Comparison of Methods in the Detection of *ALK* and *ROS1* Rearrangements in Lung Cancer

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Introduction: The use of targeted therapies toward specific oncogenic driver mutations has become a critical factor in the treatment of patients with lung cancer. It is therefore essential to utilize tests with high performance characteristics. Fluorescence in situ hybridization (FISH) is the standard method for detecting anaplastic lymphoma kinase *(ALK*) and *ROS1* rearrangements in non–small-cell lung cancer but the utility of other methods such as immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) is unclear.

Methods: Three hundred and sixty-two lung cancer patients were tested with FISH, CISH, and IHC using three ALK antibodies (ALK1, 5A4, D5F3) and one ROS1 antibody in the detection of *ALK* and *ROS1* rearrangements.

Results: There was a 97.4% concordance (298 of 306) between FISH and CISH for detection of *ALK* rearrangements. The *ROS1* rearrangement status had a 97% (291 of 300) concordance between CISH and FISH. ALK protein expression was observed in 6 of 341 samples with the ALK1 and 5A4 antibodies and 5 of 341 samples with D5F3. All three antibodies stained each of the *ALK* FISH-positive samples (100% sensitivity). ROS1 protein expression was observed in 2 of 322 samples. One of three samples with a *ROS1* rearrangement by FISH showed ROS1 protein expression (33.3% sensitivity).

Conclusion: Our findings show good correlation between FISH versus CISH in the detection of *ALK* and *ROS1* rearrangements. FISH versus IHC showed good correlation in the detection of *ALK* rearrangements but showed weak correlation in the detection of *ROS1* rearrangements. These results suggest CISH and IHC could be complimentary detection methods to FISH in the detection of *ALK* and *ROS1* rearrangements.

Key Words: *ALK, ROS1*, Non–small-cell lung cancer, Fluorescence in situ hybridization, Immunohistochemistry, Chromogenic in situ hybridization.

(*J Thorac Oncol.* 2015;10: 611–618)

Lung cancer remains one of the most common leading causes of cancer-related deaths worldwide.[1] Over recent years, there has been a shift in strategies for the treatment of lung cancers toward the use of tyrosine kinase inhibitors specifically targeted at an oncogenic driver mutation identified in tumor tissue. The prototypical example is in patients with non–small-cell lung cancer (NSCLC), who harbor activating mutations in the epidermal growth factor receptor gene who may have dramatic responses with tyrosine kinase inhibitors.[2,3] Recently, anaplastic lymphoma kinase (*ALK*) and *ROS1* rearrangements have been identified as a distinct subset of NSCLC in 3–5% and 1–2% respectively, which have also shown sensitivity to treatment with the tyrosine kinase inhibitor, crizotinib.

ALK rearrangements in NSCLC were first discovered in 2007 when a fusion was demonstrated between *ALK* and echinoderm microtubule-associated protein-like 4 (*EML4*)[4,5] due to an inversion on the short arm of chromosome 2. *EML4* is a widely expressed cytoplasmic protein involved in the formation of microtubules.[6] These *ALK* fusions in NSCLC are usually a result of an intrachromosomal rearrangement in which the intracellular kinase domain of *ALK* fuses to the *N*-terminal of the *EML4* gene.[4] There are multiple known *ALK-EML4* variants which all occur in the same region of the *ALK* gene but involve different breakpoints within the *EML4* gene with other rare fusion partners including a kinesin family member 5B (*KIF5B*), TRK-fused gene (*TFG*), and kinesin light chain 1 (*KLC1*).[5,7–9]

ROS1 is a receptor tyrosine kinase, which is part of the insulin receptor family. *ROS1* rearrangements were first described in the glioblastoma cell line U118MG but have since been described in cholangiocarcinoma at a frequency of approximately 9%.[10] *ROS1* rearrangements were described in NSCLC in 2007 in the lung cell line HCC78 with fusion partner *SLC34A2* and in one patient's tumor with fusion partner

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Disclosure: LAH, SSi, SSt, JG, ER are employees of Ventana. GW has been asked to be on a board for Pfizer but did not receive any payments. All other authors declare no conflict of interests.

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DOI: 10.1097/JTO.0000000000000465

ISSN: 1556-0864/15/1004-0611

CD74.[5] Since then multiple other fusion partners have been identified such as *FIG* (also known as *GOPC*), *SDC4, TPM3, EZR,* and *LRIG3*.[11,12] All *ROS1 r*earrangements involve the same 3′ region of the kinase domain of the *ROS1* gene which fuses to the 5′ region of the partner gene.[13]

There are currently several methods available for the detection of *ALK* and *ROS1* rearrangements such as immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH), and reverse-transcription polymerase chain reaction (RT-PCR). However, FISH is the current standard procedure for testing and is the only Food and Drug Administration-approved method for the detection of *ALK* rearrangements. FISH is a specialized test requiring expensive equipment and extensive experience with interpretation due to heterogeneity within the tissue and morphology. FISH is therefore not readily available in many pathology laboratories. On the other hand, IHC is a routine method used in many pathology laboratories but is dependent upon the sensitivity and specificity of the antibody clone used. RT-PCR is also a highly sensitive specialized technique not readily available in most pathology laboratories that is reliant on extraction of good quality RNA from formalin-fixed paraffin-embedded tissue. Its main limitation is that with the high variability of the fusion partners, novel rearrangements may be missed.

