless, to the best of our knowledge, it includes all known sample variations for comparing two antibodies.

In conclusion, the ROS1 IHC assay is a useful screening test for ROS1 immunoreactivity. We found that the ROS1 SP384 clone was more sensitive than D4D6. The higher average IHC score of SP384 than the D4D6 antibody led to easier assessment. However, before using ROS1 antibodies in clinical applications, it is important to know the variable diagnostic performance of different ROS1 antibodies. Although SP384 is more sensitive than D4D6, it can also cause false-positive results as D4D6. Therefore, IHC-positive results alone will not be sufficient to detect ROS1 positivity in clinical applications. IHC-positive results should be confirmed by a molecular method, such as FISH and RT-PCR, in determining ROS1 positivity. Furthermore, we found EGFR mutation and RET mutation positivity in IHC ROS1-positive and FISH-negative cases (unpublished data). Therefore, patients

with positive ROS1 IHC and negative FISH may have molecular alterations in the lung that are sensitive to other tyrosine kinase inhibitors.

Ethics Committee Approval: Ethical approval for this study was obtained from the Cerrahpaşa, Clinical Research Ethical Committee of Cerrahpaşa Medical Faculty, İstanbul University-Cerrahpaşa.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- O.D., B.Ö.; Design- O.D., B.Ö.; Data Collection or Processing- O.D., B.Ö.; Analysis or Interpretation- O.D., B.Ö.; Literature Search- O.D., B.Ö.; Writing- O.D., B.Ö.

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