Analysis of Receptor Tyrosine Kinase ROS1-Positive Tumors in Non–Small Cell Lung Cancer: Identification of a FIG-ROS1 Fusion

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Abstract

Purpose: To deepen our understanding of mutant ROS1 expression, localization, and frequency in non–small cell lung cancer (NSCLC), we developed a highly specific and sensitive immunohistochemistry (IHC)-based assay that is useful for the detection of wild-type and mutant ROS1.

Experimental Design: We analyzed 556 tumors with the ROS1 D4D6 rabbit monoclonal antibody IHC assay to assess ROS1 expression levels and localization. A subset of tumors was analyzed by FISH to determine the percentage of these tumors harboring ROS1 translocations. Using specific and sensitive IHC assays, we analyzed the expression of anaplastic lymphoma kinase (ALK), EGFR L858R, and EGFR E746-A750del mutations in a subset of lung tumors, including those expressing ROS1.

Results: In our NSCLC cohort of Chinese patients, we identified 9 (1.6%) tumors expressing ROS1 and 22 (4.0%) tumors expressing ALK. FISH identified tumors with ALK or ROS1 rearrangements, and IHC alone was capable of detecting all cases with ALK and ROS1 rearrangements. ROS1 fusion partners were determined by reverse transcriptase PCR identifying CD74-ROS1, SLC34A2-ROS1, and FIG-ROS1 fusions. Some of the ALK and ROS1 rearranged tumors may also harbor coexisting EGFR mutations.

Conclusions: NSCLC tumors with ROS1 rearrangements are uncommon in the Chinese population and represent a distinct entity of carcinomas. The ROS1 IHC assay described here is a valuable tool for identifying patients expressing mutant ROS1 and could be routinely applied in clinical practice to detect lung cancers that may be responsive to targeted therapies.

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cell growth of Baf3 cells overexpressing HG-ROS1 (S) and (LJ)(15).

In addition to glioblastoma and cholangiocarcinoma, ROS1 fusions have also been identified in NSCLCs. Phosphoproteomic studies surveying the tyrosine phosphorylation space in 41 NSCLC cell lines and 150 tumors identified 1 cell line (HCC78) and 1 tumor with high ROS1 phosphopeptides, implicating ROS1 as a possible oncogetic driver. Two novel ROS1 fusion partners, CD74 and solute carrier protein (SLC34A2), were identified from the tumor and HCC78 cell line, respectively. Unlike FIG-ROS1, CD74-ROS1 and SLC34A2-ROS1 result from translocation of the ROS1 locus. Collectively, studies in glioblastoma, NSCLCs, and cholangiocarcinoma associate ROS1gene rearrangements with the formation of solid tumors.

A recent report describes early evidence of clinical response to crizotinib in ROS1-rearranged NSCLCs (16). With the current need to rapidly identify these patients for potential therapies, we developed a highly sensitive and specific ROS1 antibody to evaluate ROS1 protein expression in NSCLCs. This study describes the validation of this ROS1 antibody for immunohistochemistry (IHC) and our analysis of ROS1 and ALK gene fusions in NSCLCs.

Translational Relevance
Approximately 2% of the non–small cell lung cancer (NSCLC) tumors harbor ROS1 gene fusions, and emerging preclinical and clinical data indicate that these tumors are responsive to inhibitors that target anaplastic lymphoma kinase (ALK). However, the extent of ROS1 protein expression in NSCLC and normal tissues remains unknown. Using a novel ROS1 immunohistochemical assay, we determined that ROS1 protein expression is restricted to NSCLC tumors harboring ROS1 fusions. This assay facilitates the routine identification of ROS1 rearranged NSCLCs in clinical practice and detects lung cancers that may be responsive to targeted therapies.

Materials and Methods
Cell culture, antibodies, and Western blot
All cell culture reagents were purchased from Invitrogen. U-118 MG and HEK 293T cells were purchased from the American Type Culture Collection. BaF3, Karpass-299, and HCC78 cells were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). ALK (D5F3), XP (#3633; Cell Signaling Technology), and ROS1 (D4D6) rabbit monoclonal antibodies (mAb) were developed at Cell Signaling Technology. ROS1 D4D6 recognizes amino acids at the carboxy terminus of human ROS1. Western blot analyses were conducted following Cell Signaling Technology protocols. All other antibodies and reagents for Western blotting were from Cell Signaling Technology. Transfection of myc/DDK-ROS1 (RC220652, Origene) was conducted using FuGENE 6 (Roche) for more than 48 hours in HEK 293T cells.

