ROS1 Immunohistochemistry Among Major Genotypes of Non—Small-Cell Lung Cancer

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Abstract

Identification of *ROS1* rearrangements in patients with lung cancer allows them to benefit from targeted therapy. We compared immunohistochemistry (IHC) with more cumbersome methods such as fluorescence in situ hybridization and reverse transcriptase polymerase chain reaction for identification of *ROS1* rearrangements in patients with lung adenocarcinoma (n = 33). Our results showed that IHC is a sensitive (100%) and specific (100%) method to identify *ROS1* rearrangements in patients with lung cancer.

Background—*ROS1* gene fusions cause several cancers by constitutively activating the ROS1 tyrosine kinase receptor. ROS1-targeted inhibitor therapy improves survival in the approximately 1% to 2% of patients with lung adenocarcinoma with *ROS1* gene fusions. Although fluorescence in situ hybridization (FISH) is the standard diagnostic procedure for detecting *ROS1* rearrangements, we studied immunohistochemistry (IHC).

Materials and Methods—ROS1 IHC was performed on a selected cohort of 33 lung adenocarcinoma whole tissue specimens with alterations in the *EGFR* (n = 5), *KRAS* (n = 5), *ERBB2* (*HER2*) (n = 3), *ROS1* (n = 6), *ALK* (n = 5), and *RET* (n = 3) genes and pan-negative (n = 6) detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and FISH.

Results—In the cohort of 33 specimens, both *ROS1* gene fusion using RT-PCR and high ROS1 protein expression using IHC were detected in 6 specimens. Of these 6 specimens, 5 were also positive by FISH for *ROS1* gene rearrangements. All 27 lung cancer specimens that were negative for *ROS1* rearrangements by genetic testing had no to low ROS1 protein expression.

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Conclusion—We have optimized ROS1 IHC and scoring to provide high sensitivity and specificity for detecting *ROS1* gene rearrangements in whole tissue. ROS1 IHC could be a practical and cost-effective method to screen for *ROS1* gene rearrangements.

Keywords

Adenocarcinoma; Biomarker; Gene rearrangement; NSCLC; ROS1

Introduction

Lung cancer is at the forefront of precision therapy for solid tumors with a rapidly advancing understanding of the genetic drivers. Many of these genetic changes are mutually exclusive, with just 1 genetic change as the cancer driver. Practical methods and standards for the identification of mutations in lung cancer specimens have become increasingly important to ensure that patients with genetic alterations are identified and receive the most effective therapy.[1-3]

ROS1 is a receptor tyrosine kinase that is overexpressed when the *ROS1* gene is constitutively activated by gene rearrangement. *ROS1* gene rearrangements were initially identified in glioblastoma[1] and cholangiocarcinoma[2] and, in 2007, for lung cancer.[3] *ROS1* rearrangements have also been identified in cases of gastric cancer,[4] colorectal cancer,[5] ovarian cancer,[6] and angiosarcoma.[7] In these cancers, fusions of the *ROS1* gene with multiple gene partners have been observed.[8] The treatment of patients with *ROS1* gene rearrangements with crizotinib and other directed therapies has shown high clinical efficacy.[9-12]

Although *ROS1* rearrangements have been identified in only 1% to 2% of non—small-cell lung carcinoma (NSCLC) cases,[10,13] they are present in a greater percentage of tumors that lack other genetic changes associated with lung cancer.[14] Limited data have also suggested that, similar to *EGFR* mutations and *ALK* rearrangements, *ROS1* rearrangements are more common in certain subsets of the population, such as young Asian patients with a negative smoking history.[10,15] A study of never smoker patients with lung adenocarcinoma treated at Severance Hospital in Seoul, Korea, detected *ROS1* rearrangements by fluorescence in situ hybridization (FISH) in 5.7% (6 of 105) of patients who were "triple negative" for *EGFR*, *KRAS*, and *ALK* alterations.[14] Likewise, a study of a selected population of white patients with "triple-negative" NSCLC found a 7.4% (9 of 121) positivity rate for *ROS1* rearrangements using FISH and immunohistochemistry (IHC).[16]

