

## Lung Cancer

# Correlation of *ROS1* (D4D6) Immunohistochemistry with *ROS1* Fluorescence In Situ Hybridization Assay in a Contemporary Cohort of Pulmonary Adenocarcinomas

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## Abstract



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## Keywords

- ▶ *ROS1*
- ▶ D4D6 clone
- ▶ immuno-histochemistry
- ▶ FISH
- ▶ pulmonary adenocarcinoma

**Objective** Repressor of Silencing (*ROS1*) gene rearrangement in the lung adenocarcinomas is one of the targetable mutually exclusive genomic alteration. Fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), next-generation sequencing, and reverse transcriptase polymerase chain reaction assays are generally used to detect *ROS1* gene alterations. We evaluated the correlation between *ROS1* IHC and FISH analysis considering FISH as the gold standard method to determine the utility of IHC as a screening method for lung adenocarcinoma.

**Materials and Methods** A total of 374 advanced pulmonary adenocarcinoma patients were analyzed for *ROS1* IHC on Ventana Benchmark XT platform using D4D6 rabbit monoclonal antibody. FISH assay was performed in parallel in all these cases using the Vysis *ROS1* Break Apart FISH probe.

**Statistical Analysis** The sensitivity, specificity, positive and negative likelihood ratios, positive and negative predictive values, and accuracy were evaluated.

**Results** A total of 17 tumors were positive either by IHC or FISH analysis or both (true positive). Four tumors were positive by IHC (H-score range: 120–270), while negative on FISH analysis (false positive by IHC). One tumor was IHC negative, but positive by FISH analysis (false negative). The sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, positive predictive value, negative predictive value, and

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accuracy were 94.4% (confidence interval [CI]: 72.71–99.86%), 63.6% (CI: 30.79–89.07%), 2.6 (CI: 1.18–5.72), 0.09 (CI: 0.01–0.62), 80.95% (CI: 65.86–90.35%), 87.5% (CI: 49.74–98.02%), and 82.76%, respectively.

**Conclusion** *ROS1* IHC has high sensitivity at a cost of lower specificity for the detection of *ROS1* gene rearrangement. All IHC positive cases should undergo a confirmatory FISH test as this testing algorithm stands as a reliable and economic tool to screen *ROS1* rearrangement in lung adenocarcinomas.

## Introduction

The Repressor of Silencing (*ROS1*) oncogene, tyrosine kinase phosphorylation, and fusion proteins as drivers in nonsmall-cell lung cancer (NSCLC) were initially identified in 2007.<sup>1–3</sup> The *ROS1* translocation/rearrangement is observed in 1 to 2% of NSCLC patients.<sup>4,5</sup> Interchromosomal and intrachromosomal rearrangements of the *ROS1* result in a fusion that leads to a constitutively active kinase that activates the MAP kinase, STAT3, and phosphoinositide-3-kinase pathways that drive cellular transformation.<sup>6–8</sup> Histopathologic and clinical profiles that are associated with the *ROS1* translocation include adenocarcinoma histology, younger age, and nonsmokers, a profile similar to the anaplastic lymphoma kinase (ALK)-rearranged NSCLCs.<sup>2,6</sup> The U.S. Food and Drug Administration has approved crizotinib, a small molecule oral tyrosine kinase inhibitor (TKI), in the *ROS1*- and *ALK*-rearranged NSCLC patients due to a high degree of homology between the *ROS1* and *ALK* tyrosine kinase domains.<sup>5</sup> Crizotinib has demonstrated high overall response rates of 72% in metastatic NSCLC (mNSCLC) with the *ROS1* rearrangement,<sup>9</sup> a subset in which the responses to traditional chemotherapy regimens have been less than 10%.<sup>10</sup> Hence, assays detecting the *ROS1* gene rearrangement as an actionable target are now being performed routinely in frontline mNSCLC.<sup>11</sup>

