

Canadian Anaplastic Lymphoma Kinase Study

A Model for Multicenter Standardization and Optimization of ALK Testing in Lung Cancer

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Introduction: Fluorescence in situ hybridization (FISH) is currently the standard for diagnosing anaplastic lymphoma kinase (ALK)-rearranged (ALK+) lung cancers for ALK inhibitor therapies. ALK immunohistochemistry (IHC) may serve as a screening and alternative diagnostic method. The Canadian ALK (CALK) study was initiated to implement a multicenter optimization and standardization of laboratory developed ALK IHC and FISH tests across 14 hospitals.

Methods: Twenty-eight lung adenocarcinomas with known ALK status were used as blinded study samples. Thirteen laboratories performed IHC using locally developed staining protocols for 5A4, ALK1, or D5F3 antibodies; results were assessed by H-score. Twelve centers conducted FISH using protocols based on Vysis' ALK break-apart FISH kit. Initial IHC results were used to optimize local IHC protocols, followed by a repeat IHC study to assess the results of standardization. Three laboratories conducted a prospective parallel IHC and FISH analysis on 411 consecutive clinical samples using post-validation optimized assays.

Results: Among study samples, FISH demonstrated 22 consensus ALK+ and six ALK wild type tumors. Preoptimization IHC scores from 12 centers with 5A4 and the percent abnormal cells by FISH from 12 centers showed intraclass correlation coefficients of 0.83 and 0.68, respectively. IHC optimization improved the intraclass correlation coefficients to 0.94. Factors affecting FISH scoring and outliers were identified. Post-optimization concurrent IHC/FISH testing in 373 informative cases revealed 100% sensitivity and specificity for IHC versus FISH.

Conclusions: Multicenter standardization study may accelerate the implementation of ALK testing protocols across a country/region.

Our data support the use of an appropriately validated IHC assay to screen for ALK+ lung cancers.

Key Words: ALK testing, Immunohistochemistry, Standardization, Fluorescence in situ hybridization, reverse-transcriptase polymerase chain reaction

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It is estimated that in approximately 3–5% of advanced lung adenocarcinoma patients, anaplastic lymphoma kinase (*ALK*) gene rearrangement is the oncogenic driver of tumor progression.^{1–3} Agents that inhibit the activity of aberrant ALK fusion receptor proteins have been shown to yield impressive clinical responses.^{4–7} The development of efficient and reliable laboratory tests is critical in the selection of patients likely to respond to these targeted agents. The standard criterion for detection of *ALK* gene rearrangements is by fluorescence in situ hybridization (FISH) using the US FDA-approved ALK break-apart FISH Probe kit (Abbott Molecular, Abbott Park, IL). However, this assay is resource and cost-intensive, especially as a screening tool, given the low prevalence of *ALK* rearrangements in non-squamous non-small-cell lung cancer (NSCLC) patients. Recent evidence suggests that ALK immunohistochemistry (IHC) protocols can be optimized to be highly sensitive and can identify most ALK-rearranged (ALK+) tumors expressing the fusion ALK protein,^{8–10} potentially providing a less costly testing platform that is available widely and used extensively in routine pathology practice worldwide.

In anticipation of the availability of ALK inhibitor therapy in Canada, a network of pulmonary and molecular pathologists working in cancer centers across Canada was formed to address the challenge of standardization and optimization of tests for detection of ALK+ lung cancers. The main goal of the Canadian ALK (CALK) study was to establish the feasibility of implementing optimal clinical testing methods and algorithms for routine diagnostic detection of ALK+ lung cancer based on a multi-laboratory and countrywide collaborative approach. We describe here the strategy and processes used to generate reference materials and to optimize and standardize protocols across a large number of participating centers, and we report the results of routine clinical ALK testing across Canada for advanced non-squamous NSCLC after CALK study completion.

MATERIALS AND METHODS

The overall CALK study schema is shown in Supplementary Figure S1 (Supplemental Digital Content 1, <http://links.lww.com/JTO/A623>). Phase 1 included identification of study cases, phase 2 involved protocol optimization and standardization of both FISH and IHC, and phase 3 conducted prospective concurrent screening of clinically advanced non-squamous NSCLC cases using the standardized IHC and FISH assays.

