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## Multicenter Evaluation of a Novel ROS1 Immunohistochemistry Assay (SP384) for Detection of ROS1 Rearrangements in a Large Cohort of Lung Adenocarcinoma Patients

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1       **Multicenter evaluation of a novel ROS1 immunohistochemistry**  
2       **assay (SP384) for detection of *ROS1* rearrangements in a large**  
3       **cohort of lung adenocarcinoma patients**

4  
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33

34 **Abstract**

35 **INTRODUCTION:** The detection of a *ROS1* rearrangement in advanced and  
36 metastatic lung adenocarcinoma (LUAD) lead to a targeted treatment with tyrosine  
37 kinase inhibitors, with favorable progression-free survival and overall survival of the  
38 patients. Thus, it is mandatory to screen for the *ROS1* rearrangement in all these  
39 patients. *ROS1* rearrangements can be detected using break-apart fluorescence *in*  
40 *situ* hybridization (FISH), which is the “gold standard”; however, ROS1  
41 immunohistochemistry (IHC) can be used as a screening test since it is widely  
42 available, easy and rapid to perform, and cost-effective.

43 **METHODS:** We evaluated the diagnostic accuracy and inter-pathologist agreement  
44 of two anti-ROS1 IHC clones, SP384 (Ventana, Tucson, AZ) and D4D6 (Cell  
45 Signaling, Danvers, MA), in a training cohort of 51 positive *ROS1* FISH LUAD cases,  
46 and then in a large validation cohort of 714 consecutive cases of LUAD from six  
47 routine molecular pathology platforms.

48 **RESULTS:** In the two cohorts, the SP384 and D4D6 clones demonstrate variable  
49 sensitivity and specificity rates on the basis of two cutoff points  $\geq 1+$  (all % tumor cells  
50 and  $\geq 2+$  ( $>30\%$  stained tumor cells). In the validation cohort, the D4D6 yielded the  
51 best accuracy for the presence of a *ROS1* rearrangement by FISH. Inter-pathologist  
52 agreement was moderate to good (ICC, 0.722–0.874) for the D4D6 clone and good  
53 to excellent (ICC, 0.830–0.956) for the SP384 clone.

54 **CONCLUSIONS:** ROS1 IHC is an effective screening tool for the presence of ROS1  
55 rearrangements. However, users must be acutely aware of the variable diagnostic  
56 performance of different anti-ROS1 antibodies prior to implementation into routine  
57 clinical practice.

58

59 **Keywords:** ROS1; SP384; D4D6; immunohistochemistry; FISH; lung  
60 adenocarcinoma.

61

## INTRODUCTION

Patients with advanced and metastatic lung adenocarcinoma (LUAD) harboring a *ROS1* rearrangement respond effectively to crizotinib, as well as to the other tyrosine kinase inhibitors that are currently used in clinical trials.<sup>1-5</sup> *ROS1* screening is thus recommended in most guidelines. *ROS1* rearrangement detection using fluorescence *in situ* hybridization (FISH) is the typical approach that is consistently performed to identify patients with LUAD who would be eligible for these treatments. Based on its high sensitivity and specificity, this method is considered to be the “gold standard” for determining *ROS1* positivity.<sup>6, 7</sup> However, given the low incidence of *ROS1* rearrangements in lung adenocarcinoma (1% to 2%),<sup>6</sup> detection of *ROS1* protein expression by immunohistochemistry (IHC) can be used as a surrogate, cost-effective screening test that is preferable to FISH analysis for screening the *ROS1* status.<sup>8</sup> Furthermore, the expression of the *ROS1* protein, as detected by IHC, may indicate a functional gene product, which is necessary for effective drug targeting.<sup>9</sup> Moreover, FISH testing requires high-level expertise for technical and diagnostic issues, which is not widely available in all laboratories. Finally, the turnaround time for getting the final result can sometimes delay an urgent treatment decision.<sup>10</sup>

Considering these latter constraints, *ROS1* IHC was recommended as a frontline screening test prior to confirmatory FISH analysis in *ROS1*-positive cases and to avoid unnecessary FISH analysis in *ROS1*-negative cases.<sup>8</sup> To date, a single commercially available antibody (D4D6 clone, Cell Signaling Technology, Danvers, MA, USA) has been used in several studies and is currently used worldwide in routine clinical practice.<sup>11-13</sup> Given the high sensitivity of IHC relative to FISH or other molecular approaches, such as next generation sequencing (NGS) technologies, 97-98% of tumors that clearly lack *ROS1* staining can be safely interpreted as negative for *ROS1* rearrangement.<sup>6, 11-13</sup>

Conversely, the interpretation of a positive *ROS1* D4D6 IHC test may be challenging and can lead to false-positive results (related to a patchy pattern and weak intensity of the staining) that do not harbor an underlying *ROS1* rearrangement as detected by FISH.<sup>14-16</sup> Therefore, the variability of specificity reported for the *ROS1* D4D6 IHC assay requires caution and subsequent confirmation of the *ROS1* status by a validated method, irrespective of the staining intensity and the percentage