The detection of *ROS1* rearrangements in lung cancer specimens has been hampered by issues similar to those described for the detection of *ALK* rearrangements.[17] Both *ROS1* and *ALK* gene rearrangements are present in a low percentage of cases and can occur with multiple fusion partners. FISH can detect multiple rearrangements by a split signal but is a cumbersome and expensive method. Reverse transcriptase polymerase chain reaction (RT-PCR) is also possible but requires multiple primer sets, and rare rearrangements can be missed. ROS1 IHC requires less labor, is less expensive, and is more widely available than FISH and RT-PCR. ROS1 proteins are not highly expressed in normal lung tissue, and gene rearrangements have been associated with high ROS1 protein expression. Thus, IHC is an

ideal method to screen for lung cancer cases with *ROS1* gene rearrangements.[18-21] In the present study, we have built on the work of others to examine the correlation of ROS1 protein expression with the presence of *ROS1* gene rearrangements. We describe our criteria for ROS1 IHC positivity using the histology score (H-score) and demonstrate how IHC can be an effective method to screen for *ROS1* gene rearrangements. In addition, we have described the clinicopathologic features of lung cancers associated with *ROS1* gene rearrangements.

Materials and Methods

Case Selection

Our "selected cohort" was split into 2 rounds of testing of whole tissue lung adenocarcinoma specimens. The first round included 20 specimens enriched for *ROS1* gene rearrangements (n = 6), *EGFR* mutations (n = 5), and *KRAS* mutations (n = 3) previously detected by RT-PCR and/or FISH. Six specimens were pan-negative for *ROS1*, *EGFR*, *KRAS*, *BRAF*, *ERBB2* (*HER2*), *ALK*, and *RET* gene abnormalities. The H-score results of this first round of IHC were correlated with the RT-PCR and FISH results to define an H-score cutoff for "positive" versus "negative." A second round of testing with 13 additional adenocarcinoma specimens was performed to validate the H-score cutoff defined in the first round of testing. The specimens in this second round were positive for *ALK* rearrangements (n = 5), *KRAS* mutations (n = 2), *ERBB2* (*HER2*) mutations (n = 3), and *RET* gene rearrangements (n = 3) and all were negative for *ROS1* rearrangements using RT-PCR. The present study was approved by the institutional review board of the Aichi Cancer Center.

Immunohistochemistry

Optimization for ROS1 IHC was performed using clone D4D6 (Cell Signaling Technology, Danvers, MA). The HCC78 cell line with the *SLC34A2-ROS1* gene fusion was selected as a positive control. The HCC1703 cell line was used for a negative control.

IHC was performed on unstained paraffin-embedded tissue sections. The whole tissue slides from the selected cohort were stained with an automated procedure. The slides were dried in a 60°C oven for 1 hour, labeled with a bar-coded, standardized, antibody-specific protocol, and loaded into a Benchmark XT automated stainer (Ventana Medical Systems, Tucson, AZ). The slides were treated with standard cell conditioning 1 reagent for 60 minutes. The primary antibody (clone D4D6, Cell Signaling Technology, Danvers, MA) was manually applied at 1:100 and 1:250 dilutions, and the slides were incubated at 37°C for 1 hour. Amplification and detection were performed using the UltraView Amplification and DAB detection kits. The slides were counterstained with hematoxylin for 4 minutes and post-counterstained with bluing agent for 4 minutes. The slides were then washed with mild detergent and dehydrated in a series of 70% to 100% alcohol baths, cleared in a xylene bath, and cover slipped.

The ROS1-stained slides were evaluated with the H-score by a pathologist (T.B.), who was unaware of the RT-PCR and FISH results in all the studies. The H-score is a semiquantitative score system that calculates a score from 0 to 300 according to both the

intensity of tumor cytoplasmic staining and the percentage of cells stained. For the present study, intensity was considered 0 for absent expression, 1+ for weak staining, 2+ for moderate staining, and 3+ for strong staining. The H-score was calculated as follows: H-score = (0 × percentage of cells with absent cytoplasmic staining) + (1 × percentage of 1+ cells) + (2 × percentage of 2+ cells) + (3 × percentage of 3+ cells).