Study Samples

Surgically resected NSCLC tumors were selected for this study, as they provide the large quantities of tumor tissue required

for multicenter studies. After approval by the Research Ethics Boards of participating institutions, eight pathology departments participated in retrieving ~2000 resected lung adenocarcinoma paraffin blocks to construct tissue microarrays (TMAs) for rapid screening of ALK+ cases by IHC and FISH (supplementary Figure S1, Supplemental Digital Content 1, <http://links.lww.com/JTO/A623>). A reference study set of 28 cases cases, 22 ALK+ and six ALK wild type (–) was assembled from the original blocks at a central laboratory, by re-embedding a 1 × 1 cm original tissue into new blocks and labeled as CALK 1–28. Twenty samples of non-neoplastic lung tissue containing bronchi or bronchioles were assembled into five control blocks labeled CALK N1–N5.

IHC Studies

Participating centers used one or more automated IHC staining systems manufactured by Dako (Carpinteria, CA), Ventana (Tucson, AZ), and Leica (Buffalo Grove, IL) already available in their respective clinical laboratories, and one or more of the three commercially available antibodies: 5A4 (Novocastra, Newcastle, United Kingdom), ALK1 (Dako), and D5F3 (Cell Signaling, Danvers, MA) in combination with a variety of amplified detection methods (Supplementary Table S1, Supplemental Digital Content 2, <http://links.lww.com/JTO/A623>). For initial screening of the TMAs, *N*-Histofine ALK Detection Kit (Nichirei Biosciences, Tokyo, Japan) was also used.

The first round of preoptimization IHC on the study set was performed using protocols developed by each laboratory independently, based on published methods.^{11–13} For each case, the participating center pathologist recorded the percentage (%) of tumor cells showing each staining intensity (I: 0, 1+, 2+, and 3+). The H-score was calculated using the formula of: $\sum = 1 \times (\%1+) + 2 \times (\%2+) + 3 \times (\%3+)$, resulting in scores ranging from 0 to 300. After each site pathologists submitted their scores to the study lead investigator (M.S.T.) and biostatistician (M.P.) for analysis, the pathologists met and compared their respective stained sections. After it was determined that results of several laboratories could be further improved, a protocol optimization procedure was developed. An optimization TMA block that included selected samples with negative (0), weakly (1+), moderately (2+), and strongly (+3)-positive ALK staining as determined by an IHC protocol demonstrating optimal performance for FISH results was also prepared and distributed for local adjustment. The protocols were considered optimized after all laboratories achieved comparable results using the standardized controls.

FISH Studies

ALK FISH testing was performed using the Vysis ALK break-apart probe set (Abbott Molecular, Abbott Park, IL) and the FDA-approved protocol.¹⁴ Before the initiation of FISH analysis, technologists and pathologists directly involved in FISH scoring were provided hands-on training by the manufacturer at a centralized training site. A detailed summary of scoring procedures, score sheets, and unstained whole section slides of the study cases were distributed to each participating

site. To conserve tissue, a DVD containing whole-slide images (.svs format) of the hematoxylin and eosin (H&E)-stained sections of each case, scanned using the Aperio ScanScope system (Vista, CA), was provided. The participating laboratories were instructed to use these H&E images to guide FISH analysis, by having a pathologist print the low-power images and circle the tumor area to be scored.

Fluorescent signal analysis was performed manually on fluorescence microscopes at each participating site. Red, green, and fusion signals (defined as red and green signal ≥ 2 signal-widths apart) were counted in a total of 200 (100/person) non-overlapping, intact interphase nuclei per tumor, by two trained technologists and/or pathologists per center. The scoring protocol involved scanning the slide and choosing two or more representative high-power fields to score, including areas suspicious for rearrangement if possible, and scoring eligible tumor nuclei in a consecutive manner within each field, before moving to the next field. Combined counts at each participating site were provided to the study biostatistician for data analysis.

