Correlation of ROS1 Immunohistochemistry With ROS1 Fusion Status Determined by Fluorescence In Situ Hybridization

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Context.—The ability to determine ROS1 status has become mandatory for patients with lung adenocarcinoma, as many global authorities have approved crizotinib for patients with ROS1-positive lung adenocarcinoma.

Objective.—To present analytical correlation of the VENTANA ROS1 (SP384) Rabbit Monoclonal Primary Antibody (ROS1 [SP384] antibody) with ROS1 fluorescence in situ hybridization (FISH).

Design.—The immunohistochemistry (IHC) and FISH analytical comparison was assessed by using 122 non–small cell lung cancer samples that had both FISH (46 positive and 76 negative cases) and IHC staining results available. In addition, reverse transcription–polymerase chain reaction (RT-PCR) as well as DNA and RNA next-generation sequencing (NGS) were used to further examine the ROS1 status in cases that were discrepant between FISH and IHC, based on staining in the cytoplasm of 2+ or above in more than 30% of total tumor cells considered as IHC positive. Here, we define the consensus status as the most frequent result across the 5 different methods (IHC, FISH, RT-PCR, RNA NGS, and DNA NGS) we used to determine ROS1 status in these cases.

Results.—Of the IHC scoring methods examined, staining in the cytoplasm of 2+ or above in more than 30% of total tumor cells considered as IHC positive had the highest correlation with a FISH-positive status, reaching a positive percentage agreement of 97.8% and negative.
percentage agreement of 89.5%. A positive percentage agreement (100%) and negative percentage agreement (92.0%) was reached by comparing ROS1 (SP384) using a cutoff for staining in the cytoplasm of 2+ or above in more than 30% of total tumor cells to the consensus status.

Conclusions.—Herein, we present a standardized staining protocol for ROS1 (SP384) and data that support the high correlation between ROS1 status and ROS1 (SP384) antibody.


Annually, lung cancer affects 222,500 patients and accounts for 155,870 deaths in the United States. Non–small cell lung cancer (NSCLC) is a subset of lung cancer that accounts for approximately 85% of all lung cancers. Several targeted therapies have become part of the standard of care for patients with NSCLC, with a subsequent need for biomarkers to identify the subset of patients who will respond to these therapies. The current testing paradigm in NSCLC is to test for ALK rearrangements, EGFR mutations, and programmed death ligand-1 (PD-L1) expression. Most recently, ROS1 status testing for all patients with lung adenocarcinoma has also become part of the standard of care as described in the 2018 guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology.

ROS1 is a receptor tyrosine kinase that is constitutively activated when ROS1 is rearranged. The prevalence of the rearrangement is approximately 1% to 2% in NSCLC. Most rearrangements occur interchromosomally and rarely intrachromosomally. In ROS1 rearrangements, the kinase domain of ROS1 (3’ region) is conserved and fuses with one of the multiple known fusion partners (eg, CD74, EZR, FIG1, CCD6, KDELR2, LRB, SDC4, SLC34A2, TPM3, and TPD52L1). Testing for ROS1 status is important to be able to identify patients who may have a good response to crizotinib and possibly to other ROS1 therapies.

Multiple technologies can be used to test for ROS1 positivity; however, the gold standard for testing ROS1 positivity is fluorescence in situ hybridization (FISH). Next-generation sequencing (NGS) is an emerging technology, with Thermo Fisher’s Ion Torrent Oncomine Dx Target Test being the only US Food and Drug Administration–approved companion diagnostic for detection of ROS1 fusion. A third molecular technique that is used to determine ROS1 fusions is reverse transcription–polymerase chain reaction (RT-PCR). The fourth technique used clinically is immunohistochemistry (IHC), whereby instead of detecting genetic alterations in the tumor cells, the protein (the final product of molecular biology and biological effector) is detected. Here, IHC has been used as a comparator for ROS1 fusions.

Current commercially available IHC assays do not have a standardized recommended staining protocol, nor do they have a large cohort of specimens with correlating IHC data to other ROS1 detection methods. Here, we present data on VENTANA ROS1 (SP384) Monoclonal Primary Antibody (ROS1 [SP384] antibody), using different proposed scoring methods to determine ROS1 IHC status when compared to FISH on 122 NSCLC cases, using a standardized IHC protocol. Additionally, RT-PCR and NGS were performed to elucidate discrepancies between IHC and FISH.