Human NSCLC tumor tissues
Institutional Review Board approval was granted by the Second Xiangya Hospital of Central South University (Chansha, Hunan, PR China). All NSCLC specimens were derived from tumor resections and subsequently embedded in paraflin blocks provided by Second Xiangya hospital. Out of the 556 NSCLC samples used in this study, 409 were constructed into tumor microarrays for IHC screening. The remaining samples were screened as whole sections. Any ALK- or ROS-positive core from the tumor microarray was subsequently confirmed using whole sections from the original blocks. Two independent pathologists evaluated all tumors as ALK and ROS1 IHC–positive tumors to confirm diagnoses. When needed, IHC staining for p63, TTF-1, mucin, and CK 5/6 was conducted.

Immunohistochemistry
Four- to six-micrometer tissue sections were deparaffinized, rehydrated, and then subjected to antigen retrieval in a Decloaking Chamber (Biocare Medical) using 1.0 mmol/L EDTA (pH 8.0). Slides were quenched in 3% H2O2 for 10 minutes, washed in diH2O, and then blocked with TBS/0.1% Tween-20. Slides were incubated overnight at 4 °C with ROS1 (D4D6) rabbit mAb at 0.19 μg/mL, or ALK (D5F3) XP rabbit mAb at 1.2 μg/mL, both diluted in SignalStain Antibody Diluent (#8112; Cell Signaling Technology). Detection was conducted with EnVision+ (HRP), rabbit; Cell Signaling Technology, #8114, #2085, and #4267, respectively; Cell Signaling Technology (HRP), rabbit; Cell Signaling Technology, #8114 for 30 minutes. All slides were exposed to NovaRED (Vector Laboratories) and coverslips were mounted. Images (× 20) were acquired using an Olympus CX41 microscope equipped with an Olympus DP70 camera and DP Controller software.

FISH
FISH was conducted on 4-μm thick formalin-fixed, paraffin-embedded (FFPE) tissue sections. ROS1 break-apart probe was developed using bacterial artificial chromosomes: RP1-179P9, RP1-323017, RP1-213A17, and RP1-94G16 (Invitrogen). Bacteria artificial chromosomes (BAC) were labeled with spectrum orange and spectrum green dUTPS with the Nick Translation DNA Labeling Kit (Enzo Life Sciences). The LSI ALK Dual Color, ALK Break Apart Rearrangement Probe was purchased from Abbott Molecular. FISH-positive cases for both ALK and ROS1 were defined as more than 15% split signals in tumor cells. At least 100 nuclei per sample were scored. The Nikon C1 confocal microscope, ×60 objective, and triflter
[4', 6-diamidino-2-phenylindole (DAPI), tetramethyl rhodamine isothiocyanate (TRITC) and, fluorescein isothiocyanate (FITC)] were used for scoring each case. For image acquisition, the Olympus BX-51 widefield fluorescence microscope with ×40 objective and Metamorph software was used to generate multicolor images.

**Reverse transcriptase PCR from FFPE tumor samples**
RNA from three 10-μm sections was extracted following standard protocols (RNeasy FFPE Kit, Qiagen). First strand cDNA was synthesized from 500 ng of total RNA with the use of the SuperScript III First-Strand Synthesis System (Invitrogen) and gene specific primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ROS1 primers were purchased from Qiagen. All primer sequences can be found in Supplementary Methods.

**Transfection and cell proliferation assay**
Transfections were carried out using FuGENE 6 (Roche Diagnostics), and retrovirus was harvested 48 hours after transfection. BaF3 cells were transduced with retroviral supernatant containing either the MSCV-Neo/FIG-ROS(L) or MSCV-Neo/FIG-ROS1 (S) vector, and selected for G418 (0.8 mg/ml). IL-3–independent growth was assessed by plating transduced BaF3 cells in IL-3–free medium, after the cells were washed 3 times in PBS. For dose–response curves, cells were incubated for 72 hours in the presence of crizotinib (ChemieTek), and the number of viable cells was determined with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega).