RT-qPCR Assay

RT-qPCR was carried out as previously reported,¹⁵ with detailed methods and primer sequences provided in Supplementary Materials (SDC, <http://links.lww.com/JTO/A623>).

Multicenter Prospective Parallel IHC and FISH Screening

To prospectively assess the sensitivity and specificity of ALK IHC for detecting ALK FISH-positive (FISH+) tumors, three centers conducted parallel IHC (5A4 antibody) and FISH testing of 411 consecutive clinical cases using the post-standardization protocols. The generally accepted criterion of $\geq 15\%$ abnormal ALK FISH signals was used to diagnose ALK FISH+ tumors. In almost all cases, the optimization resulted in background IHC staining being completely absent.

Statistical Analysis of Multicenter results

Descriptive statistics were used to describe quantitatively the main features of the data collection. Variability of scores for both IHC and FISH was estimated by intraclass correlation (ICC), where an ICC of 0.85 or higher is considered to indicate an acceptably reliable test.

RESULTS

Multicenter IHC Correlation

Altogether 13 centers participated, with 12 centers electing to use the 5A4 antibody and one center to use only the ALK1 and D5F3 antibody. Seven centers used the Ventana Benchmark XT/Ultra, three used the Leica Bond III/Max system, and four used the Dako Autostainer (Supplementary Table S1, Supplemental Digital Content 2, <http://links.lww.com/JTO/A623>). One center also evaluated the ALK-1 antibody using the Ultraview system on BenchMark, whereas three centers evaluated the D5F3 antibody using the Autostainer and Advanced or Flex+ systems or the BenchMarkXT. Representative images of

tumors with different staining intensities are shown in Fig. 1A. The overall ICC of H-scores for the preoptimization scores between centers that evaluated 5A4 was 0.866 (Fig. 1B). After optimization, background staining with 5A4 was largely absent, with positive tumors showing diffuse staining involving practically all tumor cells. For eight centers that performed the second evaluation after optimization, the ICC of 0.867 from preoptimization scores improved to 0.949 after optimization (Fig. 1C and Supplementary Figure S2, Supplemental Digital Content 3, <http://links.lww.com/JTO/A623>).

For centers that evaluated two antibodies, the Pearson's correlations between the H-scores for 5A4 with ALK-1 and D5F3 were 0.844 and 0.972, respectively (Supplementary Figure S3, Supplemental Digital Content 4, <http://links.lww.com/JTO/A623>). For three centers that evaluated D5F3, correlation between their respective D5F3 H-scores with the mean of 5A4 scores from across 12 centers or with their own 5A4 scores ranged from 0.863 to 0.978 (Supplementary Figure S4, Supplemental Digital Content 5, <http://links.lww.com/JTO/A623>). Some background staining was noted often with D5F3, especially in macrophages and airway epithelial cells.

Multicenter FISH Correlation

FISH counts were obtained from 12 participating centers. Results were obtained from all centers for 21 of 28 (75%) of tumors and 19 of 20 (95%) of the normal tissues. The FISH patterns seen in the tumors can be roughly grouped into five major patterns: negative/normal (Fig. 2A), negative with extra copies of ALK (Fig. 2B), positive with classical split signals (Fig. 2C), and positive with loss of the 5' (green) probe (Fig. 2D). A consensus FISH result was clear in all 28 tumor samples, with complete concordance in 19 (68%) tumors (Supplementary Table S4, Supplemental Digital Content 8, <http://links.lww.com/JTO/A623>). Overall, 22 of 28 tumors were positive for ALK rearrangement and six of 28 tumors were negative, with the distribution of the tumors among these FISH patterns shown in Supplementary Table S2 (Supplemental Digital Content 6, <http://links.lww.com/JTO/A623>). Using the standard cutoff of $\geq 15\%$ abnormal cells and the consensus FISH result as the reference, we observed only 14 of 317 (4.4%) FISH outlier results (Supplementary Table S3, Supplemental Digital Content 7, <http://links.lww.com/JTO/A623>). The overall diagnostic sensitivity of FISH across all laboratories in our study was 95.2%, and specificity was 95.8%, with a positive predictive value of 99%, and negative predictive value of 89%. Figure 2E shows the distribution of % abnormal FISH signals for all 28 tumors and 20 normal lung tissue across 12 centers. The overall ICC among all samples including the normal was 0.83, but was only 0.68 among the tumors, illustrating a considerable variation in the percent abnormal tumor cells scored among the 12 laboratories.