The *ROS1* fusion in the tumor cells can be detected using a variety of techniques including fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), reverse transcriptase polymerase chain reaction (RT-PCR), and next-generation sequencing. The *ROS1* Break Apart FISH assay is used in the pivotal crizotinib trials as a gold standard to detect *ROS1* rearrangement.<sup>12–14</sup> However, FISH assay is not universally performed in all laboratories, is relatively labor intensive, expensive, and has a comparatively longer turn-around time (TAT). IHC to detect *ROS1* fusion has a low specificity and hence a follow-up confirmatory testing with FISH is necessary for utilizing *ROS1* IHC as a screening modality.<sup>11</sup> Yet, IHC is performed across many laboratories, does not need expertise, is inexpensive, and has a shorter TAT, thus rendering it as a screening modality for *ROS1* testing in comparison to FISH testing. There are a few studies comparing IHC with FISH assay to detect the *ROS1* rearrangement. The overall sensitivity and specificity of IHC in comparison to FISH ranges from 97.8 to 100% and 72.6 to 96.67%, respectively.<sup>13–21</sup>

We sought to evaluate the correlation between *ROS1* IHC and FISH analyses to determine the utility of IHC as a

screening method for lung adenocarcinoma. Currently, there is a lack of Indian literature describing the use of *ROS1* IHC as a screening technique for lung adenocarcinoma and comparison between the IHC and FISH results.

## Materials and Methods

A total of 374 advanced pulmonary adenocarcinoma patients (January 2017 to November 2017) were analyzed to detect the *ROS1* rearrangement by both IHC and FISH following approval by the institutional review board. *ROS1* IHC was performed using the rabbit monoclonal D4D6 antibody clone (Cell Signaling Technology, RTU) on the automated Ventana Benchmark XT platform. A positive and a negative control were run with each case. Appendix was used for both positive and negative controls. Primary antibody was not put on the negative control slides. Rest all steps were similar. However, there is no established benign tissue that can be utilized as the positive control. The tumor cell lines with already proven *ROS1* gene rearrangement were used as the representative tissues for validating IHC as positive control. Furthermore, the staining pattern differs with the fusion partner of *ROS1* gene. The analysis was based on an H-score system that calculates a score from 0 to 300 taking both the intensity (0 = no staining; 1 = weak; 2 = moderate; 3 = strong) of tumor cell (cytoplasmic) staining and the percentage of tumor cells stained into consideration. The following formula was used for calculating the H-score: (1 × [percentage of tumor cells with 1+ Staining] + 2 × [percentage of tumor cells with 2+ Staining] + 3 × [percentage of tumor cells with 3+ Staining]). An H-score of ≥100 was considered positive.<sup>13</sup>

FISH analysis for *ROS1* gene rearrangement was performed on the formalin fixed paraffin embedded tissue section with 4 μm thickness. A Vysis Break Apart probe was designed to detect *ROS1* rearrangements mapping to chromosome band 6q22.1. The following standard procedures were performed for performing the FISH analysis. The tumor areas were highlighted on the hematoxylin and eosin-stained slides, excluding the necrotic areas, and were evaluated on the hybridized slides to determine the specificity of hybridization, probe signal intensity, and signal to hybridization ratio for optimum analysis. The low-power (×10) analyses were based on abundance of abnormal cells, even distribution and the presence of very few overlapping abnormal nuclei, and the presence of heterogeneity (presence of subclonal changes), whereas the high-power (×60 or