**Results**

**ROS1 expression in cell lines, xenografts, and normal human tissues**
To study ROS1 protein expression in NSCLCs, we developed a ROS1 rabbit mAb clone D4D6. We first validated ROS1 D4D6 on cell lines and xenograft models and then investigated ROS1 expression in normal human tissues. To our knowledge, the only cell lines that express ROS1 fusion proteins are HCC78 (SLC34A2-ROS1) and U-118 MG (FIG-ROS1). ROS1 D4D6 recognizes ROS1 fusion proteins in both cell lines by Western blot (Fig. 1A) and IHC (Fig. 1B and C). Specificity by Western blot was further confirmed using antibodies recognizing ROS1 epitopes that are distinct from ROS1 D4D6. Western blot signals detected in HCC78 and U-118 MG can be blocked when ROS1 D4D6 is incubated together with its immunogen (data not shown). Strong cytoplasmic and punctate staining was observed in HCC78 and U-118 MG xenografts by IHC (Fig. 1F). X-magnifications are shown in panels H and I, respectively. Other negative tissues include: heart (J), ovary (K), pancreas (L), testis (M), and stomach (N). Tissues staining with ROS1 rabbit mAb include: colon (O), kidney (P), and cerebellum (Q).

![Figure 1. ROS1 expression in lung cancer cell lines, xenografts, and normal tissues. A, a panel of lung cell lines was analyzed by Western blot with the ROS1 D4D6 antibody. ROS1 fusion proteins in HCC78 (SLC32A2-ROS1, 60–80 kDa) and U-118 MG (FIG-ROS1, 110 kDa) were detected. Lung cancer cell lines were screened to evaluate cross-reactivity with other RTKs. ROS1 or cross-reactive RTKs were not detected in these cell lines. All of these cell lines were also screened by IHC. ROS1 was detected in (B) HCC78, (C) U-118 MG, (D) HEK 293T-ROS1-DDYK (FLAG) myc and, but absent, in (E) H3122. Xenografts were also examined by IHC to confirm cell pellet staining. F, strong cytoplasmic ROS1 staining was present in the HCC78 xenograft. G, as a negative control, a H3122 xenograft was stained with the ROS1 D4D6 antibody by IHC. Representative images of lung tissue at ×20 and ×40 magnifications are shown in panels H and I, respectively. Other negative tissues include: heart (J), ovary (K), pancreas (L), testis (M), and stomach (N). Tissues staining with ROS1 rabbit mAb include: colon (O), kidney (P), and cerebellum (Q).](https://www.aacrjournals.org/doi/10.1158/1078-0432.CCR-11-3351)
was observed in the HCC78 cell line (Fig. 1B), whereas cytoplasmic staining in U-118 MG cell line was relatively weak (Fig. 1C), requiring the use of SignalStain Boost IHC Detection Reagent to enhance the signal. We were unable to identify cell lines expressing full length ROS1; however, full-length ROS1 is detectable by IHC when exogenously expressed (Fig. 1D). To address RTK cross-reactivity, we screened a panel of lung cancer cell lines known to express various RTKs including MET, Axl, EGFR, FGFR, PDGFR, ALK, DDR1, and IGF-1R. No cross-reactivity was detected by Western blot (Fig. 1A) or IHC (data not shown). We also examined ROS1 expression by IHC in xenograft models including HCC78, H1299, H1975, HCC827, H1650, H2228, and H441. ROS1 staining was only observed in HCC827 and HCC78 xenografts. ROS1 protein in HCC827 is undetectable by Western blotting (Fig. 1A), whereas the ROS1 transcript is present (data not shown), suggesting that low levels of ROS1 protein can be detected using a sensitive IHC assay. ROS1 is highly expressed in both the membrane and cytoplasmic compartments in HCC78 xenografts (Fig. 1F). ROS1 was not detected in the H3122 (EML4-ALK) cell pellet or xenograft (Fig. 1E and G).