CISH including silver in situ hybridization (SISH) are common methods used in determining the *HER2* status in breast and gastric cancers, which have shown comparable results with FISH.[14,15] The use of CISH in the detection of rearrangements is not common as the fusion signals are often difficult to distinguish.[16] However, improvements in *ALK* probe design have made the use potentially feasible as recent studies have shown.[17–19]

In this study, we assessed the concordance between a break-apart dual-color CISH assay and the more commonly used FISH assay for the detection of A*LK* and *ROS1* rearrangements and investigated the reliability of an IHC method of detection for these rearrangements using three commercially available ALK antibodies and one ROS1 antibody. The aim of this study was to assess whether dual-color CISH and/or IHC are sufficiently reliable and robust to be used as alternate methods for the identification of *ALK* and *ROS1* rearrangements to FISH so that they can potentially be adopted by pathology laboratories without access to FISH. This would enable local as opposed to reference testing, facilitating access to molecular testing for all patients in an equitable manner without the requirement of specialist techniques.

MATERIALS AND METHODS

Patients, Histological Evaluation, and Tissue Microarrays

Tissue microarrays (TMA) from 362 lung tumors from the Peter MacCallum Cancer Centre and St Vincent's Hospital were constructed as previously described.[20] All 362 patients had a histopathologic diagnosis of primary lung cancer defined according to the 2004 World Health Organization of Lung Tumors[21] with adenocarcinomas additionally classified according to the new IASLC/ATS/ERS international multidisciplinary lung adenocarcinoma classification.[22] All tumors were staged according to the 7th edition AJCC tumor, node, metastasis classification. Clinical information was obtained from detailed prospective clinical databases. The definition of a never-smoker was a person with lifetime equivalent consumption of fewer than 100 cigarettes. Ethics approval was obtained from the Human Research and Ethics Committees at Peter MacCallum Cancer Centre and St Vincent's Hospital, Melbourne (PMCC: 03/90 and SVH: A03/12).

Fluorescence In Situ Hybridization

Three micron thickness formalin-fixed paraffin-embedded sections were de-waxed, taken to water then placed in Heat Pretreatment Solution (Invitrogen Corporation, Camarillo, CA) in the Pascal pressure cooker (DakoCytomation, Carpinteria, CA) at 124°C for 2 minutes. Slides were washed in distilled water after which they were covered with Enzyme Reagent (Invitrogen Corporation, Camarillo, CA) at room temperature for 30 minutes. Slides were washed in distilled water and dehydrated in successive percentages of alcohol (70%, 85%, and 100%) and air-dried at room temperature. Ten microliters of the appropriate probe: *ALK* Break Apart Rearrangement Probe (Abbott Molecular, Des Plaines, IL) or *ROS1* Break Apart Rearrangement (Bacterial Artificial Chromosome clones: RP11-1036C2-9.40 [chromosome 6q *ROS1* 3′ locus] Spectrum Orange, RP11-378F24-9.43 [chromosome 6q *ROS1* 5′ locus] Spectrum Green and RP11-835I21-9.39 [chromosome 6q *ROS1* 5′ locus] Spectrum Green; gift from Translational Research Laboratory, Massachusetts General Hospital) was placed onto the tissue sections. Slides were denatured at 85°C for 5 minutes and then hybridized at 37°C for 17 hours on a Dako Hybridiser (Dako, Fort Collins, CO). Following hybridization, slides were washed with buffers then coverslipped.

Fifty tumor nuclei per case were assessed for each case using the following criteria: for *ALK* FISH, cells were considered positive (rearranged) if there was a split two or more signal widths apart between the orange and green signals or there was a single orange signal (3′ end) without a corresponding green signal (5′ end) in conjunction with a fused and/or split signals. A case was considered positive (rearranged) if 15% or greater of tumor cell nuclei were rearranged. For *ROS1*, FISH cells were considered positive (rearranged) if there was a split two or more signal widths apart between the orange and green signals or there was a single orange signal (3′ end) without a corresponding green signal (5′ end) in conjunction with a fused and/or split signals. A case was considered to have an atypical pattern if there was a single green signal (5′ end) without a corresponding orange signal (3′ end) in conjunction to a fused and/or split signals. A case was considered positive (rearranged) if 15% or greater of tumor cell nuclei were rearranged. For statistical analysis samples that showed an atypical signal pattern by *ROS1* FISH were considered negative.