Of the nine samples with at least one discrepant result, six were ALK FISH+, two were ALK FISH-, and one was an atypical case (CALK-11) to be discussed further below. The false-positive results for the two FISH- cases ranged between 20% and 32% abnormal nuclei and for false-negative

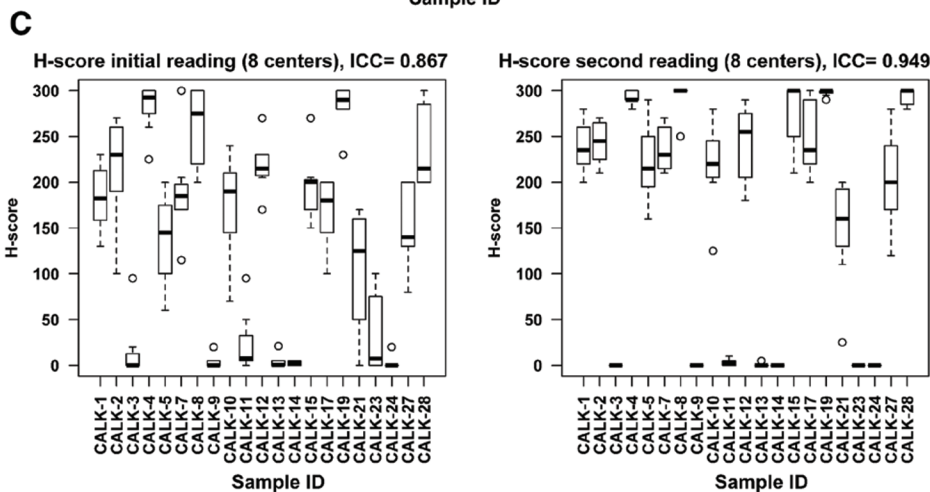
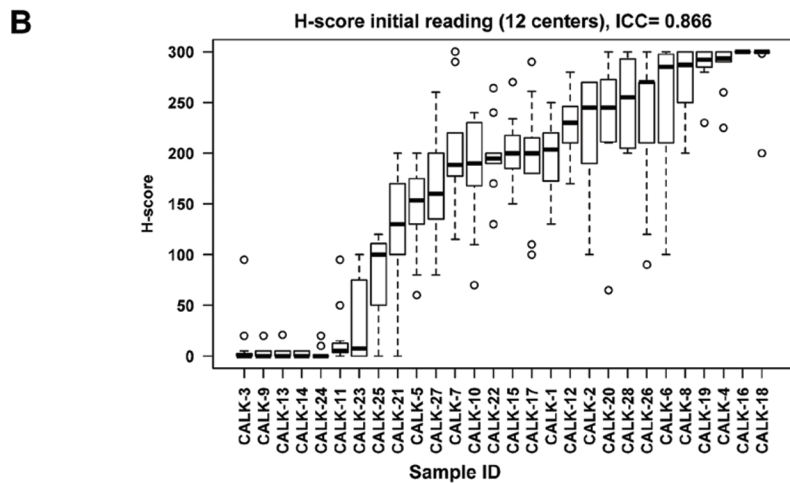
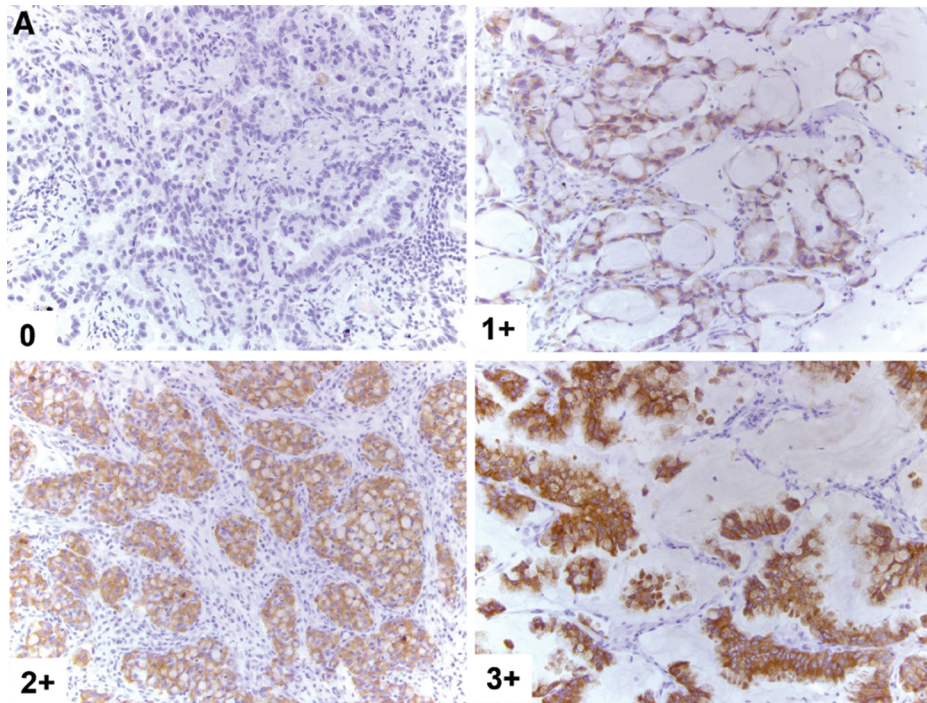