Knowledge about wild-type ROS1 protein expression and regulation in normal human tissues is limited. However, expression of ROS1 transcripts in kidney, testis, lung, and intestines has been described (17–20). To examine the extent of wild-type ROS1 expression in normal tissues, we screened a normal human tissue array and conducted FISH on all 9 ROS1-expressing tumors and a subset of negative tumors (n = 138). The break-apart FISH assay previously described (21) detects all known ROS1 fusions including FIG-ROS1, CD74-ROS1, and SLC34A2-ROS1. Negative tumor samples were completely devoid of ROS1 protein expression by IHC (Fig. 2A and B) and normal by FISH. In 7 of 9 ROS1-expressing tumors, the FISH probes were broken, indicative of translocation of the ROS1 locus (Fig. 2C). The probes 5’ to the common breakpoint region of ROS1 were not detected in one tumor, suggesting deletion of this chromosomal region equivalent to the FIG-ROS1 fusion pattern in U-118 MG (Supplementary Fig. S1F). We were unable to analyze one tumor due to high background and low signal intensity.

Table 1. Histopathology and genotypes of ROS1 IHC–positive samples

<table>
<thead>
<tr>
<th>Tumor ID</th>
<th>Diagnosis</th>
<th>Histologic pattern (%)</th>
<th>IHC Score</th>
<th>ROS1 fusion</th>
<th>EGFR mutation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>147</td>
<td>Adenocarcinoma</td>
<td>BAC (40), papillary (30), Acinar (20), Solid (10)</td>
<td>3+ cytoplasmic</td>
<td>+</td>
<td>SLC34A2-ROS1</td>
</tr>
<tr>
<td>306</td>
<td>Adenocarcinoma</td>
<td>Acinar (70), papillary (20), and solid (10)</td>
<td>3+ cytoplasmic</td>
<td>+</td>
<td>CD74-ROS1</td>
</tr>
<tr>
<td>570</td>
<td>Adenocarcinoma</td>
<td>Acinar (90), BAC (5), micropapillary (5)</td>
<td>3+ cytoplasmic, punctae</td>
<td>+</td>
<td>CD74-ROS1</td>
</tr>
<tr>
<td>760</td>
<td>Adenocarcinoma</td>
<td>Signet cells</td>
<td>3+ cytoplasmic, membrane</td>
<td>+</td>
<td>Insufficient material</td>
</tr>
<tr>
<td>400037</td>
<td>Adenocarcinoma</td>
<td>Acinar</td>
<td>2+ cytoplasmic, punctae</td>
<td>+</td>
<td>CD74-ROS1</td>
</tr>
<tr>
<td>575</td>
<td>Large Cell</td>
<td>Solid (80), Acinar (10), BAC (10)</td>
<td>2+ cytoplasmic</td>
<td>Not scoreable</td>
<td>Unknown</td>
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<tr>
<td>702</td>
<td>Adenocarcinoma</td>
<td>Papillary (40), Acinar (30), Solid (30)</td>
<td>1+ cytoplasmic</td>
<td>+</td>
<td>SLC34A2-ROS1</td>
</tr>
<tr>
<td>749</td>
<td>Adenocarcinoma</td>
<td>Solid (80), Acinar (20)</td>
<td>1+ vesicular</td>
<td>+</td>
<td>FIG-ROS1 (S)</td>
</tr>
</tbody>
</table>

ROS1 D4D6 rabbit mAb detects ROS1 fusion proteins in formalin-fixed tumor samples