MATERIALS AND METHODS

Specimen Cohort

A total of 122 cases with conclusive IHC and FISH results were included in the analysis of this study and these cases came from a variety of different sources (Figure 1).

A cohort consisting of a total of 1380 formalin-fixed, paraffin-embedded (FFPE) NSCLC specimens from resections and biopsies were procured from the following sources: (1) 1335 specimens were retrieved from the internal tissue bank at Ventana Medical Systems Inc, to screen for ROS1 (SP384) staining; (2) 40 were received from expert pathologists and of these 40 cases, 36 cases had known ROS1 FISH positivity; and (3) 5 specimens with known FISH positivity were procured from US Biomax Inc (commercial tissue bank vendor).

Immunohistochemistry Staining

Four-micron-thick tissue sections were cut from each case in the cohort and mounted on positively charged glass slides. The slides were stained with ROS1 (SP384) antibody (Ventana Medical Systems Inc, Tucson, Arizona) in conjunction with OptiView DAB IHC Detection Kit (P/N 760-700, Ventana Medical Systems)
on the BenchMark instrument, using the recommended staining conditions for ROS1 (SP384) (Table 1). Rabbit Monoclonal Negative Control Ig (P/N 790-4789, Ventana Medical Systems) was used as the negative reagent control. Samples were counterstained with Hematoxylin II (P/N 790-2208, Ventana Medical Systems) and Bluing Reagent (P/N 760-2037, Ventana Medical Systems). Slides stained with hematoxylin-eosin used either VENTANA HE 600 system or Sakura Tissue-Tek Prisma Plus and Film Automated Slide Stainer & Coverslipper (Sakura Finetek U.S.A. Inc, Torrance, California) according to the manufacturer’s instruction.

**Fluorescence In Situ Hybridization**

The 1335 cases from the internal data base were assessed for overall staining intensity of tumor cells. Specimens were scored on the basis of the following stain intensity descriptions: strong, moderate, weak-moderate, weak, trace-weak, trace, and negative (absence of staining). Based on the initial assessment of 1335 cases, 114 cases representing a range of staining intensity and percentages (110 cases from the internal tissue bank and 4 cases from expert pathologists without FISH data) were sent to Cancer Genetics Incorporated (CGI; Clinical Laboratory Improvement Amendments–certified laboratory) in Los Angeles, California, for FISH testing. The IHC staining intensity of the 1380 tumors (1335 cases from the internal data base, 40 from expert pathologists, and 5 cases with known status from US Biomax) ranged from no staining, weak staining, and moderate staining to strong staining and had variable staining percentages from 0% to 100% tumor cell staining. ROS1 Dual Color Probe from Kreatech (Leica, Buffalo Grove, Illinois) was used for FISH testing (Part No. 06Q003B550 and 06Q0028495).

Of the 114 cases tested for FISH at CGI, 82 cases (71.9%) had conclusive results. However, only 81 of the 82 cases that had conclusive FISH results were included in this study (5 FISH positive and 76 FISH negative). One case was not included owing to invalid IHC results.

In total, 122 cases with conclusive FISH results were included in the study: 81 specimens for which FISH status was determined through CGI (3 of which were from expert pathologists); 36 specimens with known FISH status, received from expert pathologists; and 5 specimens from US Biomax that were positive for FISH by standard FISH testing methodologies.

**Scoring**

A board-certified pathologist performed H-scores for the 122 cases for which FISH data were available. H-score was determined by using the following formula: 

\[
H = (1 \times [\text{Percentage of Relevant Cells With 1+ Staining}]) + 2 \times [\text{Percentage of Relevant Cells With 2+ Staining}] + 3 \times [\text{Percentage of Relevant Cells With 3+ Staining}].
\]

The pathologist was blinded to the FISH status. Separate H-scores were obtained for the nuclear, cytoplasm, and membrane compartments of tumor cells. Staining intensity was defined as strong staining (3+), moderate staining (2+), weak staining (1+), and absence of staining (0).

**IHC Versus FISH Analysis**

The IHC and FISH analytical comparison was assessed with 122 NSCLC samples that had both FISH and IHC staining results. Samples stained with ROS1 (SP384) antibody were H-scored as described above. FISH results were obtained from CGI, expert pathologists, or US Biomax. The FISH status served as a reference standard to calculate the positive percentage agreement (PPA), negative percentage agreement (NPA), and overall percentage agreement (OPA) when compared to IHC. Two-sided 95% CIs were calculated by using the Wilson score method. Using different percentages of total tumor cells with staining of 2+ or above in the cytoplasm of tumor cells, multiple IHC scoring algorithms were developed. The results (ROS1 IHC status) from the different IHC scoring algorithms for each case were compared with the FISH status for that case.