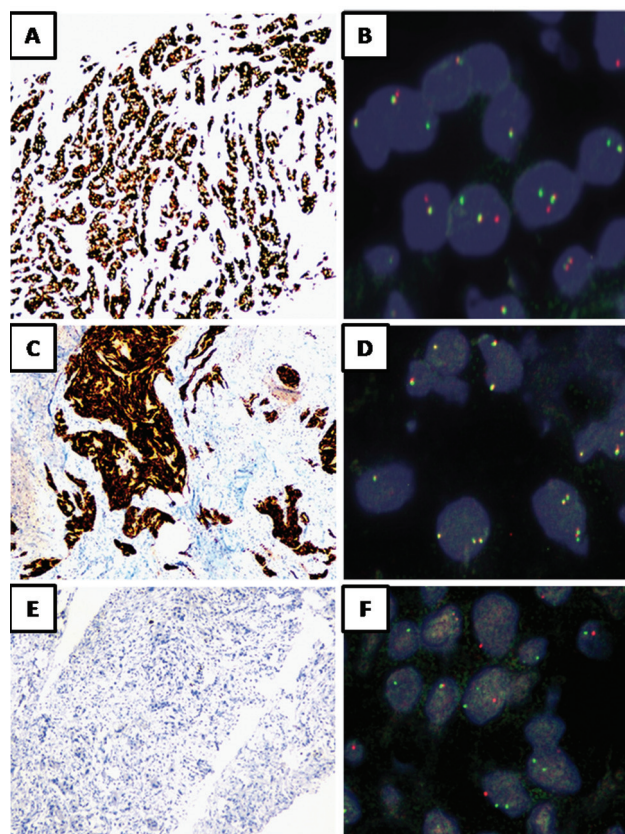
×100) analyses helped in the assessment of nonoverlapping, distinct, and nondisrupted nuclei with bright uniform 4',6-diamidino-2-phenylindole staining, a score of nuclei of a similar size to avoid truncation effect, and avoidance of autofluorescent structures. The slides and areas that passed the aforementioned criteria were enumerated for fluorescent signals. *ROS1* Break Apart (red/orange and green) signals were enumerated on their own using a single band-pass filter. It was started with one probe, followed by enumeration of the signals in each cell, and then was proceeded to the green filter for the other. This was followed by checking under the dual band-pass filter to look for a fused yellow signal. The number of signals in the nucleus was recorded on the score sheet. Inconclusive cells were not counted. Around 100 to 200 abnormal cells were counted. A valid preparation showed bright signals in >90% of the cells. Similar-sized nuclei were chosen to avoid truncation effect and autofluorescent bodies were distinguished. Once the abnormal cells were scored, the number of fused (yellow, normal pattern) and discrete individual (red/orange and green, split signal) signals/cells were counted. If the average percentage of positive tumor cells with a split signal was 10% (10/100), the sample was considered positive. The FISH result was considered noninformative in the following cases: slides having less than 50 scorable abnormal cells, slides with no or patchy hybridization, and slides with high background or autofluorescence that interfered with signal enumeration.<sup>13–15</sup>

## Results

A total of 17 tumors were positive either by IHC or FISH analysis or both (true positive). Four tumors were positive by IHC (H-scores 120, 150, 190, and 270), while negative on FISH analysis (false positive by IHC). One tumor was IHC negative, but positive by FISH analysis (false negative; →Fig. 1 and →Table 1). The sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, positive predictive value, negative predictive value, and accuracy were 94.4% (confidence interval [CI]: 72.71–99.86%), 63.6% (CI: 30.79–89.07%), 2.6 (CI: 1.18–5.72), 0.09 (CI: 0.01–0.62), 80.95% (CI: 65.86–90.35%), 87.5% (CI: 49.74–98.02%), and 82.76%, respectively (→Table 2).

## Discussion

*FIG-ROS1*, *SLC34A2-ROS1*, *CD74-ROS1*, *SDC-ROS1*, *EZR-ROS1*, *LRIG3-ROS1*, and *TPM3-ROS1* are various *ROS1* gene fusions studied in 1 to 2% of nonsmall-cell lung carcinoma.<sup>2,8,22</sup> Most clinical trials conducted to establish the role and efficacy of TKIs in patients with *ROS1*-rearranged NSCLC have looked at the FISH analysis results. IHC, however, may be used as a cheaper alternative and a surrogate for *ROS1* rearrangement. IHC holds some advantages over FISH for determining *ROS1* status. It is cost-effective, easier to train on, and fast to perform. IHC is especially useful as a screening tool with low-prevalence biomarkers such as *ROS1*. In our study, *ROS1* IHC using antibody D4D6 has high sensitivity (94.4%) but the