To address the frequency of ROS1 expression in NSCLC tumors and examine ROS1 cellular localization, we screened 556 NSCLC tumors by IHC with ROS1 D4D6 rabbit mAb. The NSCLC cohort is composed of 246 adenocarcinomas, 64 bronchioalveolar carcinomas, 226 squamous, and 20 large cell carcinomas. ROS1 was expressed in 1.6% (9 of 556) of NSCLC tumors. Eighty-nine percent of ROS1-expressing tumors were adenocarcoma, similar to what was observed in tumors expressing ALK and mutant EGFR proteins. One tumor expressing ROS1 was of the large cell carcinoma subtype. Overall ROS1 was expressed in 3.3% (8 of 246) of all adenocarcinoma tumors (Table 1). To determine whether the ROS1 protein detected by ROS1 D4D6 is due to a fusion or wild-type expression, we conducted FISH on all 9 ROS1-expressing tumors and a subset of negative tumors (n = 138). The break-apart FISH assay previously described (21) detects all known ROS1 fusions including FIG-ROS1, CD74-ROS1, and SLC34A2-ROS1. Negative tumor samples were completely devoid of ROS1 protein expression by IHC (Fig. 2A and B) and normal by FISH. In 7 of 9 ROS1-expressing tumors, the FISH probes were broken, indicative of translocation of the ROS1 locus (Fig. 2C). The probes 5’ to the common breakpoint region of ROS1 were not detected in one tumor, suggesting deletion of this chromosomal region equivalent to the FIG-ROS1 fusion pattern in U-118 MG (Supplementary Fig. S1F). We were unable to analyze one tumor due to high background and low signal intensity.
Although few differences were observed by FISH, a wide variety of ROS1 staining patterns were observed by IHC. ROS1 localized diffusely to the cytoplasm in 5 of 9 (55%) cases (Fig. 2D); however, strong perinuclear aggregates were observed in 2 of 9 tumors (Fig. 2E). In the large cell carcinoma sample, ROS1 was localized to the cytoplasm (Fig. 2G and H). Both membrane (Fig. 2F) and vesicular localization of ROS1 (Fig. 2I) were also observed. Generally, normal adjacent tissue did not stain with ROS1 D4D6 (Fig. 2K). In rare cases, nonneoplastic cells such as macrophages and bronchial epithelial cells (Fig. 2L) stained with ROS1 D4D6.

This cohort was also screened for ALK expression using the rabbit mAb ALK D5F3 IHC assay (11). In these tumors, ALK was expressed throughout the cytoplasmic compartment with varying intensities amongst tumors. Concurrent expression of ALK and ROS1 was never observed. We found 22 of 556 (4.0%) tumors expressing ALK protein with 21 confirming FISH positivity (Table 2). In all 21 cases, the FISH pattern was consistent with an inversion phenotype. Because of inadequate tissue availability, we were only able to interrogate the ALK fusion partners for a small subset (n = 7) of samples. The most common fusions in NSCLC, EML4-ALK variant 1 and variant 3 were assessed by FFPE reverse transcriptase PCR (RT-PCR). EML4-ALK variant 3 was detected in 5 of 7 samples, and neither EML4-ALK variant 1 nor 3 were detected in the other 2 samples (data not shown).

Identification of ROS1 fusion partners by FFPE RT-PCR

Both IHC and FISH are limited in their ability to distinguish one ROS1 fusion partner from another. To identify ROS1 fusion partners, we extracted total RNA from FFPE sections and conducted RT-PCR using primers specific to known ROS1 fusions: CD74-ROS1, SCL34A2-ROS1, and FIG-ROS1. As a control, we amplified the 3′ portion of ROS1 to detect any fused or wild-type ROS1 transcript. All samples with ROS1 translocations were analyzed for SCL34A2-ROS1 (S) and (L) isoforms and CD74-ROS1. We identified fusion partners for 7 of 9 ROS1-expressing tumors (Table 1 and Supplementary Table S1). CD74-ROS1 and SCL34A2-ROS1 fusions were detected with CD74-ROS1 being the most prevalent. All amplicons were sequence verified (data not shown). We were unable to identify fusion partners for 2 tumors due to lack of material and low RNA quality.

One sample, #749, was tested for FIG-ROS1 fusions because of the observed vesicular localization of ROS1 (Fig. 2J) and deletion of green FISH probes (Supplementary Fig. S1F), which suggest the presence of a FIG-ROS1 fusion. RT-PCR followed by sequencing confirmed the presence of FIG-ROS1 (S) (Fig. 3A). In addition, we devised an alternative FISH assay to visualize the FIG-ROS1 fusion in whole sections from paraffin blocks (Supplementary Fig. S1). To our knowledge, FIG-ROS1 fusions have not been previously described in NSCLCs.