95 of positive tumor cells.<sup>15, 17</sup> Only the confirmation of a *ROS1* rearrangement by a  
96 molecular or cytogenetic method (e.g., FISH, NGS) can be used prior to considering  
97 a patient with LUAD for *ROS1*-targeted therapy.<sup>8, 18</sup>

98 In this context, the development of a new anti-*ROS1* antibody (SP384 clone,  
99 Ventana, Tucson, AZ, USA) prompted the evaluation of its diagnostic performance to  
100 predict the presence of a *ROS1* rearrangement in a routine clinical setting.

101 This study aimed to investigate the correlation between two *ROS1* IHC assays  
102 and paired *ROS1* FISH analysis as well as inter-pathologist agreement for IHC  
103 interpretation in two large-scale multicenter cohorts of LUAD cases.

104

## 105 **PATIENTS AND METHODS**

### 106 **Case selection**

107 The training cohort was comprised of retrospective archival formalin-fixed  
108 paraffin-embedded (FFPE) tumor samples from 51 patients with stage IIIB/IV LUAD  
109 and *ROS1* FISH positive. None of the *ROS1* positive cases harbored concurrent *ALK*  
110 rearrangements nor *EGFR* or *BRAF* activating mutations. In *ROS1* positive patients,  
111 tumor response was evaluated per RECIST 1.1 on target lesions, 3 months after the  
112 initiation of crizotinib and was compared with baseline results.

113 In the validation cohort, 714 cases of consecutive LUAD diagnosed in the  
114 Laboratory of Clinical and Experimental Pathology (Pasteur Hospital, Université Côte  
115 d'Azur, Nice, France) between 2006 and 2017 were integrated in tissue microarray  
116 (TMA) FFPE blocks. Three tissue cores of 600 µm in diameter were taken from  
117 different tumor areas within the same FFPE block for each case.

118 A signed informed consent was obtained from all patients. The study was  
119 approved by the local ethics committee (Human Research Ethics Committee, Nice  
120 University Hospital Center/hospital-related Biobank BB-0033-00025) and was  
121 performed in accordance with the guidelines of the Declaration of Helsinki. *ROS1*  
122 FISH positive tissue samples were provided by 6 pathology laboratory centers in  
123 France (University Hospitals of Bordeaux, Caen, Nancy, Nice, Rouen and Toulouse)  
124 and centralized for IHC and FISH analysis in the Laboratory of Clinical and  
125 Experimental Pathology (Nice, France), which is accredited by COFRAC (French  
126 National Authority for Accreditation delivery, n°8-3034; www.cofrac.fr) for FISH and  
127 IHC testing according to the ISO 15189 norm.

128 **ROS1 FISH analysis**

129 Tumor samples were fixed in formaldehyde 10% and paraffin, embedded at  
130 each participating center before centralization. Tissue sections (4 µm thick) were  
131 stained with hematoxylin eosin and the percentage of tumor cells was assessed  
132 independently by three senior thoracic pathologists (VH, MI, PH). Briefly, FISH  
133 analysis was conducted using the Abbott Molecular FISH probe (Abbott Molecular  
134 Inc., Des Plaines, IL, USA), LSI ROS1 (Tel) SpectrumOrange Probe and LS1 ROS1  
135 (Cen) SpectrumGreen Probe. Additionally, some tumors were assessed for *ROS1*  
136 rearrangement using the ZytoLight SPEC ROS1 Dual Colour Break Apart Probe  
137 (ZytoVision, Bremerhaven, Germany). FISH was performed according to the  
138 manufacturer's recommendations. Interphase signals were manually counted in at  
139 least 100 tumor nuclei per case using an epi-fluorescence microscope (Eclipse 80i,  
140 Nikon, Champigny-sur-Marne, France) supported in parallel by the PathScan®  
141 Viewer software (Excilone, Elancourt, France).

142 A positive case was defined if more than 15% of tumor cells in more than 100  
143 tumor cells demonstrated a split signal at least 2 signal distances apart or an isolated  
144 centromeric 3' (green signal) pattern.