**Additional Molecular Testing on Discordant Cases Between IHC and FISH**

Additional molecular testing, including ROS1 RT-PCR, ROS1 RNA NGS, and ROS1 DNA NGS, was performed on all cases that were discordant between ROS1 FISH and ROS1 IHC (based on the scoring guidance of ≥2+ staining in cytoplasm in >30% of total tumor cells as ROS1 IHC positive, and ≥2+ staining in cytoplasm in ≤30% of total tumor cells as ROS1 IHC negative).

**Detection of ROS1 Fusions by Quantitative RT-PCR.**—RNA was isolated from 10-μm FFPE tissue sections mounted on glass slides or provided as curls according to the High Pure FFPE RNA Isolation Kit (Roche, Pleasanton, California) instructions. Total RNA was quantified by using Nanodrop (Thermo Fisher Scientific, Waltham, Massachusetts). ROS1 fusion status was determined by a proprietary ROS1 quantitative RT-PCR (qRT-PCR) multiplex assay on the user-defined channel of the cobas 4800 system (Roche). The qRT-PCR assay detects 13 ROS1 fusions (CD74 exon 6–ROS1 exon 34, CD74 exon 6–ROS1 exon 32, E2R exon 10–ROS1 exon 34, TPM3 exon 8–ROS1 exon 35, SDC4 exon 4–ROS1 exon 34, SDC4 exon 2–ROS1 exon 32, SDC4 exon 2–ROS1 exon 34, SDC4 exon 4–ROS1 exon 32, SLC34A2 exon 13–ROS1 exon 34, SLC34A2 exon 3–ROS1 exon 32, SLC34A2 exon 4–ROS1 exon 35, and LRIG3 exon 16–ROS1 exon 35) and uses 50 ng RNA input for 1-step real-time RT-PCR amplification using a custom PCR mastermix and positive and negative controls. Primers for each fusion were designed against the ROS1 exon 1 and fusion partner exon sequences at the site of the fusion junction. TaqMan probe sequences were designed in ROS1 exon 32, 34, and 35.

**Detection of ROS1 Fusions by Targeted RNA Amplicon-Sequncing.**—FFPE tissue RNA was also tested by a proprietary targeted RNA amplicon-sequencing NGS assay. Briefly, cDNA was synthesized from 50 ng FFPE RNA by using the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific), followed by multiplex PCR for targeted amplicon-sequencing to detect 13 ROS1 fusions (CD74 exon 6–ROS1 exon 34, CD74 exon 6–ROS1 exon 32, E2R exon 10–ROS1 exon 34, TPM3 exon 8–ROS1 exon 35, SDC4 exon 4–ROS1 exon 34, SDC4 exon 2–ROS1 exon 32, SDC4 exon 4–ROS1 exon 34, SDC4 exon 2–ROS1 exon 34, SLC34A2 exon 13–ROS1 exon 34, SLC34A2 exon 3–ROS1 exon 32, SLC34A2 exon 4–ROS1 exon 35, and LRIG3 exon 16–ROS1 exon 35). The sample libraries were pooled and sequenced along with positive controls using the 2X150 bp paired-end sequencing protocol on the MiSeq platform (Illumina, San Diego, California).
and fragmented for library preparation. Sequencing libraries were prepared by using a hybrid capture-based target enrichment method with the AVENIO Expanded Panel. The libraries were pooled and sequenced on the Illumina NextSeq500 in high output mode using 2X150 bp paired-end sequencing. The AVENIO Tumor Tissue Expanded Kits were designed to detect ALK, ROS1, RET, FGFR2, FGFR3, and NTRK1 fusions. ROS1 introns 31, 32, 33, 34, and 35 were tiled in the panel to detect ROS1 fusion partner genes SLC34A2, CD74, SDC4, TPM3, LRG3, EZR, FIG, GOPC, MSN, and CLTC.

RESULTS

IHC H-Score Compared With Number of Cases

Of the NSCLC cases that had any staining, most were in the cytoplasm, a few were in the membrane (Figure 2), and 1 case had staining in the nucleus (H-score, 120). In addition, a range of staining intensities was observed (Figure 3, A through H).