Dual-Color Chromogenic In Situ Hybridization

Rearrangements of the *ALK* and *ROS1* genes were identified using *ALK* and *ROS1* rearrangement DNA probe sets

(Ventana Medical Systems Inc, Tucson, AZ), respectively. Target sequences for probe design for each gene region were analyzed using an in-house bioinformatics software package. The sub-sequences within the each genomic target region were further tested using a Roche NimbleGen Comparative Genomic Hybridization custom array. DNA target sequence hybridization to a human genomic probe, and a Cot1 DNA probe were analyzed and sequences with high hybridization scores following the Comparative Genomic Hybridization analysis were eliminated. Specificity of each probe was confirmed on a metaphase spread. The dual-color brightfield CISH assays were conducted with the BenchMark XT and Ultra automated slide processing systems (Ventana Medical Systems Inc, Tucson, AZ). For detection of the *ALK* rearrangements, DNP-3′-ALK and DIG-5′-ALK probes were hybridized to targets and detected using *ultra*View SISH DNP and *ultra*View Red ISH DIG detection kits, respectively (Ventana Medical Systems Inc, Tucson, AZ). *ROS1* rearrangements were measured with the same detection method using DNP-3′-ROS1 and DIG-5′-ROS1. The scoring criteria for both *ALK* and *ROS*1 dual-color CISH were the same as those described for FISH.

Immunohistochemistry

ALK IHC was performed with the three ALK antibodies as previously described.[20] ROS1 IHC was performed using a Ventana Benchmark Ultra automated immunostainer (Ventana Medical Systems, Tucson, AZ). Heat pretreatment was performed using CC1solution (Ventana Medical Systems) for 64 minutes. The primary ROS1 rabbit monoclonal antibody, Clone D4D6 (Cell Signaling Technology, Danvers, MA) was diluted to 1:50 and incubated at 37°C for 32 minutes. The optiView universal DAB detection system (Ventana Medical Systems) was used.[23] Each case was assessed using the following scoring criteria without knowledge of FISH and dual-color CISH results and an IHC score was assigned: Staining intensity; 0 = no staining; 1+ = mild staining; 2+ = moderate staining; 3+ = heavy/marked staining and then extent of staining; 0 = 0%; 1+ = between 1% and 20% of tumor cells; 2+ = between 21% and 50% of tumor cells; 3+ = greater that 51% of tumor cells.

Statistical Analysis

Analysis was performed using IBM SPSS version 22.0 (IBM Corporation, New York, NY). Concordance between each of the antibody clones and FISH, and concordance between FISH and dual-color CISH were calculated using kappa (κ) statistics. Interpretation was as follows: greater than 0.8 representing very good agreement, greater than 0.7 representing good agreement, and greater than 0.5 representing moderate agreement.

RESULTS

Patient and Tumor Characteristics

The clinicopathological and tumor characteristics of the 362 patients are outlined in Table 1. The *ALK* rearrangement status could be determined in 329 samples (90.9%) by FISH and in 308 samples (85.1%) by dual-color CISH. ALK protein expression could be determined in 343 samples (94.8%) for the ALK1 clone, 356 samples (98.3%) for the 5A4 clone, and 350 samples (96.7%) for the D5F3 clone. The *ROS1* rearrangement status could be determined in 317 samples (87.6%) by FISH and in 309 samples (85.4%) by dual-color CISH. ROS1 protein expression could be determined in 322 samples (89.0%). The reasons for loss of data included missing tumor cores ($n = 139$), non tumor tissue within the tissue cores ($n = 83$), no in situ hybridization signals ($n = 32$), and non specific background staining ($n = 8$) with the flow of patients throughout the study documented as per the ReMARK guidelines[24] in Table 2.

TABLE 1. Clinicopathological Characteristics of Cohort[20]

	$n = 362$
Age	
Median (yrs)	73.4 (36–97)
Sex	
Men	226 (62.4%)
Women	136 (37.6%)
Histology	
Adenocarcinoma	221 (61.0%)
Squamous cell carcinoma	90 (24.9%)
Large-cell carcinoma	28 (7.7%)
Adenosquamous cell carcinoma	8 (2.2%)
Pleomorphic carcinoma	6 (1.7%)
Other[a]	9 (2.5%)
AJCC stage	
Ia	98 (27.1%)
Ib	98 (27.1%)
IIa	52 (14.4%)
IIb	46 (12.7%)
IIIa	51 (14.1%)
IIIb	6 (1.7%)
IV	11 (3.0%)
Smoking history	
Never	45 (12.4%)
Ever	317 (87.6%)

[a]Typical and atypical carcinoid and carcinosarcoma.
AJCC, American Joint Committee on Cancer.

Fluorescence In Situ Hybridization

ALK rearrangements were detected in 4 of 329 samples (1.2%), three of which were adenocarcinoma and one of which was pleomorphic carcinoma. If we were analyzing exclusively adenocarcinoma for the cohort then the frequency of *ALK* rearrangements would be 3 of 199 (1.5%). Two of these showed the typical break-apart signal pattern, whereas the other two samples showed deletion of the 5′ region (isolated orange signal in addition to a fused signal). There was also one case which contained a narrow split, less than two signal widths, classified as *ALK* FISH negative but positive on IHC, as noted below.