145 **ROS1 IHC analysis**

146 ROS1 IHC analysis was performed using the D4D6 clone (Cell Signaling  
147 Technology, Danvers, MA, USA; CC1 buffer for 64 min, 1:50 dilution for 32 min,  
148 ultraView Universal DAB Detection Kit, Ventana, Tucson, AZ, USA) and the SP384  
149 clone (Ventana, Tucson, AZ, USA; CC1 buffer for 64 min, 1:50 dilution for 16 min,  
150 OptiView DAB IHC Detection Kit, Ventana) and processed on the Ultra Benchmark  
151 autostainer (Ventana) according to the manufacturer's recommendations.  
152 Immunostainings were interpreted independently by three senior lung pathologists  
153 (VH, MI, PH), blinded to FISH results, for the following criteria: signal intensity (0; no  
154 staining, 1+; weak, 2+; moderate; and 3+; strong) and percentage of stained cells,  
155 topography and characteristics of the signal (cytoplasmic – diffuse or focal and/or  
156 granular; cytoplasmic and membranous; membranous). Results were quantified by  
157 multiplying the percentage of ROS1 positive tumor cells by the intensity (H-score,  
158 range, 0–300). For each case included on the TMA FFPE blocks, the mean H-score  
159 of the three cores was recorded. Non-specific staining of macrophages and type II

160 pneumocytes was disregarded. ROS1 negative and positive controls were put on  
161 each IHC slide (HCL0024, HistoCyte Laboratories Ltd, Newcastle University, UK).

## 162 **Statistical analysis**

163 Statistical analyses assessing agreement between the D4D6 and SP384  
164 antibodies with the ROS1 FISH analysis (gold standard) were performed on  
165 dichotomized variables using two positivity cutoff points: IHC ROS1 positive (intensity  
166  $\geq 1+$ , any percentage of tumor cells) and IHC ROS1 strongly positive (intensity  $\geq 2+$ ,  
167  $>30\%$  tumor cells), as recently described.<sup>17, 19</sup> Sensitivity, specificity, positive  
168 predictive value (PPV), negative predictive value (NPV), and accuracy were  
169 calculated for the comparison between the dichotomized results using each of the  
170 two antibodies relative to the FISH results. The Spearman's rank correlation test (*rho*)  
171 was used for a correlation analysis between the H-scores as continuous variables of  
172 each clone. Intra-class correlation coefficients were calculated for the comparison of  
173 H-scores as continuous variables from each of the ROS1 clones, and  $\kappa$  scores were  
174 calculated for dichotomized results based on two positivity cutoff points, as described  
175 above, to assess the inter-pathologist agreement for each of the clone. The paired  
176 Student's t-test was used to assess the correlation between the ROS1 IHC status  
177 and RECIST 1.1. All statistical analyses were carried out using R software (version  
178 3.2.2, R Core Team, Vienna, Austria). Any *P*-value  $<0.05$  was assumed to indicate a  
179 statistically significant difference.

180

## 181 **RESULTS**

### 182 **Training cohort**

183 In a clinically selected cohort enriched for tumors with FISH-identified ROS1  
184 rearrangements, the frequency of split or isolated centromeric signals averaged 64%  
185 and ranged from 15% to 100%. IHC SP384 showed strong 3+ expression in 40/51  
186 (78%), 2+ moderate in 10/51 (20%) and 1+ weak in 1/51 (2%) of cases (**Table 1,**  
187 **Figure 1**). IHC D4D6 showed a wider range of intensity in 15/51 (30%) with 3+  
188 staining, 16/51 (31%) with 2+ staining and 20/51 (39%) with 1+ staining (**Table 1,**  
189 **Figure 1**). Inter-pathologist agreement was high in interpreting both ROS1 IHC  
190 clones (**Supplementary Table S1**).

191 On the basis of the  $\geq 1+$  expression cutoff, both ROS1 IHC clones are 100%  
192 sensitive, whereas if  $\geq 2+$  tumors are considered positive, the SP384 clone is 98%

193 sensitive and D4D6 is only 61% sensitive for the detection of a ROS1 rearrangement  
194 by FISH (**Table 1**).

195 H-scores ranged from 5 to 300 and from 50 to 300 when using the D4D6 and  
196 SP384 clones, respectively. However, the Spearman's rank correlation was not linear  
197 ( $\rho=0.508$ ; 95% CI, 0.19–0.72) between the two clones (**Figure 1, Supplementary**  
198 **Figure S1**).

199 Neither the intensity nor the percentage of ROS1 protein expression detected  
200 with both SP384 and D4D6 antibodies correlated with the percentage of ROS1 split  
201 signals by FISH (**Supplementary Figure S2**).

202 In most of the cases analyzed with the SP384 clone, a diffuse cytoplasmic  
203 staining was observed with or without membranous staining (**Figure 2**). Less  
204 frequently, a granular cytoplasmic or a membranous staining alone was noted  
205 (**Figure 2**). Corresponding D4D6 IHC staining was weaker and in some cases the  
206 percentage of positive tumor cells was lower than those noted in the corresponding  
207 tumors stained with the SP384 clone (**Figure 1, Supplementary Figure S1**). ROS1  
208 protein expression was also seen with both D4D6 and SP384 antibodies in non-  
209 neoplastic alveolar epithelium at the tumor periphery, in sub-pleural pneumocytes or  
210 in areas of type II pneumocyte hyperplasia and bronchiolar metaplasia. However, the  
211 expression in this epithelium was weak in intensity in most cases.