FIGURE 1. ALK immunohistochemistry (IHC) staining (A) representative images of ALK staining intensities (0 to 3+) for scoring the sections stained before multicenter protocol optimization. (B) Distribution of the H-scores obtained from 12 laboratories across 28 study cases. Seven cases were considered ALK IHC negative by consensus, despite the occasional outlier scores. (C) Box-plot of H-scores from eight centers that provided both preoptimized and post-optimized IHC scores.

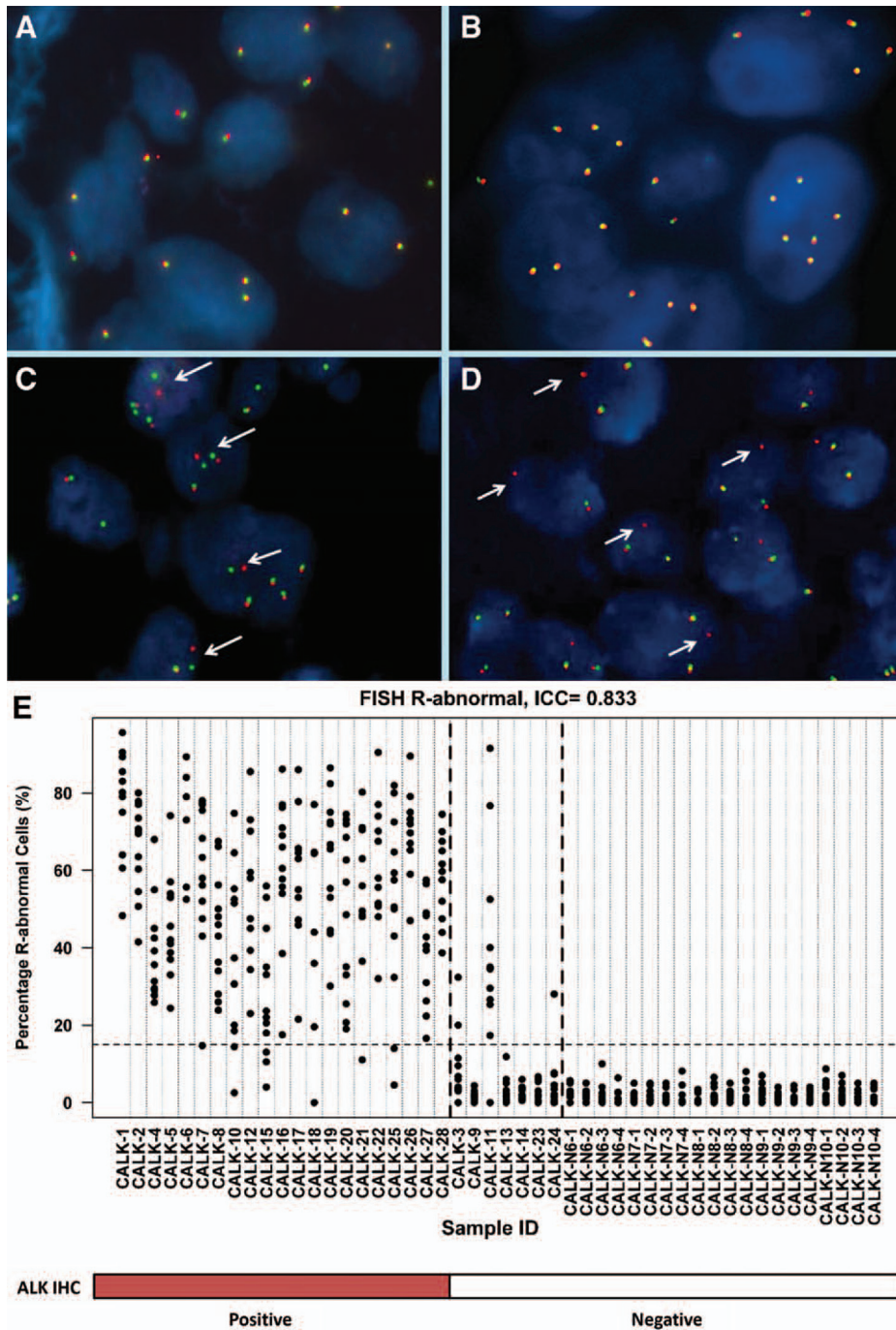


FIGURE 2. Representative often observed ALK break-apart fluorescence in situ hybridization images and distribution of break-apart signals among Canadian Anaplastic Lymphoma Kinase (CALK) samples. *A*, A straightforward negative pattern. *B*) A negative for ALK rearrangement case with multiple copies of ALK arising from polysomy and/or unbalanced structural rearrangements involving chromosome 2. *C*) A typical ALK inversion pattern, with variably extra copies of the ALK gene. Arrows point to characteristic split signals in adjacent cells. *D*) A common ALK rearrangement pattern where the 5' ALK probed region has been lost, leaving solitary red signals without solitary green signals. *E*) Distribution of all abnormal ALK signals from 12 centers according to their percentages counted in 200 nuclei (by two technologists).