ROS1 rearrangements were detected in 3 of 317 samples (0.9%), which consisted of adenocarcinoma, adenosquamous

TABLE 2. Flow of Patients throughout the Study as per ReMARK Guidelines

	Results Obtained	Missing Core	Non Tumor Tissue	No In Situ Hybridization Signals	Non Specific Background	Total
ALK IHC—Clone ALK1	343	14	5	N/A	0	362
ALK IHC—Clone 5A4	356	4	2	N/A	0	362
ALK IHC—Clone D5F3	350	10	2	N/A	0	362
ALK FISH	329	20	13	0	0	362
ALK dual-color CISH	308	23	16	13	2	362
ROS1 IHC—Clone D4D6	322	24	16	N/A	0	362
ROS1 FISH	317	20	15	10	0	362
ROS1 dual-color CISH	309	24	14	9	6	362

IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization; *ALK*, anaplastic lymphoma kinase.

carcinoma, and squamous cell carcinoma. If we were analyzing exclusively adenocarcinoma for the cohort then the frequency of *ROS1* rearrangements would be 1 of 183 (0.5%). For the *ROS1*-rearranged samples, one showed the typical break-apart signal pattern, whereas the other two samples showed deletion of the 5′ region (isolated orange signal in addition to a fused signal). In addition, there was 1 of 317 cases (0.3%) which showed an atypical signal pattern.

Dual-Color Chromogenic In Situ Hybridization

ALK rearrangements were detected in 9 of 308 samples (2.9%). Of these one showed the typical break-apart signal pattern and the remaining eight samples had deletion of the 5′ region (isolated black signal in addition to a fused signal). In the group of eight samples with a deletion of the 5′ region, five samples had a low positivity percentage of tumor cells with a rearrangement (three samples with 16% and two samples with 22%).

ROS1 rearrangements were detected in 5 of 309 samples (1.6%). Of the positive cases, one contained the typical break-apart signal pattern and the remaining four cases had deletion of the 5′ region (isolated red signal in addition to a fused signal). In the group of cases which contained a *ROS1* rearrangement, one case, which had deletion of the 5′ region, had a low positivity percentage of tumor cells with a rearrangement of 20%. In addition, there were 9 of 309 samples (2.9%) with an atypical signal pattern. In the group of nine samples which showed an atypical signal pattern, four samples had a low positivity percentage of tumor cells with this atypical signal pattern (two with 16% and two with 24%).

Immunohistochemistry

ALK protein expression was evaluable with all three ALK antibodies in 341 samples. Cytoplasmic staining of varying intensities was observed in 6 of 341 samples (1.8%) for the ALK1 and 5A4 antibodies and 5 of 341 samples (1.5%) for the D5F3 antibody with the extent of staining ranging from 50% to 100%. The remaining samples all showed no immunoreactivity for ALK protein. All three antibodies stained the *ALK* FISH-positive samples giving 100% sensitivity. All three ALK antibodies had a high specificity with the ALK1 and 5A4 clones with 99.4% specificity and the D5F3 clone with 99.7% specificity. In general, the Cell Signaling Technology D5F3 antibody had the strongest intensity as represented in Figure 1. All three antibodies showed 2+ in one sample, which was negative with *ALK* FISH, although this case contained a narrow split on FISH that did not meet the *ALK* FISH scoring criteria for positivity. An additional case showed 1+ positivity with the ALK1 clone which could not be confirmed by FISH as there was no remaining tumor tissue within the core and another case showed 1+ positivity with the 5A4 antibody which was FISH negative.

ROS1 protein expression was evaluable in 322 samples. Cytoplasmic staining was observed in 2 of 322 samples (0.6%). The remaining samples showed no immunoreactivity for ROS1 protein expression. One sample had

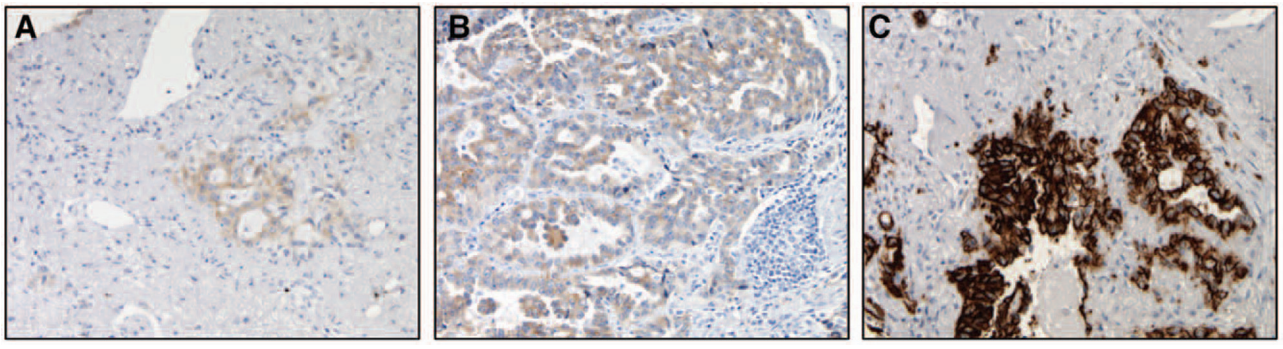

FIGURE 1. Comparison of staining intensities for three ALK antibodies of one sample (×10). *A*, DAKO ALK1 clone. *B*, Novocastra 5A4 clone. *C*, Cell Signaling Technology D5F3 clone. ALK, anaplastic lymphoma kinase.