212 In this retrospective study, at the time of clinical care, the vast majority of  
213 ROS1 positive patients, as confirmed by FISH, were treated with crizotinib in the  
214 second and third line settings. After 3 months, 18% of patients had a complete  
215 response, 19% demonstrated a partial response, 19% had stable disease, and 44%  
216 had progressive disease (**Supplementary Figure S3**). In total, 56% of patients had  
217 disease control after 3 months on crizotinib. However, no significant difference  
218 existed between the two IHC clones (**Supplementary Figure S3**) nor between the %  
219 of rearranged tumor cells by FISH and the response by RECIST (t-test,  $P$ -  
220 value=0.306). In order to identify putative resistance mechanisms<sup>20</sup>, an NGS analysis  
221 (Ion AmpliSeq Cancer Hotspot Panel, ThermoFisher Scientific, San Francisco, CA,  
222 USA) was performed in two patients with progressive disease and available tumor  
223 material. In one patient, we found a mutation in the gene encoding for  $\beta$ -catenin  
224 (CTNNB1 p.S45F, VAF 43%). The NGS analysis detected no copy-number



225 variations. Moreover, these cases had no *ROS1* copy-number gain, as determined by  
226 FISH.<sup>20</sup>

### 227 **Validation cohort**

228 A total of 714 cases of LUAD with an unknown *ROS1* rearrangement status  
229 were screened on TMA blocks using paired a *ROS1* IHC and FISH assay. *ROS1*  
230 rearrangements were detected in this screening cohort by FISH at a rate of 9 of 714  
231 cases (1.3%).

232 On the basis of the  $\geq 1+$  expression cutoff, both *ROS1* IHC clones are 100%  
233 sensitive for the presence of a *ROS1* translocation detected by FISH, while the D4D6  
234 clone was deemed more specific and accurate (**Table 1, Figure 3**). Conversely, on  
235 the basis of the  $\geq 2+$  expression cutoff, the SP384 clone was more sensitive than the  
236 D4D6 clone, while the D4D6 was more specific and accurate than SP384 (**Table 1,**  
237 **Figure 3**).

238 Inter-pathologist agreement ranged from moderate to good (ICC, 0.722–0.874)  
239 in interpreting the D4D6 IHC assay and from good to excellent (ICC, 0.830–0.956) for  
240 the SP384 IHC assay (**Supplementary Table S1**).

241

242

## 243 DISCUSSION

244 This is the first study to assess the diagnostic performance of a newly  
245 developed anti-ROS1 antibody (SP384 clone) by comparison to two diagnostic ROS1  
246 assays that are validated for a clinical setting (e.g., D4D6 IHC and ROS1 break-apart  
247 FISH assays) in two large-scale multicenter cohorts of LUAD cases.

248 We considered two cutoffs for positivity and showed that none of the ROS1  
249 IHC assays demonstrated 100% accuracy in detection of *ROS1* rearrangements as  
250 identified by FISH. In the validation cohort of ROS1-positive LUAD cases, while these  
251 two clones demonstrate similar sensitivity around the  $\geq 1+$  cutoff, the sensitivity rate  
252 dropped at 61% for the D4D6 clone when the  $\geq 2+$  cutoff was applied. In addition, the  
253 intensity and the percentage of stained tumor cells were higher when using the  
254 SP384 clone than with the D4D6 clone. Inter-reader agreement varied from moderate  
255 to good when interpreting the D4D6 assay, while it was deemed good to excellent for  
256 the SP384 clone, as recently reported.<sup>19</sup> Most of the cases analyzed with the SP384  
257 clone showed diffuse cytoplasmic staining, with or without membranous labeling.

258 It is noteworthy that the percentage and the intensity of SP384 and D4D6 IHC  
259 did not correlate with the percentage of *ROS1* FISH split signals, as had previously  
260 been reported.<sup>12</sup> No significant difference was noted between the different ROS1 IHC  
261 assays and the RECIST 1.1 subgroups in patients treated with crizotinib.

262 In our screening cohort, which was drawn from a clinical molecular diagnostics  
263 practice, ROS1 rearrangement was detected in 1.3% of tumors, compared to 1–2%  
264 of unselected LUAD cases, as previously reported.<sup>6</sup> Around the  $\geq 1+$  cutoff, sensitivity  
265 was similar for both IHC assays; however, the specificity of the D4D6 clone (97.87%)  
266 was higher than that of the SP384 clone (87.38%). We also explored the recently  
267 described  $\geq 2+$  cutoff in  $>30\%$  tumor cells.<sup>17, 19</sup> Whereas the sensitivity of the D4D6  
268 clone declined to 66.67% under these criteria, and was 88.89% for the SP384  
269 antibody, the specificity was still higher for the D4D6 assay (99.57%) than the SP384  
270 clone (91.06%).

