Correlation of \textit{ROS1} (D4D6) Immunohistochemistry with \textit{ROS1} Fluorescence In Situ Hybridization Assay in a Contemporary Cohort of Pulmonary Adenocarcinomas

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Abstract

Objective Repressor of Silencing (\textit{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 \textit{ROS1} gene alterations. We evaluated the correlation between \textit{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 \textit{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 \textit{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...
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 2007.1–3 The ROS1 translocation/rearrangement is observed in 1 to 2% of NSCLC patients.4,5 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.6–8 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.2,6 The U.S. Food and Drug Administration has approved crizotinib, a small molecule 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.5 Crizotinib has demonstrated high overall response rates of 72% in metastatic NSCLC (mNSCLC) with the ROS1 rearrangement,9 a subset in which the responses to traditional chemotherapy regimens have been less than 10%.10 Hence, assays detecting the ROS1 gene rearrangement as an actionable target are now being performed routinely in frontline mNSCLC.11

The ROS1 fusion in the tumor cells can be detected using a variety of techniques including fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), reverse transcription 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.12–14 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 testing.11 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.13–21

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.13

FISH analysis for ROS1 gene rearrangement was performed on the formalin fixed paraffin embedded tissue section with 4µm 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
Correlation of ROS1 IHC with FISH in the Adenocarcinoma of Lungs  

Sharma et al.  

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× 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.13–15

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. 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 al13 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).13 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.14 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.15 Sholl et al showed 100% sensitivity and 92% specificity between ROS1 IHC and FISH, using D4D6 clone in their cohort of pulmonary adenocarcinomas.16 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 | Partial |
| 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, Repressor of Silencing.
In their 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. Using D4D6 clone with the positivity defined as IHC score of 2+,

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 al15       | ROS1 (D4D6) rabbit monoclonal antibody       | 6q22 ROS1 (Tel) Spectrum Orange Break Apart Probe | 100                    | 96.67                  |
| Shan et al14      | ROS1 (D4D6) rabbit monoclonal antibody       | ZytoLight SPEC ROS1 Dual Color Break Apart Probe  | 100                    | 93.6                   |
| Huang et al13     | ROS1 (SP384) antibody (Ventana)              | ROS1 Dual Color Probe                              | 97.8                   | 89.5                   |
| Sholl et al16     | ROS1 (D4D6) rabbit monoclonal antibody       | ROS1 Gene (RP11–59K17 and RP1–92C8)               | 100                    | 92                     |
| Cha et al17 (retrospective analysis) | ROS1 (D4D6) rabbit monoclonal antibody | Vysis LSI Dual Color, Break Apart Rearrangement Probe | 100 | 93.4 |
| Cha et al17 (prospective analysis) | ROS1 (D4D6) rabbit monoclonal antibody | Vysis LSI Dual Color, Break Apart Rearrangement Probe | 100 | 72.6 |
| Mescam-Mancini et al18 | ROS1 (D4D6) rabbit monoclonal antibody | Aquarius Pathology ROS1 Break Apart Probe and/or the ZytoLight SPEC ROS1 Dual Color Break Apart Probe | 100 | 96.9 |
| Selinger et al20  | ROS1 (D4D6) rabbit monoclonal antibody       | ZytoLight SPEC ROS1 Dual Color Break Apart Probe and the LSI ROS1 (Tel) Spectrum Orange Probe and LSI ROS1 (Cen) Spectrum Green Probe | 100 | 76 |
| Viola et al21     | ROS1 (D4D6) rabbit monoclonal antibody       | Cytocell ROS1 Dual Color Break Apart FISH Probe   | 100                    | 83                     |
| Our study         | ROS1 (D4D6) rabbit monoclonal antibody       | Vysis ROS1 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. Using D4D6 clone with the positivity defined as IHC score of 2+,

Mescam-Mancini et al have shown a sensitivity of 100% and the specificity 96.9%. Wu et al have demonstrated a high congruence between FISH and IHC, when IHC showed a diffuse (≥60% tumor cells) and moderate to strong (2–3+) correlation with FISH.
cytoplasmic staining. Selinger et al had a sensitivity of 100% and specificity of 76% in their study, using D4D6 clone for ROS1 IHC. Viola et al had 100% sensitivity and 83% specificity when they used an overall H-score higher than 100 to define positivity. (Table 3).

Further, occasional IHC positive, FISH negative cases have been shown to harbor ROS1 translocations. 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 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: 72.6–96.6%). 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. 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. 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 design. 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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Correlation of ROS1 IHC with FISH in the Adenocarcinoma of Lungs

Sharma et al.

255

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