Fluorescence In Situ Hybridization Results

Of the 114 cases that were sent to CGI for FISH testing, 32 cases (28.1%) had inconclusive results, and 82 cases (71.9%) had conclusive results. Eighty-one of those 82 cases were included in this study. Summary of FISH results from CGI are shown in Table 2.

IHC Correlation With FISH

Of the 4 scoring algorithms examined, staining in the cytoplasm of 2+ or above in more than 30% of total tumor cells had the highest correlation with a FISH-positive status (Table 3).

Discrepant Cases With 5 Methodologies to Test for ROS1 Status

Nine cases showed discordant status between FISH and IHC (based on the scoring algorithm of ≥2+ staining in cytoplasm in >30% of total tumor cells as positive and ≥2+ staining in cytoplasm in ≤30% of total tumor cells as negative). Eight cases were FISH+/IHC+ and 1 case was FISH+/IHC−. The results of the orthogonal molecular testing performed on these cases are summarized in Table 4. Of the 9 discordant cases, 2 had IHC status concordant with the consensus status, and 6 cases had FISH status concordant with the consensus status, and 1 case did not have enough DNA or RNA material to yield conclusive results with the orthogonal methods.

DISCUSSION

One of the major advantages of using IHC to determine biomarker status is the ease, speed, and cost of performing and interpreting an IHC assay. This is especially important with low-prevalence biomarkers, such as ROS1, where using the various molecular technologies greatly increases the resources needed to test all patients with NSCLC. Currently, one of the more commonly used IHC assays in the clinic is the ROS1 D4D6 rabbit monoclonal antibody (Cell Signaling Technologies, Danvers, Massachusetts), which is a research-use-only (RUO) assay. This with an RUO product, a variety of protocols, detection kits, and staining platforms are used. This leads to variation in staining and interpretation of ROS1 IHC. Furthermore, with the D4D6 clone, the correlation data between IHC and FISH in NSCLC are variable, likely due to the variability inherent in an RUO product. Also, individual studies with the D4D6 clone have small cohorts of FISH-positive cases, the largest study having only 17 cases. This leads to a deficiency in studies with large cohorts of ROS1-FISH positive cases available to support analytical correlation between IHC with FISH. Here, we provide a solution to many of the obstacles in adopting IHC as a screening tool for ROS1 positivity by providing a standardized assay with a recommended protocol for ROS1 (SP384) antibody that is supported by correlation data from a large cohort of FISH-positive and FISH-negative cases.

All diagnostic technologies have their advantages and shortcomings. For FISH, if the break-apart probes are clearly separated, then it is relatively specific for a fusion. However, often the cases are equivocal, or the probes do not function properly, leading to indeterminate results as exemplified by our comparatively high FISH failure rate of 28.1% in the cohort of cases that were sent for FISH testing. Additionally, certain FISH tests are not as sensitive in identifying intrachromosomal rearrangements. Despite the limitations with FISH, we used this technique as the criterion standard in this study because it is the current gold standard in

Figure 2. Immunohistochemistry H-score (25 increments) versus number of cases for cytoplasm and membrane compartments.
clinical practice, owing to its usefulness in clinical trials for determining ROS1 positivity. An RT-PCR assay, on the other hand, is highly sensitive for detecting specific fusions, which the assay was designed to detect. However, owing to the more than 25 known different fusions and additional unknown fusions, it is difficult to design a multiplex PCR that is able to detect all possible rearrangements. Lastly, NGS can be designed to detect both the RNA and DNA of

Figure 3. Non–small cell lung carcinoma with hematoxylin-eosin (left column: [A], [C], [E], and [G]) and corresponding ROS1 IHC (SP384) (right column: [B], [D], [F], and [H]) tissue with a range of staining intensities: (B) no staining, (D) 1+ staining, (F) 2+ staining, and (H) 3+ staining (original magnification ×10 [A through H]).
Abbreviations: CGI, Cancer Genetics Incorporated.

a Three of the cases that were sent to CGI and included in the study (1 case without FISH data from the expert pathologist was not included in the study owing to inconclusive FISH results from CGI) were received from expert pathologists without FISH data.

b Thirty-six expert pathologist cases include cases that were confirmed to be FISH positive by the respective expert pathologists.