271 The average H-score of the SP384 clone was higher than the D4D6 antibody,  
272 which led to easier interpretation. However, if an algorithm for *ROS1* testing that is  
273 similar to that established for *ALK* status testing were used and if the  $\geq 1+$  cutoff were  
274 to be used for both IHC clones, this could lead to an increase of nearly 10% in  
275 unnecessary FISH testing based on screening with the SP384 clone. Moreover, in

276 our study, the  $\geq 2+$  cutoff in  $>30\%$  tumor cells did not improve the sensitivity and  
277 specificity rates of either IHC assay.

278 When newly developed diagnostic assays for the detection of rare events such  
279 as *ROS1* rearrangements are considered for implementation in a routine clinical  
280 setting, our findings highlight the importance of their evaluation in large-scale cohorts,  
281 and the necessity of both positive and negative cases. As accurate detection of  
282 *ROS1* rearrangements in clinical tumor samples is mandatory for optimal targeted  
283 treatment, investigation of new testing modalities should not be limited to positive  
284 samples only.<sup>18</sup>

285 Previous studies reported *ROS1* D4D6 IHC as being highly sensitive, but less  
286 specific compared with FISH for the detection of *ROS1* rearrangements. *ROS1*  
287 D4D6-positive tumors, especially when the tumor is stained with moderate to strong  
288 intensity or a diffuse pattern, are recommended to undergo FISH to confirm the  
289 presence of *ROS1* rearrangement.<sup>12-14</sup> Besides the assessment of the *ROS1*  
290 rearrangement status, it is mandatory to systematically detect other genomic  
291 alterations in advanced and metastatic lung adenocarcinoma. This can be performed  
292 by molecular biology (e.g., *EGFR* mutation) and by molecular and/or IHC approaches  
293 (e.g., *BRAFV600* mutation, *ALK* rearrangement). Moreover, PD-L1 IHC is now  
294 required in this population. Additionally, the evaluation of other genomic alterations  
295 (e.g., *RET* rearrangement, *MET* mutations, *HER2* mutations, *NTRK* rearrangement)  
296 can be required if the tumors are *EGFR*, *ALK*, *ROS1* and *BRAF* wild-type and with  
297 less than 50% of PD-L1 positive tumor cells. Moreover, recent clinical trials for  
298 immunotherapy associated with the assessment of the tumor mutation burden (TMB)  
299 have indicated some promising results for the systemic use of this predictive  
300 biomarker in the near future.<sup>21, 22</sup> In this context, one challenge is to be able to  
301 perform these analyses on smaller tissue biopsies and/or samples with low  
302 proportions of tumor cells (less than 20%). Considering that a minimum number of  
303 tissue sections are required for the diagnosis of NSCLC, it is critical to conserve  
304 tissue as much as possible for ancillary analyses (different predictive IHC, targeted  
305 sequencing analyses or NGS panels – potentially including TMB assessment). In this  
306 context, it is of interest to economize and only use tissue sections for *ROS1* FISH  
307 analysis in IHC positive cases. More importantly, the feasibility of using *ROS1* IHC for  
308 *ROS1* status assessment has many advantages in comparison with the *ROS1* FISH.

309 IHC is available in all pathology laboratories and can easily be conducted. The  
310 turnaround time to get the results is much shorter than the FISH approach. IHC is a  
311 cost-effective technology. Finally, shipment and archiving of stained slides can be  
312 done at room temperature.

313 Different testing approaches, such as DNA-based hybrid capture library  
314 preparation followed by NGS, RNA-based anchored multiplex polymerase chain  
315 reaction library preparation followed by NGS or nCounter technologies could allow  
316 the simultaneous detection of gene rearrangements such *ALK*, *ROS1*, *RET*, or  
317 *NTRK*.<sup>18, 23, 24</sup> Thus, it might be questionable whether the development of IHC assays  
318 for the detection of these alterations should continue, in particular for *ROS1*  
319 rearrangement. However, some difficulties can be encountered for the NGS  
320 approach in comparison with the IHC method in a clinical setting.<sup>25</sup> Depending on the  
321 quantity and quality of nucleic acids present in the FFPE tissue biopsy, NGS can give  
322 false negative results, in particular in small specimens and/or when a low percentage  
323 of tumor cells are present. Moreover, the technology is not available in all  
324 laboratories and requires specific and high-level expertise. The time lag for results is  
325 also much longer than for IHC testing. Finally, the accreditation is certainly easier to  
326 obtain for an IHC test than for a test developed on an NGS platform.