cases ranged between 0 and 14.7 % (Supplementary Table S4, Supplemental Digital Content 8, <http://links.lww.com/JTO/A623>). Upon group review of the cases and feedback on consensus results, reasons determined for false FISH– results were poor signal quality and tumor cells being lost in deeper sections. Other potential reasons for false negatives included the lack of physically marked H&E images to guide scoring, as some laboratories reported that the.svs files were too awkward to refer to. Potential reasons for false positives were poor signal quality, nonadherence to more than or equal to two

signal-widths for a break-apart, selective scoring of abnormal nuclei, and elevated ALK copy number resulting in increased artifactual split signals.

Concordance Between IHC and FISH

Consensus FISH and IHC results agreed for 27 of 28 tumors (Fig. 2E). All normal tissues were scored as negative for both FISH and IHC by all centers. Tumor CALK-11 was consensus positive by FISH and mostly negative by IHC, with very focal weak positive staining noted at a few centers. Upon

review, CALK-11 demonstrated a unique FISH pattern, with three to five fusion signals containing a tiny green component, and three to five solitary green signals (Supplementary Figure S5, Supplemental Digital Content 9, <http://links.lww.com/JTO/A623>). As these tiny green components of the fusion signals were poorly visible, all but one laboratory counted these signals as solitary red signals, and the consensus result was positive for ALK-rearrangement, with a polysomy pattern. This tumor was the only FISH+ case in our study with a negative IHC result.