ROS1 rearrangement events, and it is relatively easy to detect all known possible fusions owing to the high throughput and the ability to perform ultra-deep sequencing with NGS. However, NGS in its current form is more resource intensive and has a slower turnaround time when compared to some of the aforementioned technologies. In this study, we first used H-score as a data collection tool to obtain staining percentage and intensity in tumor cells stained with ROS1 (SP384) antibody. In our initial analysis, we found that a H-score of 150 or more within the cytoplasmic compartment was highly correlative with FISH-positive cases. Interestingly, in other studies with different cutoffs when scoring IHC, we examined multiple scoring methodologies that are more easily translatable to practicing pathologists interpreting at the 30% cutoff. This is the rationale for using staining in the cytoplasm of 2+ or above in more than 30% of total tumor cells as the recommended cutoff for this ROS1 IHC. It is important to note that with this cutoff, there could be borderline cases around the 30% cutoff for which the pathologist assigning a score would have difficulty determining a positive or negative status (ie, the pathologist cannot decide between a 25% versus a 30% or a 30% versus a 35% tumor cell staining, or whether the cytoplasm staining of the tumor cell is 1+ versus 2+ in intensity). In these cases, the specimen should be reflexed to a confirmatory testing method. Alternatively, another approach could be to just reflex any NSCLC case with any 2+ staining to confirmatory molecular testing. The choice of how to interpret the ROS IHC will largely depend on the institution and the clinical judgement of the pathologist scoring the ROS IHC.

All the aforementioned technologies have a certain degree of false positivity and negativity; therefore, the true ROS1 status can be difficult to determine when different testing methodologies produce inconsistent results. Hence, we

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**Table 2. Summary of Fluorescence In Situ Hybridization (FISH) Results**

<table>
<thead>
<tr>
<th>FISH Source</th>
<th>FISH-Positive Status, n/N (%)</th>
<th>FISH-Negative Status, n/N (%)</th>
<th>Total No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5/81 (6.2)</td>
<td>76/81 (93.8)</td>
<td>81</td>
</tr>
<tr>
<td>Expert pathologists&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36/36 (100)</td>
<td>0/36 (0)</td>
<td>36</td>
</tr>
<tr>
<td>US Biomax</td>
<td>5/5 (100)</td>
<td>0/5 (0)</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>46/122 (37.7)</td>
<td>76/122 (62.3)</td>
<td>122</td>
</tr>
</tbody>
</table>

Abbreviation: CGI, Cancer Genetics Incorporated.

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**Table 3. ROS1 (SP384) Antibody Immunohistochemistry Versus Fluorescence In Situ Hybridization (FISH) Analytical Comparison Data**

<table>
<thead>
<tr>
<th>FISH Status</th>
<th>Measure</th>
<th>% (n/N)</th>
<th>95% CI&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥2+ staining in cytoplasm in any tumor cell</td>
<td>PPA</td>
<td>97.8 (45/46)</td>
<td>(88.7, 99.6)</td>
</tr>
<tr>
<td>≥2+ staining in cytoplasm not present in any tumor cell</td>
<td>NPA</td>
<td>76.3 (58/76)</td>
<td>(65.6, 84.5)</td>
</tr>
<tr>
<td>Total</td>
<td>OPAn</td>
<td>84.4 (103/122)</td>
<td>(77.0, 90.9)</td>
</tr>
<tr>
<td>≥2+ staining in cytoplasm in ≥25% of total tumor cells</td>
<td>PPA</td>
<td>97.8 (45/46)</td>
<td>(88.7, 99.6)</td>
</tr>
<tr>
<td>≥2+ staining in cytoplasm in ≤25% of total tumor cells</td>
<td>NPA</td>
<td>85.5 (65/76)</td>
<td>(75.9, 91.7)</td>
</tr>
<tr>
<td>Total</td>
<td>OPAn</td>
<td>90.2 (110/122)</td>
<td>(83.6, 94.3)</td>
</tr>
<tr>
<td>≥2+ staining in cytoplasm in ≥30% of total tumor cells</td>
<td>PPA</td>
<td>97.8 (45/46)</td>
<td>(88.7, 99.6)</td>
</tr>
<tr>
<td>≥2+ staining in cytoplasm in ≤30% of total tumor cells</td>
<td>NPA</td>
<td>89.5 (68/76)</td>
<td>(80.6, 94.6)</td>
</tr>
<tr>
<td>Total</td>
<td>OPAn</td>
<td>92.6 (113/122)</td>
<td>(86.6, 96.1)</td>
</tr>
<tr>
<td>≥2+ staining in cytoplasm in ≥50% of total tumor cells</td>
<td>PPA</td>
<td>91.3 (42/46)</td>
<td>(79.7, 96.6)</td>
</tr>
<tr>
<td>≥2+ staining in cytoplasm in ≤50% of total tumor cells</td>
<td>NPA</td>
<td>93.4 (71/76)</td>
<td>(85.5, 97.2)</td>
</tr>
<tr>
<td>Total</td>
<td>OPAn</td>
<td>92.6 (113/122)</td>
<td>(86.6, 96.1)</td>
</tr>
</tbody>
</table>