327 Assuming that the implementation of the SP384 IHC assay in the testing  
328 algorithm of advanced and metastatic LUAD patients can be considered, following  
329 the results showed in the present work and probably other forthcoming studies,  
330 independent validation studies need to be performed. In addition, it will be of interest  
331 to further assess the performance of the SP384 clone on cytology samples such as  
332 cellblocks.<sup>26, 27</sup> Finally, as a perspective, the ROS1 IHC assay could be a good  
333 candidate to be associated with other “predictive” antibodies such as the anti-ALK,  
334 anti-BRAFV600E and anti-PD-L1 in a chromogenic multiplex IHC assay for small  
335 tissue biopsies.<sup>28</sup>

336 In conclusion, ROS1 IHC testing is an effective screening tool for the presence  
337 of *ROS1* rearrangements. However, users must be aware of the variable diagnostic  
338 performance of different commercialized anti-ROS1 antibodies before incorporation  
339 into the diagnostic molecular pathology workup of LUAD cases.

340  
341

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346

347 **Conflict of interests:**

348 I.R. has received honoraria from AstraZeneca, MSD, Bristol-Myers Squibb,  
349 Boehringer-Ingelheim, Roche and Takeda.

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361 Myers Squibb, Novartis, MSD, Qiagen, Thermofisher, Biocartis, and Merck & Co.

362

363 The remaining authors have declared no conflict of interests.

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376 **Legend to Figures**

377

378 **Figure 1. Representative images of ROS1 IHC using the D4D6 and the SP384**  
379 **antibodies in the training cohort. (A)** Strong diffuse staining with the SP384  
380 antibody, and **(B)** weak to moderate diffuse staining with the D4D6 antibody in the  
381 same case as in (A). **(C)** Weak to moderate diffuse staining with the SP384 antibody,  
382 and **(D)** weak focal staining with the D4D6 antibody in the same case as in (C). A  
383 *ROS1* rearrangement was identified in both cases (A-B, and C-D) by FISH. (Scale  
384 bars are shown on the figures).

385

386 **Figure 2. Representative immunostaining localization in tumor cells using the**  
387 **SP384 antibody. (A)** Diffuse cytoplasmic. **(B)** Membranous and cytoplasmic. **(C)**  
388 Membranous. **(D)** Cytoplasmic granular. Scale bars are shown on the figures.

389

390 **Figure 3. Representative comparative images between the ROS1 IHC using the**  
391 **D4D6 and the SP384 antibodies and the ROS1 FISH analysis in the validation**  
392 **cohort.**

393 **(A)** Strong diffuse (3+) staining with the D4D6 and SP384 antibodies in a LUAD case  
394 positive by ROS1 FISH.

395 **(B)** Weak diffuse staining (1+) with the D4D6 antibody but strong staining (3+) with  
396 the SP384 clone in a LUAD case positive by ROS1 FISH.

397 **(C)** Weak diffuse staining (1+) with the D4D6 antibody and moderate staining (2+)  
398 with the SP384 clone in a LUAD case positive by ROS1 FISH.

399 **(D)** Lack of staining with both D4D6 and SP384 clones in a LUAD negative by ROS1  
400 FISH.

401 **(E)** No staining with the D4D6 clone in a LUAD case moderately stained (2+) with  
402 SP384 clone and positive by ROS1 FISH.

403 **(F)** No staining with the D4D6 clone in a LUAD case strongly stained (3+) with the  
404 SP384 clone but revealed negative by ROS1 FISH.

405 Red circles; positive tumor cells by FISH; green circles, negative tumors cells by  
406 ROS1 FISH.

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410 **Legend to Supplementary Figures**

411

412 **Supplementary Figure S1.** Distribution plot of H-scores for each anti-ROS1  
413 antibody.

414

415 **Supplementary Figure S2.** Correlation analysis between the H-scores of **(A)** D4D6  
416 or **(B)** SP384 clones and the percentage of tumor cells harboring *ROS1*  
417 rearrangement by FISH. *Note: the X-axis in (A) is manually set to have a maximum of*  
418 *300 to match with SP384.*

419

420 **Supplementary Figure S3.** Correlation analysis between response assessments by  
421 RECIST 1.1 after 3 months under crizotinib and the IHC status by D4D6 (paired  
422 Student's t-test; P=0.002) and SP384 (paired Student's t-test; P=0.006) clones.  
423 Abbreviations: Progressive Disease (PD), Stable Disease (SD), Partial Response  
424 (PR), Complete Response (CR).

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**Table 1.** Comparative performance analyses between *ROS1* FISH and SP384 and D4D6 IHC clones.