The specimens were scored only if 20 tumor cells were present. Tissue areas in which it was difficult to distinguish type II pneumocyte hyperplasia from adenocarcinoma in situ were excluded from scoring, because previous studies have warned that hyperplastic type II pneumocytes can exhibit ROS1 protein expression and can result in false positivity.[19]

RT-PCR and FISH

RT-PCR with sequencing was performed for *EGFR*, *KRAS*, *ERBB2* (*HER2*), *ROS1*, *ALK*, and *RET* alterations, as previously described.[8,10,22-25] RT-PCR for *ROS1* rearrangements was performed to detect the following known *ROS1* gene fusion partners: *CD74*, *SLC34A2*, *LRIG3*, *SDC4*, *SLCA2*, *TMP*, and *EZR* In brief, total RNA was reverse transcribed to cDNA and then subjected to amplification with fusion-specific primers. Gel electrophoresis was performed with follow-up Sanger sequencing on PCR products with visible bands. FISH assays were performed for *ALK, RET*, and *ROS1* using commercially available probe sets (Vysis), as previously described.[25]

Results

Test Cohort

The first round of ROS1 IHC testing of 20 whole tissue specimens was performed using 1:100 and 1:250 dilutions of the D4D6 antibody. Six specimens with *ROS1* rearrangements previously detected by RT-PCR exhibited strong staining (Figures 1 and 2). The calculated H-scores were > 100 for all 6 specimens (mean, 197; range, 130-270). FISH was positive in 5 of the 6 specimens with *ROS1* rearrangements detected by RT-PCR and IHC.

The histopathologic features of the 6 ROS1 IHC-positive specimens are summarized in Table 1. Three had *CD74* gene fusions, and three had different fusion partners: *SCL34, EZR*, and *SDC4*. All 6 tumors were adenocarcinoma; 4 had a predominantly solid pattern, 1 acinar, and 1 lepidic. Of the 6 tumors, 3 were mucinous, of which, 2 also had signet ring cells. All 6 tumors had at least some areas with a cribriform pattern. All had finely granular cytoplasmic staining for ROS1; however, the specimen with the *EZR* fusion partner also showed strong membrane staining (Figure 1E). Of the 3 specimens with *CD74* as a fusion partner, 2 had ROS1-positive globules within the cytoplasm of a proportion of the tumor cells (Figure 1C and 1D).

Of the specimens that were negative for the *ROS1* rearrangements by RT-PCR, all had IHC H-scores of 100 at both dilutions (mean, 17; range, 0-100). Thus, ROS1 H-score cutoffs from 100 to 130 were associated with perfect correlation of the ROS1 IHC and RT-PCR results (100% sensitivity and 100% specificity). Four specimens that were negative for *ROS1* rearrangements by FISH exhibited low ROS1 protein expression by IHC, with H-scores ranging from 5 to 100 (Figure 3). Considering the clean background at both dilutions

and a need for high sensitivity for *ROS1* rearrangement detection, a dilution of 1:100 was selected for our ROS1 IHC protocol.

A second round with testing of 13 additional specimens known to be negative for *ROS1* rearrangement by RT-PCR was performed to test the specificity of an H-score cutoff of 100. The specificity for this second round of testing was again 100%, with all H-scores < 100 (mean, 3.1; range, 0-30; Figure 2).

Discussion

Our data have described ROS1 IHC as a method for detection and screening of lung cancer whole tissue specimens for *ROS1* gene rearrangements. We used the same antibody (D4D6) for ROS1 IHC staining as others have used[19,26] and confirmed ROS1 IHC as a highly sensitive method for the detection of *ROS1* translocation in lung cancer. An algorithm with the detection of *ROS1* and *ALK* rearrangements (6%-7%), *EGFR* mutations (10%-20%), and *KRAS* mutations (approximately 25%) in lung adenocarcinoma specimens would identify driver mutations in approximately one half of all patients with lung adenocarcinoma.