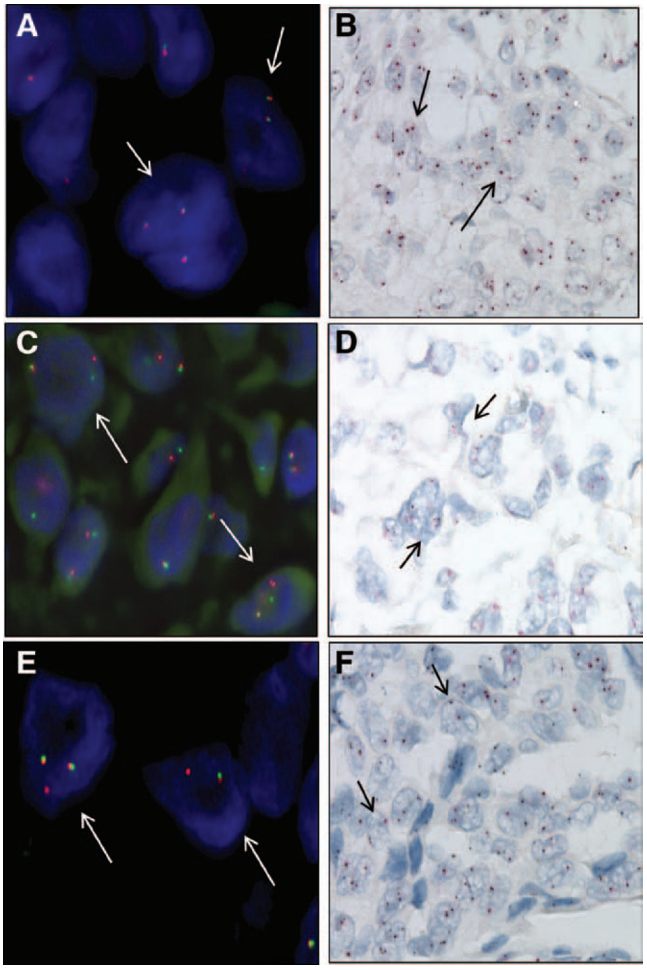

FIGURE 2. *ALK* signal patterns observed by FISH (×100) and dual-colour CISH (×63). *A* and *B*, A non-*ALK*-rearranged tumor. *C* and *D* An *ALK*-rearranged tumor with a split (greater than two signal widths apart). *E* and *F*, An *ALK*-rearranged tumor with an isolated 3′ end signal in conjunction with a fused signal. FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization; ALK, anaplastic lymphoma kinase.

a staining intensity of 3+ in 100% of tumor present, which was confirmed by FISH to contain a *ROS1* rearrangement (typical break-apart signal pattern). The second sample had 1+ staining in 20% of the tumor but showed no rearrangement by FISH. The ROS1 antibody had 33.3% sensitivity and 99.7% specificity.

Correlation between FISH and Dual-Color Chromogenic In Situ Hybridization

For *ALK* rearrangements 306 samples had both FISH and dual-color CISH results, 296 of 306 samples (96.7%) were negative for an *ALK* rearrangement by both FISH and dual-color CISH and 2 of 306 samples (0.7%) were positive for an *ALK* rearrangement by both FISH and dual-color CISH (Fig. 2). The overall concordance for *ALK* rearrangement status between FISH and dual-color CISH was 298 of 306 (97.4%; $\kappa = 0.323$). An additional 7 of 306 samples (2.3%) showed a deletion of the 5′ region by dual-color CISH but not by FISH. There was 1 of 306 samples (0.3%) with an *ALK* rearrangement by FISH that was negative by dual-color CISH (Table 3). Of the discordant results between dual-color CISH and FISH, 5 of 7 samples (71.4%) had a low number of positive cells present, 16% and 22%, by dual-color CISH. The sample positive by FISH but negative on dual-color CISH had a greater proportion of tumor cells on the FISH TMA core, compared with the dual-color CISH TMA core which had only a small number of tumor cells admixed within normal lung. This may account for the discordant result.