Training cohort	SP384 IHC				D4D6 IHC			
FISH ROS1	Strong 3+	Moderate 2+	Weak 1+	Negative 0	Strong 3+	Moderate 2+	Weak 1+	Negative 0
Positive n = 51	40 (78%)	10 (20%)	1 (2%)	0 (0%)	15 (30%)	16 (31%)	20 (39%)	0 (0%)
<b>Sensitivity</b> Cutoff ≥1+ Cutoff ≥2+	100% (95% CI, 93.02%–100%) 98.04% (95% CI, 89.55%–99.95%)				100% (95% CI, 93.02%–100%) 60.78% (95% CI, 46.11%–74.16%)			
Validation cohort	SP384 IHC				D4D6 IHC			
FISH ROS1								
Positive n = 9 (1.3%)	6 (67%)	2 (22%)	1 (11%)	0 (0%)	3 (33%)	3 (33%)	3 (33%)	0 (0%)
Negative n = 705 (98.7%)	0 (0%)	63 (9%)	26 (4%)	616 (87%)	0 (0%)	3 (1%)	12 (2%)	690 (97%)
<b>Cutoff ≥1+</b>								
Sensitivity	100% (95% CI, 66.37%–100%)				100% (95% CI, 66.37%–100.00%)			
Specificity	87.38% (95% CI, 84.70%–89.74%)				97.87% (95% CI, 96.51–98.80%)			
PPV	9.18% (95% CI, 7.69%–10.94%)				37.50% (95% CI, 26.67%–49.75%)			
NPV	100%				100%			
Accuracy	87.54% (95% CI, 84.89%–89.87%)				97.90% (95% CI, 96.56%–98.82%)			
<b>Cutoff ≥2+</b>								
Sensitivity	88.89% (95% CI, 51.75%–99.72%)				66.67% (95% CI, 29.93%–92.51%)			
Specificity	91.06% (95% CI, 88.71%–93.06%)				99.57% (95% CI, 98.76%–99.91%)			
PPV	11.27% (95% CI, 8.37%–15.01%)				66.67% (95% CI, 37.12%–87.14%)			
NPV	99.84% (95% CI, 99.02%–99.98%)				99.57% (95% CI, 98.93%–99.83%)			
Accuracy	91.04% (95% CI, 88.70%–93.03%)				99.16% (95% CI, 98.18%–99.69%)			

Abbreviations: FISH, Fluorescence in situ hybridization; NPV, Negative Predictive Value; PPV, Positive Predictive Value.