**Fig. 1** (A and B) A case of pulmonary adenocarcinoma with diffuse and strong cytoplasmic Repressor of Silencing (*ROS1*) staining by immunohistochemistry (IHC, A) with presence of *ROS1* gene rearrangement by fluorescence in situ hybridization (FISH, B). (C and D) A case of pulmonary adenocarcinoma with multifocal and strong cytoplasmic *ROS1* staining by IHC (C) while absence of *ROS1* gene rearrangement by FISH (D). (E and F) A case of pulmonary adenocarcinoma with no staining on *ROS1* IHC (E), but presence of *ROS1* gene rearrangement by FISH (F) (IHC, 4 ×, *ROS1* antibody; FISH, 60 ×, fluorescent signals were captured under Olympus fluorescent microscope with Bioview FISH software).

specificity of detection of *ROS1* gene rearrangement is low (63.6%). Cao et al<sup>15</sup> conducted a study on the comparative assessment of FISH, IHC, and RT-PCR in detecting the *ROS1* fusion. Huang et al showed a high correlation between *ROS1* FISH and IHC using SP384 clone. Using cytoplasmic IHC score of ≥2+ in more than 30% of tumor cells as the cutoff, they observed a high correlation with FISH positivity (97.8% positive percentage and 89.5% negative percentage agreement).<sup>13</sup> Shan et al in their cohort of 60 patients demonstrated that 16 (26.7%) and 13 (21.7%) patients were *ROS1* positive by IHC and FISH, respectively. They showed a sensitivity and specificity of IHC to be 100% and 93.6%, respectively.<sup>14</sup> Considering FISH as the gold standard method, Cao et al has demonstrated that the sensitivity and specificity of *ROS1* IHC with ≥1+ staining were 100% and 96.67%, respectively.<sup>15</sup> Sholl et al showed 100% sensitivity and 92% specificity between *ROS1* IHC and FISH, using D4D6 clone in their cohort of pulmonary adenocarcinomas.<sup>16</sup> Cha et al used the D4D6 *ROS1* clone for the detection of patients who harbor *ROS1* rearrangements in two separate cohorts. In the

**Table 1** Clinicopathologic characteristics of patients with ROS1 translocation

Age (y)	Gender	Stage	Smoking history (yes/no)	Histopathologic type	IHC	FISH	Response
28	F	IV	No	Adenocarcinoma, solid predominant	Positive	Positive	Partial
65	M	IV	Unknown	Adenocarcinoma, acinar predominant	Negative	Positive	Unknown
46	F	IV	No	Adenocarcinoma, solid predominant	Positive	Positive	Unknown
54	M	IV	Unknown	Adenocarcinoma, solid predominant	Positive	Negative	Partial
31	F	IV	No	Adenocarcinoma, solid predominant	Positive	Negative	Partial
63	M	IV	Unknown	Adenocarcinoma, acinar predominant	Positive	Negative	Stable
44	M	IV	Unknown	Adenocarcinoma, solid predominant	Positive	Negative	Unknown
56	M	IV	Unknown	Adenocarcinoma, solid predominant	Positive	Positive	Partial
41	F	IV	No	Adenocarcinoma, solid predominant	Positive	Positive	Stable
38	F	IV	No	Adenocarcinoma, solid predominant with macronucleoli	Positive	Positive	Partial
65	F	IV	No	Adenocarcinoma, solid predominant	Positive	Positive	Stable
51	M	IV	Yes	Adenocarcinoma, solid predominant with macronucleoli	Positive	Positive	Partial
49	F	IV	No	Mucinous adenocarcinoma	Positive	Positive	Partial
55	F	IV	No	Adenocarcinoma, solid and micropapillary patterns	Positive	Positive	Partial
65	M	IV	Yes	Adenocarcinoma, solid predominant with macronucleoli	Positive	Positive	Stable
54	F	IV	No	Adenocarcinoma, solid predominant with macronucleoli	Positive	Positive	Partial
46	F	IV	Unknown	Adenocarcinoma, solid predominant with macronucleoli	Positive	Positive	Partial

Abbreviations: F, female; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; M, male; ROS1, Receptor of Silencing.