Abbreviations: NPA, negative percentage agreement; OPA, overall percentage agreement; PPA, positive percentage agreement.

<sup>a</sup> Two-sided 95% CI: the Wilson score method.
attempted to determine the consensus status in cases where IHC and FISH results were discrepant by using additional orthogonal testing methodologies (RT-PCR, RNA NGS, and DNA NGS). Here, we define the consensus status as the most frequent result across the 5 different methods (IHC, FISH, RT-PCR, RNA NGS, and DNA NGS) we used to determine ROS1 status in these cases or FISH when only FISH is available. A PPA (100%) and NPA (92.0%) was reached by comparing ROS1 (SP384) antibody when using a cutoff for staining in the cytoplasm of ≥ 2 above in more than 30% of total tumor cells to the consensus status (for NPA we excluded the case with insufficient nucleic acid for testing and hence no consensus status, therefore 69 of 75 = 92.0%). It is interesting to note here that case 2 in Table 4 showed a negative result for FISH, but a positive result for IHC, RT-PCR, RNA NGS, and DNA NGS, suggesting that the negative result with FISH was a false negative. While only 1 case, it suggests that FISH is not as sensitive as the other molecular techniques and perhaps using the non-FISH molecular techniques as the confirmatory method might be better practice. However, this is only 1 case and higher-powered studies need to be performed to evaluate which molecular confirmatory methodology is the most ideal.

As shown in Figure 1, we obtained our NSCLC cases from a variety of sources owing to the low prevalence of ROS1 fusion positivity. By amassing a large cohort of ROS1 FISH-positive cases in this way, we created some potential bias, since we actively selected for FISH-positive cases and, moreover, the prevalence in our study did not mimic the actual prevalence in the clinical population. However, we chose to actively find ROS1 FISH-positive cases to validate our hypothesis that ROS1 IHC is highly sensitive for ROS1 FISH positivity and hence can be used as a screening tool. Herein, we present the ROS1 (SP384) antibody and data supporting the high correlation between our assay and FISH, using our proposed scoring algorithm (cutoff: ≥ 2 staining in cytoplasm in >30% of total tumor cells). The ROS1 (SP384) antibody will provide a standardized tool to examine the ROS1 biomarker at the protein level for the many ROS1 inhibitors currently under investigation. If future clinical trials investigating ROS1 inhibitors begin to use our standardized assay, we will be able to determine if ROS1 protein expression, DNA, or RNA alterations correlate best with clinical outcomes. Although the data presented here demonstrate a high degree of agreement between ROS1 IHC and ROS1 FISH, this is insufficient to establish clinical utility for ROS1 IHC. To truly understand the clinical utility of ROS1 IHC as it relates to ROS1 inhibitors, clinical trial data would be needed.

### Table 4. Results of 5 ROS1 Testing Methodologies for Discordant Cases Between Fluorescence In Situ Hybridization (FISH) and Immunohistochemistry (IHC)

<table>
<thead>
<tr>
<th>Case No.</th>
<th>FISH Results</th>
<th>IHC Results</th>
<th>RT-PCR Results</th>
<th>RNA NGS Results</th>
<th>DNA NGS Results</th>
<th>Consensus Status</th>
<th>IHC Concordant With Consensus Status</th>
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</thead>
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<tr>
<td>1</td>
<td>Positive</td>
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<tr>
<td>2</td>
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<td>Yes</td>
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<tr>
<td>3</td>
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<td>9</td>
<td>Negative</td>
<td>Positive</td>
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<td>N/A</td>
<td>N/A</td>
<td>No consensus</td>
<td>N/A</td>
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</table>

Abbreviations: N/A, not applicable due to insufficient nucleic acid sample for testing; NGS, next-generation sequencing; RT-PCR, reverse transcription–polymerase chain reaction.

### References