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**Figure 1**

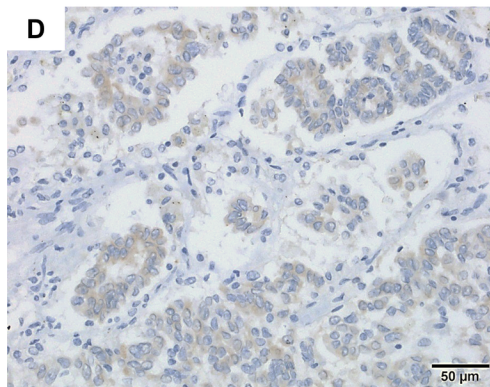
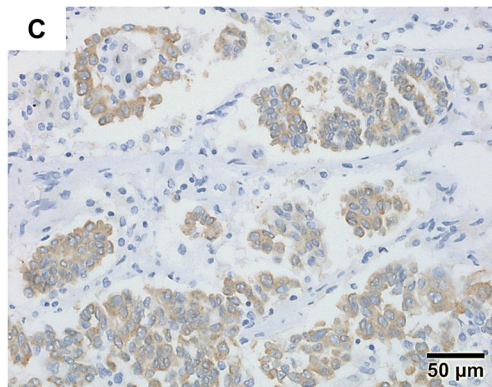
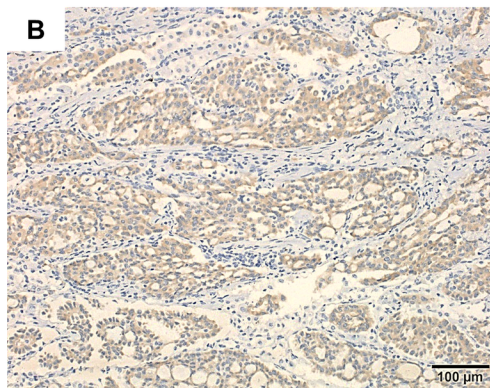
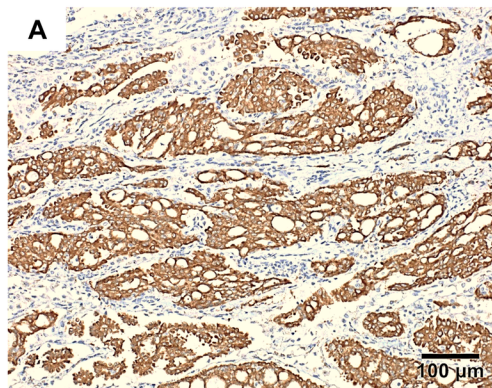
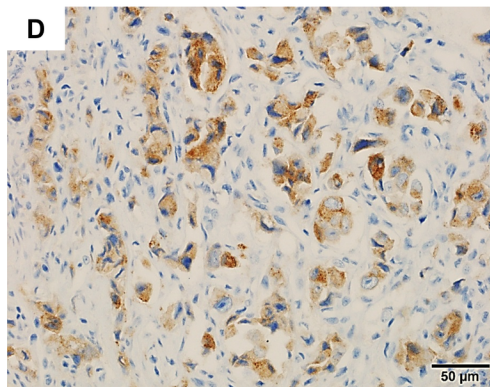
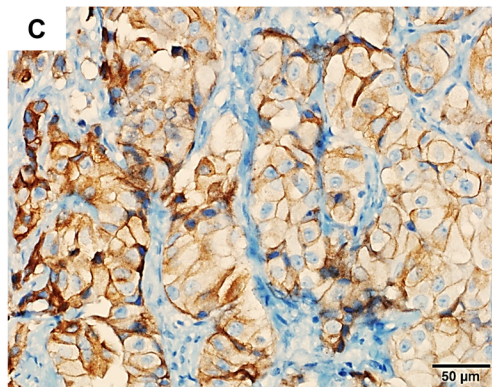
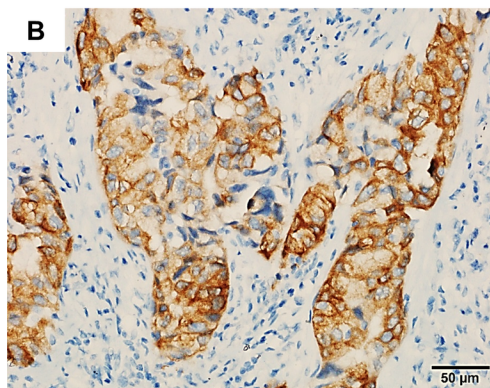
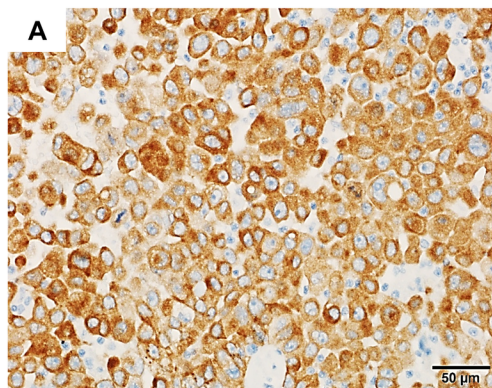
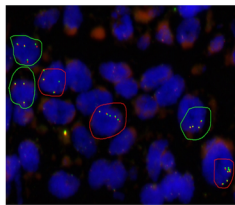
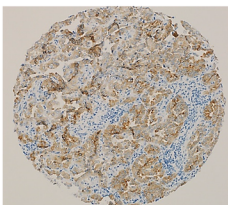
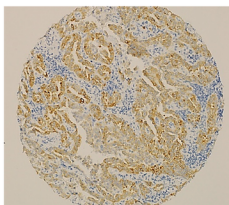
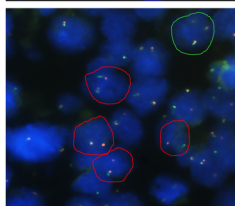
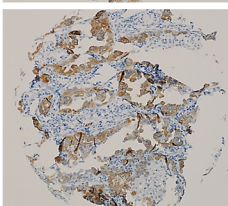
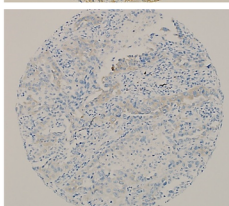
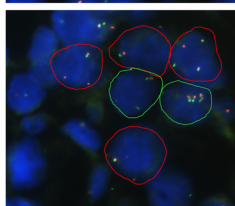
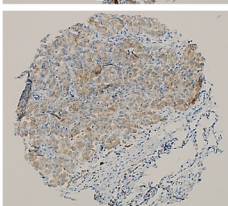
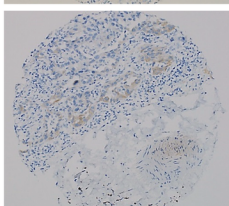
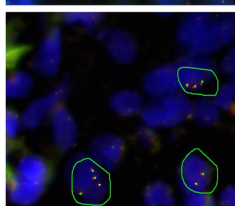
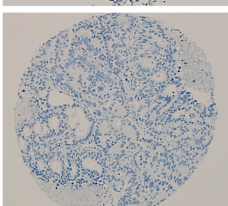
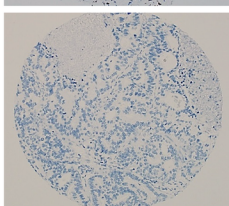
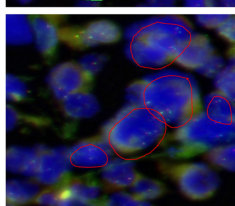
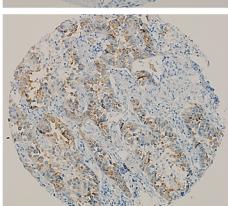
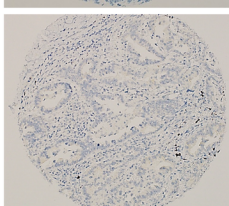


Figure 2



**Figure 3****D4D6****SP384****FISH****A****B****C****D****E****F**