**Table 2** Statistical indicators calculated from the immunohistochemistry and fluorescence in situ hybridization assays

Indicators	Values	95% confidence interval
Sensitivity	94.44%	72.71–99.86%
Specificity	63.64%	30.79–89.07%
Positive likelihood ratio	2.6	1.18–5.72
Negative likelihood ratio	0.09	0.01–0.62
Positive predictive value	80.95%	65.86–90.35%
Negative predictive value	87.50%	49.74–98.02%
Accuracy	82.76%	–

**Table 3** Comparative data on the sensitivity and specificity of *ROS1* IHC and FISH testing

Study (Reference)	IHC antibody clone used	FISH platform	Sensitivity of IHC (%)	Specificity of IHC (%)
Cao et al <sup>15</sup>	<i>ROS1</i> (D4D6) rabbit monoclonal antibody	6q22 <i>ROS1</i> (Tel) Spectrum Orange Break Apart Probe	100	96.67
Shan et al <sup>14</sup>	<i>ROS1</i> (D4D6) rabbit monoclonal antibody	ZytoLight SPEC <i>ROS1</i> Dual Color Break Apart Probe	100	93.6
Huang et al <sup>13</sup>	<i>ROS1</i> (SP384) antibody (Ventana)	<i>ROS1</i> Dual Color Probe	97.8	89.5
Sholl et al <sup>16</sup>	<i>ROS1</i> (D4D6) rabbit monoclonal antibody	<i>ROS1</i> Gene (RP11–59K17 and RP1–92C8)	100	92
Cha et al <sup>17</sup> (retrospective analysis)	<i>ROS1</i> (D4D6) rabbit monoclonal antibody	Vysis LSI Dual Color, Break Apart Rearrangement Probe	100	93.4
Cha et al <sup>17</sup> (prospective analysis)	<i>ROS1</i> (D4D6) rabbit monoclonal antibody	Vysis LSI Dual Color, Break Apart Rearrangement Probe	100	72.6
Mescam-Mancini et al <sup>18</sup>	<i>ROS1</i> (D4D6) rabbit monoclonal antibody	Aquarius Pathology <i>ROS1</i> Break Apart Probe and/or the ZytoLight SPEC <i>ROS1</i> Dual Color Break Apart Probe	100	96.9
Selinger et al <sup>20</sup>	<i>ROS1</i> (D4D6) rabbit monoclonal antibody	ZytoLight SPEC <i>ROS1</i> Dual Color Break Apart Probe and the LSI <i>ROS1</i> (Tel) Spectrum Orange Probe and LSI <i>ROS1</i> (Cen) Spectrum Green Probe	100	76
Viola et al <sup>21</sup>	<i>ROS1</i> (D4D6) rabbit monoclonal antibody	Cytocell <i>ROS1</i> Dual Color Break Apart FISH Probe	100	83
Our study	<i>ROS1</i> (D4D6) rabbit monoclonal antibody (Ventana)	Vysis <i>ROS1</i> Break Apart FISH Probe	94.4	63.6

Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; *ROS1*, Repressor of Silencing.