A comparison of the ROS1 IHC data with the RT-PCR results showed a perfect correlation (100% sensitivity and 100% specificity) in our small selected cohort when a positive result was defined as an H-score > 100. After we had established an H-score cutoff internally, Cha et al[27] published their findings on ROS1 IHC using the same antibody and methods and also determined an H-score of > 100 as their cutoff for positivity, with a sensitivity of 100% for 8 *ROS1*-rearranged cases. Thus, we have confirmed their findings and propose the IHC method and an H-score cutoff of 100 for standardized testing to screen patients for *ROS1* rearrangements.

Of the 6 specimens with a *ROS1* rearrangement detected by RT-PCR and IHC, 1 was negative by FISH. This specimen with the discrepant negative FISH result had an *EZR* fusion partner detected using RT-PCR. The *EZR* gene is on the same chromosome as the *ROS1* gene, such that detection by FISH technology might be more complicated than for fusion genes on different chromosomes. Thus, it might be difficult to distinguish between *ROS1-EZR* fusions and normal findings using FISH, because the signals for these 2 genes are located on the same chromosome, regardless of fusion. Therefore, IHC and RT-PCR might be superior to FISH in detecting *ROS1* rearrangements caused by *ROS1-EZR* fusions.

Our histopathologic findings, summarized in Table 1, add to the descriptions of others regarding the appearance of specimens with *ROS1* rearrangements. We had a high proportion of specimens with cribriform (100%), mucinous (50%), and signet ring (33%) features.[19,26] We also can support the observations of Yoshida et al[26] about 2 observations regarding fusion partners and histologic features. Yoshida et al[26] observed at least focal globular immunoreactivity in 6 of 10 *CD74-ROS1* rearrangement-positive tumors; 2 of 3 of our *CD74-ROS1*–positive tumors had globules and none of the others. Likewise, 3 of 4 *EZR-ROS1* tumors reported by Yoshida et al[26] had membrane positivity, and our 1 tumor with an *EZR-ROS1* rearrangement also showed strong membrane positivity. Their only *SLC-ROS1*– positive tumor did not have distinctive features, and neither did ours. Although these

combined numbers are still small, it does support the possibility that tumors with different fusion partners could stain differently.

Conclusion

The results of our study have shown that ROS1 IHC is a sensitive and specific method for detecting *ROS1* gene rearrangements; however, additional studies are needed to standardize specimen processing, ROS1 staining, and ROS1 scoring. It could be important to standardize tissue processing for lung cancer specimens, such as has occurred for breast tissue processing for *ERBB2* (*HER2*) testing.[26] A cost-effective algorithm for testing might be initial testing of ROS1 using IHC, with a follow-up evaluation of tumors with high ROS1 expression using RT-PCR or FISH for *ROS1* rearrangements. IHC is a cost-effective and widely available method that can identify the patients with lung cancers driven by *ROS1* rearrangements who would be likely to benefit from targeted therapy.

Acknowledgments

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Clinical Practice Points

- IHC is a cost-effective widely available method that could be used to screen patients with lung cancer for ROS1 rearrangements.

- Multiple clinical studies have demonstrated the clinical efficacy of targeted therapy in patients with *ROS1* rearrangement-driven cancer.[11]

- FISH has been the reference standard for rearrangement detection but is expensive and cumbersome.

- The most practical algorithm to identify patients who might benefit from targeted therapy would be to screen all patients with lung cancer for ROS1 overexpression using IHC and then perform FISH analysis for those patients with equivocal to high ROS1 expression to specifically identify *ROS1* genetic rearrangements.