For *ROS1* rearrangements 300 samples had both FISH and dual-color CISH results, 287 of 300 samples (95.7%) were negative for a *ROS1* rearrangement by both FISH and dual-color CISH, 3 of 300 samples (1%) were positive for a *ROS1* rearrangement by both FISH and dual-color CISH and 1 of 300 samples (0.3%) showed a deletion of the 3′ region by both FISH and dual-color CISH (Fig. 3). The overall concordance for *ROS1* rearrangement status between FISH and dual-color CISH was 291 of 300 (97%; $\kappa = 0.747$). An additional 7 of 300 samples (2.3%) showed a deletion of the 3′ region by dual-color CISH but were negative by FISH and 2 of 300 samples (0.7%) showed a deletion of the 5′ region by dual-color CISH but were negative by FISH (Table 4). Of the discordant results between dual-color CISH and FISH, 5 of 9 samples (55.6%) had a low number of positive cells present (two with 16%, one with 20%, and the remaining two with 24%) by dual-color CISH that is very close to the cutoff value (Table 4).

Correlation between FISH and IHC

Correlation between ALK IHC and FISH from this cohort has been previously described.[20] For the ALK1 clone, 321 samples had both FISH and IHC results, 316 of 321 (98.4%) were negative for an *ALK* rearrangement by FISH and showed no protein expression on IHC, 4 of 321 samples (1.2%) were positive for an *ALK* rearrangement by FISH and

TABLE 3. ALK IHC Staining and *ALK* Dual-Color CISH Comparison to *ALK* FISH

	ALK1 Clone				5A4 Clone				D5F3 Clone					
	IHC 0	IHC 1+	IHC 2+	IHC 3+	IHC 0	IHC 1+	IHC 2+	IHC 3+	IHC 0	IHC 1+	IHC 2+	IHC 3+	*ALK* CISH Positive	*ALK* CISH Negative
ALK FISH positive	0	3	1	0	0	3	0	1	0	1	1	2	2	1
ALK FISH negative	335	1	1	0	335	1	1	0	336	0	1	0	7	296
Total	335	4	2	0	335	4	1	1	336	1	2	2	9	297

FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization; ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry.

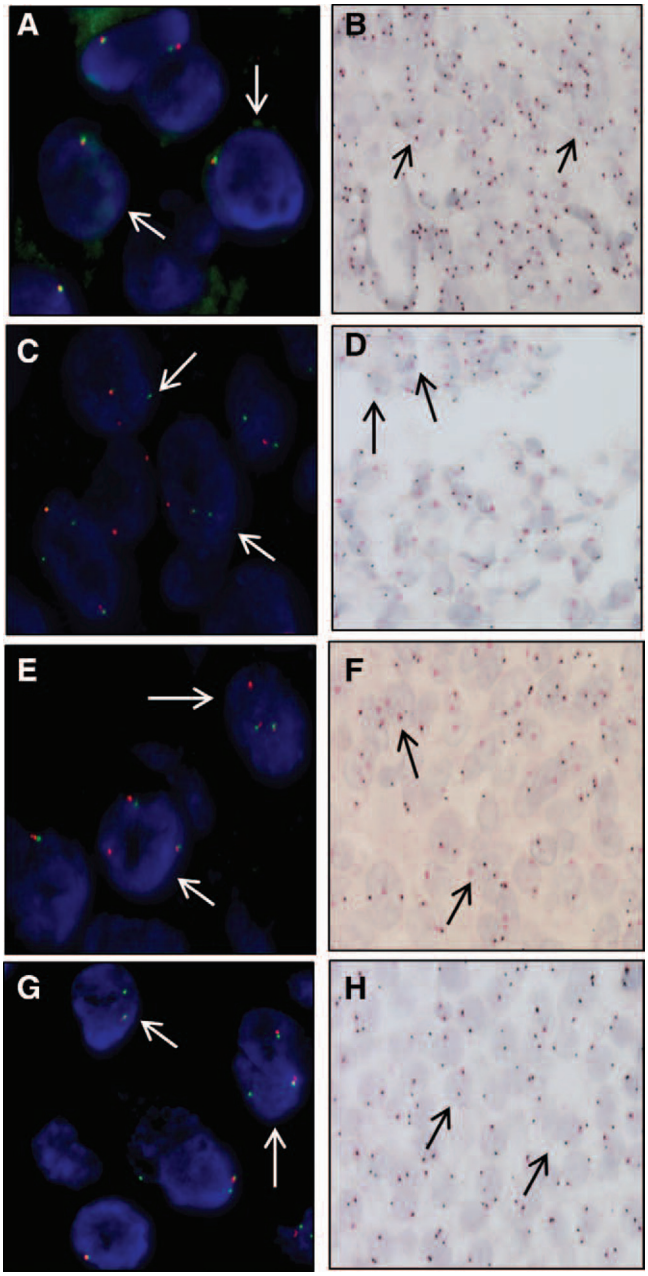

FIGURE 3. *ROS1* signal patterns observed by FISH (×100) and dual-colour CISH (×63). *A* and *B*, A non-*ROS1*-rearranged tumor. *C* and *D*, A *ROS1*-rearranged tumor with a split (greater than two signal widths apart). *E* and *F*, A *ROS1* signal pattern with an isolated 3′ end signal in conjunction with a fused signal. *G* and *H*, An atypical *ROS1* signal pattern with an isolated 5′ end signal in conjunction with a fused signal. FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization.