retrospective cohort, they observed a sensitivity of 100% and specificity 93.4%; however, the specificity was 72.6% and sensitivity 100% in their prospective cohort.<sup>17</sup> Using D4D6 clone with the positivity defined as IHC score of 2+, Mes-

cam-Mancini et al have shown a sensitivity of 100% and the specificity 96.9%.<sup>18</sup> Wu et al have demonstrated a high congruence between FISH and IHC, when IHC showed a diffuse ( $\geq 60\%$  tumor cells) and moderate to strong (2–3+)



cytoplasmic staining.<sup>19</sup> Selinger et al had a sensitivity of 100% and specificity of 76% in their study, using D4D6 clone for *ROS1* IHC.<sup>20</sup> Viola et al had 100% sensitivity and 83% specificity when they used an overall H-score higher than 100 to define positivity (–Table 3).<sup>21</sup>

Further, occasional IHC positive, FISH negative cases have been shown to harbor *ROS1* translocations.<sup>23</sup> Based on our and published observations, all the IHC positive tumors should be confirmed by FISH testing. This testing algorithm stands as a reliable and cost-effective approach to screen *ROS1* positive lung adenocarcinomas. Next-generation sequencing and other molecular techniques can help to resolve discordant cases<sup>23</sup> and this may play a role in scenarios where both IHC and FISH tests are performed with equivocal or discordant results.

The sensitivity of *ROS1* IHC in our study (94.4%) is similar to the results from studies conducted outside India (sensitivity range: 97–100%). However, the specificity of *ROS1* IHC in our study (63.6%) is less than the results from studies conducted outside India (specificity range: 72.6–96.67%). This variation in the specificity of *ROS1* IHC in our study in comparison to other studies may be explained by the clone used, the population studied, and other unexplained factors. This in fact is the limitation of our study.

Also, intensity of IHC staining should be considered when interpreting *ROS1* status. Boyle et al found that in 27 lung adenocarcinoma specimens, which were negative for *ROS1* rearrangements by FISH, four of these cases showed low-level positive staining by IHC.<sup>24</sup> An intensity score was assigned, similar to our study, and was calculated by using the intensity of tumor cytoplasmic staining and percentage of cells stained. Setting an appropriate cutoff resulted in perfect correlation between *ROS1* IHC and FISH. A particular cutoff and different antibodies may show interlaboratory variability and thus, appropriate validation should be performed prior to adopting a *ROS1* IHC assay. Staining intensity may also vary by fusion partner with granular cytoplasmic, focal granular, strongly globular, and membranous patterns all having been observed.<sup>24</sup> A higher cutoff would decrease the number of discordant cases of IHC positive/FISH negative cases requiring molecular confirmation.

In summary, alterations of the *ROS1* gene are uncommon in NSCLC but, when present, have the potential for therapeutic intervention using targeted therapies. *ROS1* FISH has high sensitivity and specificity but it is expensive and cumbersome for many laboratories and requires technical expertise and specialized equipment, particularly in resource-limited settings and developing countries. IHC is commonly and readily performed in anatomic pathology practices and can be interpreted by pathologists. This can enable an efficient workflow for detecting *ROS1* alterations. All cases of advanced pulmonary adenocarcinoma should undergo screening with *ROS1* IHC; however, the cases with a positive result must be confirmed with a *ROS1* FISH prior to initiating therapy.

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None.

#### Note

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#### Authors' Contributions

S. K. Mohanty, S. Sharma, and S. K. Mishra contributed to conceptualization and designing. S. K. Mohanty, S. K. Mishra, S. Sharma, and M. Bhardwaj were involved in the development of methodology. S. K. Mohanty, S. Sharma, M. Bhardwaj, E. Jain, A. Kumar, M. Dixit, D. Jain, and S. Jha acquired the data. S. K. Mohanty, S. Sharma, S. Kumar, S. Jha, and S. K. Mishra analyzed the data. S. Sharma, M. Geller, M. Bhardwaj, S. K. Mohanty, and S. K. Mishra interpreted the data. S. Sharma, M. Geller, M. Bhardwaj, S. K. Mohanty, S. Jha, and S. K. Mishra were involved in writing and review/revision of the manuscript. S. Kumar and M. Bhardwaj provided technical support. S. K. Mohanty and M. Geller supervised the study.

#### Conflict of Interest

None declared.

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