- We have proposed a standardized approach for ROS1 IHC staining and scoring to screen for *ROS1* rearrangements, a clinically actionable genetic change in patients with lung cancer.
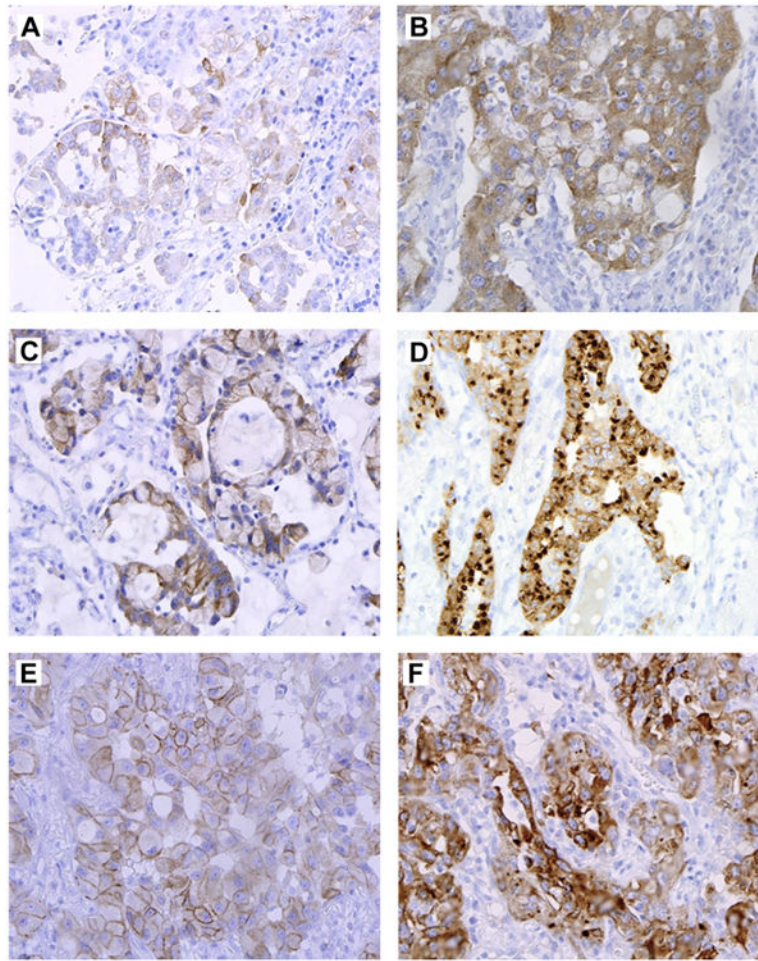
Figure 1. Microscopic Images of Specimens With Positive ROS1 Immunohistochemistry (A-F Correlate With Specimens 1-6 in Table 1, Respectively; All Original Magnification ×400)
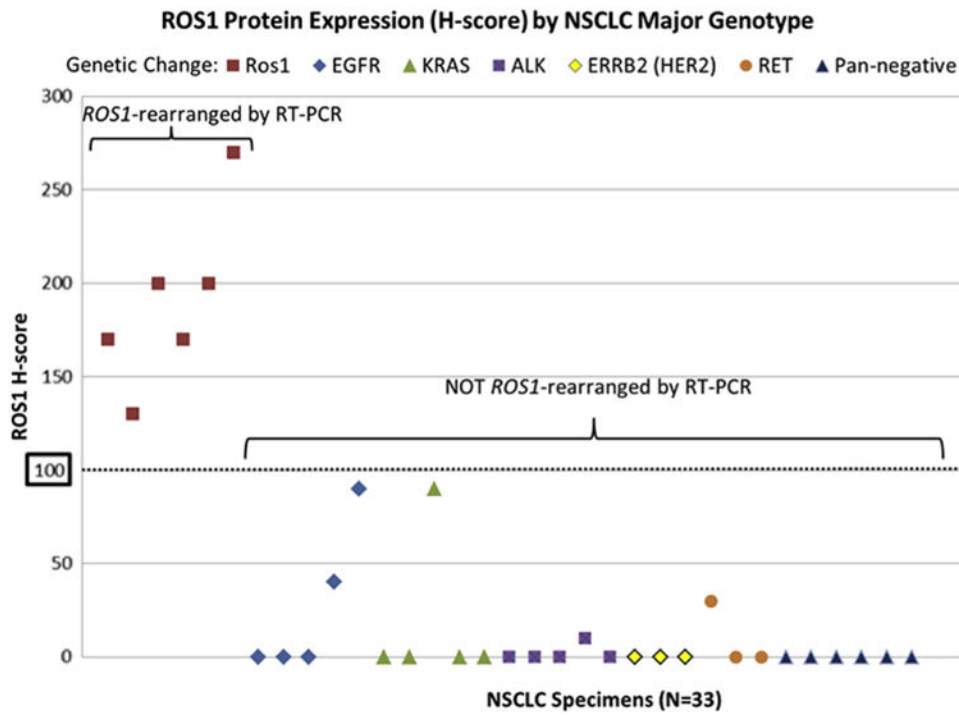
Figure 2. Histology Score (H-score) for Specimens in Selected Cohort. The First 6 Specimens Were ROS1-Rearrangement Positive. The Remainder Were ROS1-Rearrangement Negative
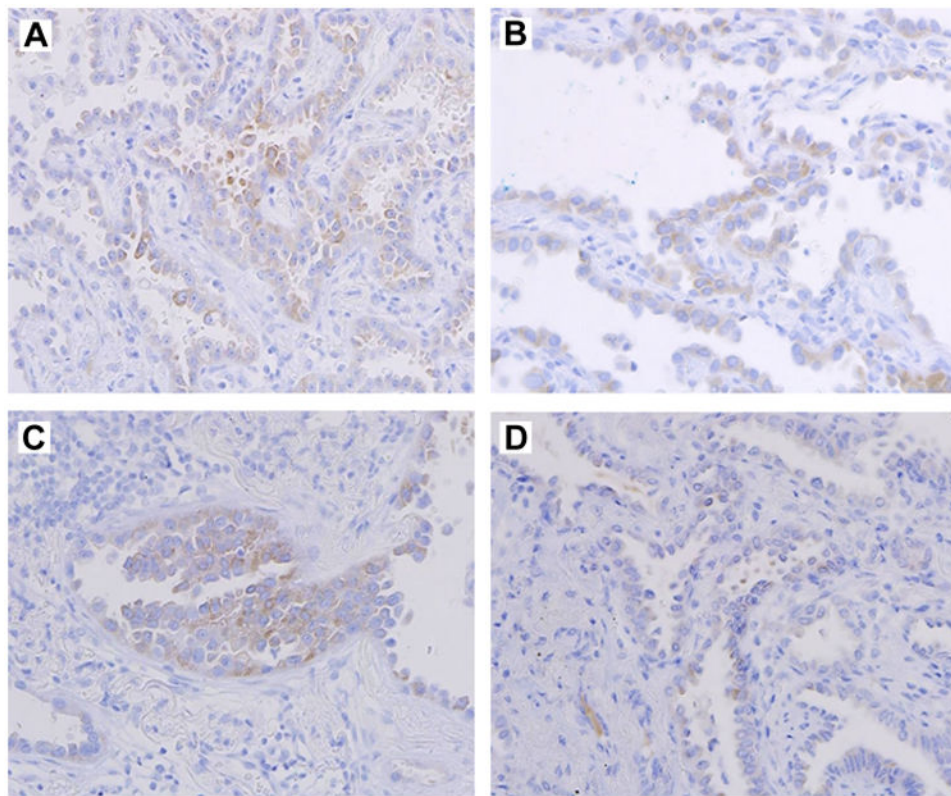
Figure 3. (A-D) Borderline Cases With Some Tumor Cells Very Weakly Positive by Immunohistochemistry (Histology Score < 100). All Were Negative for *ROS1* Gene Rearrangement (Original Magnification ×400 for All)

Table 1

Histopathologic Features of ROS1 Rearrangement-Positive Specimens (n = 6)

Specimen	Fusion Partner	H-Score	Staining Pattern	Mucinous	Signet Ring	Cribriform	Predominant Pattern
1611	SCL34	170	Granular cytoplasmic	No	No	Yes	Solid
1958	CD74	130	Granular cytoplasmic	Yes	Yes (75%)	Yes (focal)	Solid/signet ring
2006	CD74	200	Cytoplasmic; focal globular	Yes	Yes (5%)	Yes	Acinar/solid
2087	CD74	170	Strongly globular (30%)	No	No	Yes	Lepidic
2604	EZR	200	Membranous; cytoplasmic	Yes	No	Yes	Solid/lepidic
2647	SDC4	270	Granular cytoplasmic	No	No	Yes	Solid