showed 1+ to 2+ protein expression on IHC. An additional case 1 of 321 (0.3%), as mentioned above, had 2+ staining on IHC but was negative by FISH as the narrow split seen on FISH did not meet the *ALK* FISH positivity criteria. The overall concordance for *ALK* rearrangement status between FISH and the ALK1 clone was 320 of 321 (99.7%; κ = 0.887). For the 5A4 clone, 331 samples had both FISH and IHC results, 325 of 331 samples (98.2%) were negative for an *ALK* rearrangement by FISH and showed no protein expression on IHC, 4 of 331 samples (1.2%) were positive for an *ALK* rearrangement by FISH and showed 1+ to 3+ protein expression on IHC. An additional 2 of 331 samples (0.6%) showed 1+ and 2+ staining on IHC and were negative by FISH. The sample with 2+ staining, as mentioned above, contained a narrow split on FISH and did not meet the *ALK* FISH positivity criteria. The overall concordance for *ALK* rearrangement status between FISH and the 5A4 clone was 329 of 331 (99.4%; κ = 0.797). For the D5F3 clone, 324 samples had both FISH and IHC results, 319 of 324 (98.5%) were negative for an *ALK* rearrangement by FISH and showed no protein expression on IHC, 4 of 324 samples (1.2%) were positive for an *ALK* rearrangement by FISH and showed 1+ to 3+ protein expression on IHC. An additional case 1 of 324 (0.3%), as mentioned above, had 2+ staining on IHC but was negative by FISH as the narrow split seen on FISH did not meet the *ALK* FISH positivity criteria. The overall concordance for *ALK* rearrangement status between FISH and the D5F3 clone was 323 of 324 (99.7%; κ = 0.887; Table 3). Interestingly, the particular sample which was positive by IHC but did not reach the positivity criteria for FISH was detected to contain an *ALK* rearrangement by Nanostring (data not shown).

TABLE 4. Comparison of FISH and Dual-Color CISH for *ROS1*

	ROS1 FISH+	*ROS1* FISH-	*ROS1* FISH Atypical	*ROS1* FISH No Result
ROS1 dual-color CISH positive	3	2	0	0
ROS1 dual-color CISH negative	0	287	0	8
ROS1 dual-color CISH atypical	0	7	1	1
ROS1 dual-color CISH no result	0	17	0	36

FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization.

For *ROS1* rearrangements, 304 samples had both FISH and IHC results, 299 of 304 samples (98.4 %) were negative for a *ROS1* rearrangement by FISH and showed no protein expression on IHC, 1 of 304 samples (0.3%) were positive for a *ROS1* rearrangement by FISH and showed 3+ staining on IHC, 2 of 304 samples (0.7%) samples showed a deletion of the 5′ region by FISH and showed no protein expression by IHC and 1 of 304 samples (0.3%) showed a deletion of the 3′ region by FISH and showed no protein expression on IHC. There was 1 of 304 samples (0.3%) which showed 1+ staining on IHC and was negative for a *ROS1* rearrangement by FISH (Table 5). The overall concordance for *ROS1* rearrangement status between FISH and IHC was 301 of 304 (99%; κ = 0.395).

DISCUSSION

The results from this study suggest that dual-color CISH shows potential as an alternative method for the detection of

TABLE 5. ROS1 IHC Staining Comparison to *ROS1* FISH

	ROS1 FISH +	*ROS1* FISH -	*ROS1* FISH Atypical
IHC—0	2	299	1
IHC—1+	0	1	0
IHC—2+	0	0	0
IHC—3+	1	0	0
Total	3	300	1

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

ALK and *ROS1* rearrangements. Previous studies comparing dual-color CISH and FISH for the detection of *ALK* rearrangements also showed promising results but had higher concordance rates between the two methods than in this study.[16,18,19] In this study, the concordance between FISH and dual-color CISH was 97.4% with 0.7% of samples showing an *ALK* rearrangement by all methods, one sample which was positive by FISH and IHC but negative on dual-color CISH had a small number of tumor cells present within the dual-color CISH TMA core which raises the possibility for the discrepancy in the results. An additional 2.3% (7 of 306) of samples showed a deletion of the 5′ region with dual-color CISH but not by FISH. However, most of these had a low percentage of positive tumor cells. In this circumstance, an additional detection method such as IHC, RT-PCR, or FISH could be used to confirm a rearrangement. A previous study that compared *ALK* CISH and FISH by Yoshida et al.[18] used a positive cutoff value for CISH of 20% which if used would give our study a concordance rate of 98.4%. To the best of our knowledge, this is the first study to compare FISH and CISH for the detection of *ROS1* rearrangements. The overall concordance rate between *ROS1* FISH and CISH was 97%. There were 2 of 300 samples (0.7%) which were positive on CISH (deletion of the 5′ region) but negative on FISH. An additional 2.3% samples (7 of 300) showed an atypical signal pattern with CISH but were negative with FISH. Again as with *ALK*, 55.6% (5 of 9) of these discordant samples had a low number of positive cells present and if we employed the above principle as Yoshida et al.[13] then our concordance rate would increase to 98%. The selection of 15% as a cutoff for *ALK* and *ROS1* rearrangements was selected based on previously published literature for *ISH* assays.[16,19,20,25]