Previously, FIG-ROS1 (S) was identified as a target of the ALK inhibitor, TAE684 (15), suggesting that the recently FDA approved drug Xalkori (crizotinib) could also be effective in inhibiting ROS1 kinase activity. To determine

Figure 2. Detection of ROS1 fusions in NSCLC tumors by IHC and FISH. ROS1-negative images of squamous cell carcinoma (A) and bronchioloalveolar carcinoma (B) tumors are seen. We used a break-apart FISH assay to detect ROS1 fusions. A representative FISH image from one ROS1-positive adenocarcinoma is seen in C. Fused green and orange probe is indicative of the intact ROS1 loci. The yellow arrowheads point to broken orange and green probes indicative of a ROS1 translocation event. This broken orange and green phenotype was present in most ROS1-expressing tumors. ROS1 localized to different cell compartments at varying intensities. In most adenocarcinoma tumors, ROS1 was expressed diffusely in the cytoplasm. Examples of ROS1-positive samples: (D) tumor 306, 3+; (E) tumor 570, 3+; (F) tumor 760, 3+; (G) tumor 575, 2+; (H) hematoxylin and eosin staining of large cell carcinoma sample 575; (I) tumor 668, 1+; and (J) tumor 749, 1+. K, lung 570 shows ROS1 positivity in tumor and no staining in adjacent normal tissue. L, ROS1-negative tumor with staining in reactive bronchial epithelial cells.
whether the FIG-ROS1 (S) fusion could be a potential therapeutic target in NSCLC, we treated BaF3 cells overexpressing FIG-ROS1 (S) with crizotinib and evaluated ROS1 phosphorylation and cell growth. BaF3 cells expressing FIG-ROS1 (S) were treated with crizotinib ranging in concentrations from 0 to 1 \( \mu \text{mol/L} \). Cell growth was inhibited in Karpas-299 and Baf3-FIG-ROS1 upon crizotinib treatment (Fig. 3B). ROS1 and ALK phosphorylation was inhibited after 3 hours of crizotinib treatment at a concentration of 1 \( \mu \text{mol/L} \) for ROS1 and 0.3 \( \mu \text{mol/L} \) for ALK (Fig. 3C). On the contrary, BaF3 cells expressing FLT3-ITD or empty vector (Neo-Myc) were not sensitive to crizotinib.

The phosphorylation of critical signaling nodes p-STAT3 and p-ERK in Karpas-299 and FIG-ROS1 (S)–expressing BaF3 cells were also inhibited by crizotinib (Fig. 3C). No significant changes in the phosphorylation of FLT3, STAT3, or ERK were observed in the FLT3-Baf3 control (Fig. 3C). These data suggest that FIG-ROS1 (S) can be inhibited by crizotinib.

Analysis of EGFR mutations in ROS1 and ALK-positive NSCLC tumors

EGFR mutations and ALK fusions are thought to be mutually exclusive, although rare cases of coexpression in NSCLCs have been reported (22–24). Using IHC with mutation-specific EGFR antibodies (EGFR L858R and EGFR E746-A750del; ref. 25), we examined the mutational status of ROS1- and ALK-positive tumors in our cohort where tissue was available (Tables 1 and 2). As expected, all ROS1- and ALK-positive tumors expressed total EGFR. Unexpectedly,
we identified 2 EGFR L858R/ALK–positive, 1 EGFR L858R/ROSI–positive, and 1 EGFR E746-A750del/ROSI–positive tumors (Fig. 4 and Supplementary Fig. S2). Immunohistochemical analysis of mutant EGFR and ROS1 on serial sections revealed dual expression in identical areas of the tumor, suggesting coexpression in the same tumor cells (Fig. 4).

The 2 ROS1-positive tumors express SLC34A2-ROS1 fusions, although their EGFR mutations are different. Sequencing confirmed the presence of EGFR mutations in these 2 SLC34A2-ROS1–positive tumors (data not shown).

We were unable to sequence the ALK-positive tumors for ROS1 mutations.