Although less likely, the chromogenic substrates used in this study also differed to those used by others and could account for remaining discrepant results. In this study, a black and red substrate flanked at either end of the specific genes for dual-color CISH was employed. Because this is a break-apart assay it can be technically challenging to discriminate if the signals are closely fused or situated on top of one another. Red and green substrates were used by Schildhaus et al.[16] who reported a brown tinge to the signals when fused which could have made interpretation easier for that study. Yoshida et al.[18] and Kim et al.[19] used blue and red substrates which resulted in a purple tinge with signal fusion which aided interpretation when both signal colors were present.

We also assessed three ALK antibodies and one ROS1 antibody to determine whether IHC could be used to identify these respective gene rearrangements. All ALK antibodies stained the FISH-positive samples with *ALK* rearrangements, giving 100% sensitivity. The specificity of the ALK antibodies ranged from 99.4% to 99.7%. The ROS1 antibody stained 1 of 3 FISH-positive samples (33.3%) with *ROS1* rearrangements. The specificity of the ROS1 antibody was 99.7%. The staining intensity in these samples ranged from 1+ to 3+, supporting the proposal that if any immunoreactivity is observed a confirmatory *ISH* assay should be performed. The sample that contained an atypical signal pattern on *ROS1* FISH showed no immunoreactivity with ROS1 IHC. Previous studies comparing ROS1 IHC and *ROS1* FISH have shown much higher concordance and sensitivity rates than this study[13,26] for which the reasons remain unclear. We speculate that these studies do not specifically indicate the individual IHC staining patterns compared with the FISH signal pattern observed. IHC was performed on the whole tissue blocks for the two cases which were positive and the one case that was atypical by FISH and CISH which confirmed the original IHC staining result from the TMA.

The *ROS1 ISH* signal patterns differ slightly within the current literature mainly surrounding the deletion of the 5′ region being considered a positive signal pattern.[13,23,25–28] Yoshida et al.[28] performed a comprehensive study comparing RT-PCR and FISH signal patterns and showed that cases which had a deletion of the 5′ region on FISH contained a *ROS1* rearrangement by RT-PCR. We can hypothesize that patients who exhibit this signal pattern should respond to *ROS1* inhibitors as the tyrosine kinase domain is still present and patients who exhibit this pattern in *ALK* rearrangements respond to *ALK* inhibitors. However, further studies are required to confirm this hypothesis and that patients will respond to targeted therapy. Recently, clinical response data was published for 50 patients with *ROS1* rearrangements who were treated with crizotinib. One of these patients exhibited an atypical FISH pattern (deletion of the 3′ region). Further testing by next-generation sequencing revealed that the tumor was normal and did not contain a *ROS1* rearrangement. This particular patient showed progressive disease on the first restaging.[29]

This study demonstrates for the majority good correlation between IHC, dual-color CISH, and FISH but as previously mentioned there are limitations with each method. Protein expression with IHC is very dependent on many factors including tissue fixation, subjective evaluation of staining intensity, and the sensitivity and specificity of the antibody clone used. For *ALK,* there have been multiple cases identified as positive by IHC but negative by FISH.[20,30,31] Published clinical response data for two of these discordant cases have shown the patients to have a positive response to treatment with crizotinib.[30,31] We acknowledge the limited number of samples in our study resulting in sub-optimal statistical power and the potential for artificially high concordance rates due to the low number of *ALK* and *ROS1*-positive cases as previously discussed in regards to Selinger et al.[20,32,33] Therefore, further studies with a larger number of cases is required to confirm and validate our results along with our proposed testing algorithm. However, the results from our study still provide relevant data for selecting the most appropriate testing methods

in the detection of *ALK* and *ROS1* rearrangements to guide treatment selection for patients with NSCLC.

Based on this study along with previously published studies, we believe that IHC should be utilized in conjunction with an *ISH* assay in the detection of *ALK* and *ROS1* rearrangements in lung cancer.[20,30,31,34] The correlation in this study between FISH and dual-color CISH is high, however, due to the chromogenic substrates used in our dual-color CISH assay which made interpretation challenging; we conclude that FISH should be the preferred *ISH* method in the detection of *ALK* and *ROS1* rearrangements in conjunction with IHC. The combined use of these methods will effectively identify patients who harbor *ALK* and *ROS1* rearrangements in lung cancer who are likely to benefit from targeted tyrosine kinase inhibitors, such as crizotinib.

ACKNOWLEDGMENTS

The authors thank Richard Young for his help with capturing the brightfield images, David Byrne for his help in the construction of the tissue microarrays, and Nelson Alexander (Ventana) for assistance in probe design and characterization.

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