Discussion

The ability to administer molecular-targeted therapies to the appropriate subset of patients with NSCLC is essential to fulfill the promise of personalized cancer therapy. With an estimated 1.35 million newly diagnosed cases of lung cancer worldwide, the identification of novel oncogenic drivers is imperative (1). ROS1 rearrangements are known oncogenic drivers and define a unique clinically important subset of NSCLC (16, 21). To expand our understanding of ROS1 fusions in NSCLC, we developed ROS1 D4D6 rabbit mAb for IHC. With this antibody, we developed the ROS1 D4D6 IHC assay and screened 556 primary NSCLC tumors to evaluate the frequency of ROS1-expressing NSCLC tumors. We identified a small percentage of tumors expressing ROS1 (1.6%); however, the presence of ROS1 may stratify patients with NSCLC into an important subset for therapeutic response. The ROS1 IHC assay could possibly be developed into a diagnostic tool to identify ROS1-positive patients who may respond to the kinase inhibitor, crizotinib (16).

Before our study, CD74-ROS1 and SLC34A2-ROS were the only reported fusions of ROS1 known in primary NSCLC tumors (16, 21, 26). In this study, we observed CD74-ROS1 as the most common ROS1 fusion, and for the first time identified FIG-ROS1 (S) in a primary NSCLC tumor. The FIG-ROS1 fusion previously described in glioblastoma and cholangiocarcinoma had never been observed in NSCLC cell lines or tumors. All known ROS1 fusions can be detected with the ROS1 D4D6 IHC assay making it a valuable tool for the identification of the low frequency of patients with NSCLC expressing ROS1 fusions. No correlation was observed between ROS1 localization or staining intensity and the identification of ROS1 fusion partner. The importance of ROS1 levels and localization as predictive or prognostic biomarkers can only be evaluated with follow-up clinical studies in a larger set of ROS1 patients.

Like other RTKs, oncogenic ROS1 is amenable to targeted therapy. Selective and potent ROS1 kinase inhibitors have recently been developed (27). These compounds have yet to be evaluated in the ROS1 fusion–positive cell lines HCC78...
We also identified 21 NSCLC cases expressing ALK fusions. The majority of cases were of the adenocarcinoma subtype, which is consistent with previous reports. ALK expression in the large cell carcinoma and squamous cell carcinoma subtypes has been less studied as most previous studies are restricted to the adenocarcinoma subtype (8, 10, 28–30). We identified one large cell carcinoma and 5 squamous cell carcinomas expressing ALK fusion protein. The significance of this finding is unclear and larger studies are needed to confirm this finding. Like patients with adenocarcinoma, patients with large cell or squamous cell expressing ALK fusions may benefit from ALK inhibitors such as crizotinib.

In our study, we identified a high percentage of ALK- or ROS1-positive tumors also expressing mutant EGFR. The ethnicity of our cohort is exclusively Chinese, where EGFR mutations are more common than in non-Asian populations. Mutations in EGFR were determined using mutation-specific IHC antibodies, which together cover approximately 80% of the EGFR mutations seen in patients with NSCLC. It is possible that there are additional cases of double mutant NSCLC specimens, but they are likely to be few. Patients with both ALK and EGFR mutations may benefit from combination therapy targeting both ALK and EGFR. In a recent report, Sasaki and colleagues identified 3 of 50 treatment naive patients with NSCLC with ALK rearrangements and EGFR-activating mutations (22). Two of these patients underwent treatment with erlotinib and both had achieved partial responses. Interestingly, these patients were found to express mutant EGFR protein but not ALK protein. This observation is contrary to what we observed. In all 4 cases, where we observed EGFR mutations and ALK or ROS1 translocations, the proteins were expressed. The reason for this discrepancy is unclear, but we suspect that the use of different ALK IHC assays on different NSCLC cohorts is a plausible explanation. Collectively, these data warrant further examination of the therapeutic options for ALK rearranged and EGFR mutant patients. We argue that ROS1 fusion-positive patients expressing mutant EGFR should also be evaluated for responsiveness to EGFR inhibitors.

In conclusion, we have identified ROS1-expressing tumors in 1.6% of NSCLCs by first screening with a ROS1 IHC assay to capture any ROS1-expressing tumors. Subsequent molecular analysis by FISH and RT-PCR identified ROS1 rearrangements in these IHC-positive tumors. The utility of a ROS1 IHC assay is underscored by the recent clinical response data of ROS1-rearranged patients to crizotinib (16). Patients with NSCLC expressing ROS1 fusions are distinct from those expressing ALK fusions, further expanding the clinical utility of crizotinib in NSCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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