RT-qPCR

The snap-frozen tumor sample from CALK-11 was studied using alternate methods. Using primers that detect low-level 5' non-kinase ALK exons (13 of 14 and 17 of 18) in normal lung and high-level 3' kinase ALK exons (22 of 23 and exons 24 of 26) in tumor cells expressing the ALK fusion mRNA, aberrant overexpression of the 3' transcript was negative (Supplementary Figure S5, Supplemental Digital Content 9, <http://links.lww.com/JTO/A623>). The RNAseq analysis of the same tumor RNA also failed to detect the presence of any fusion transcript involving the ALK cDNA (data not shown). Based on these results, we postulate that the atypical FISH pattern with a tiny green signal fused with the "solitary" red signals may result from a breakpoint 5' to the *ALK* gene causing the abnormal FISH pattern, but not involving the *ALK* gene itself.

Prospective IHC and FISH Screening

Three centers conducted parallel IHC using the 5A4 antibody and the FISH testing of 411 consecutive clinical cases using the optimized and validated protocols. The centers scored ALK IHC+ when there was diffuse staining of the tumor cells clearly distinct from background, and equivocal when focal faint staining was noted or in rare cases with background staining that made it difficult to decide staining specificity (Supplementary Figure S6, Supplemental Digital Content 10, <http://links.lww.com/JTO/A623>), thus requiring FISH confirmation for a definitive diagnosis. Cases without tumor cell staining above background were called IHC-.

Among the 411 cases, 373 (90.8%) were informative by both methods and 38 (9.2%) failed FISH testing. FISH uninformative cases were because of inadequate residual tumor tissue or technical failure. Among the dual informative cases, 18 (4.8%) were both IHC+/FISH+, 326 (87.4%) tested IHC-/FISH-; 29 (7.8%) were IHC equivocal/FISH-, all of which defaulted to FISH testing for final diagnosis. Of 411 cases, two were initially discordant with IHC-/FISH+ (case 8: 22%) or "borderline negative" (case 13: 14.5%; Supplementary Table S5, Supplemental Digital Content 11, <http://links.lww.com/JTO/A623>). In both cases, blinded repeat FISH analyses locally and at another center were scored as negative. Among the 29 IHC equivocal cases, one case was initially FISH+ (case 47, 19% split signal), but repeat scoring (2×13%) and independent testing at another center found it negative (1% signal).

Among the 373 dual informative cases, FISH-/IHC- had mean 3.1% (95% confidence interval [CI] 2.7–3.5%) abnormal FISH signal counts, FISH+/IHC+ cases had 48.6% (95% CI 46.7–50.5%) split-signal, and IHC equivocal cases scored 4.0% (95% CI 3.6–4.3%) FISH split-signal. When considering IHC equivocal cases as positive, IHC sensitivity and specificity were 100% (95% CI 81.5–100.0%) and 91.8% (95% CI 88.5–94.5%), respectively. When equivocal cases were considered negative, the sensitivity and specificity of IHC against FISH was 100% (sensitivity 95% CI 81.5–100.0%; specificity 95% CI 99.0–100.0%).

ALK IHC for Screening ALK-Rearranged Lung Cancers

Since the completion of the study in May 2012, most CALK centers started implementing ALK IHC as the preferred method to screen clinically, in advanced non-squamous NSCLC patients. All centers performed FISH only on cases that the signing pathologist diagnosed as IHC+ or IHC equivocal, as described. Through the end of June 2013, 4927 patients were screened by IHC, representing close to half of all advanced non-squamous cases across the country (Fig. 3). Among these, 718 (14.6%) cases were FISH tested, with 124 cases confirmed as FISH+ for ALK rearrangement, representing an overall prevalence of 2.5%.

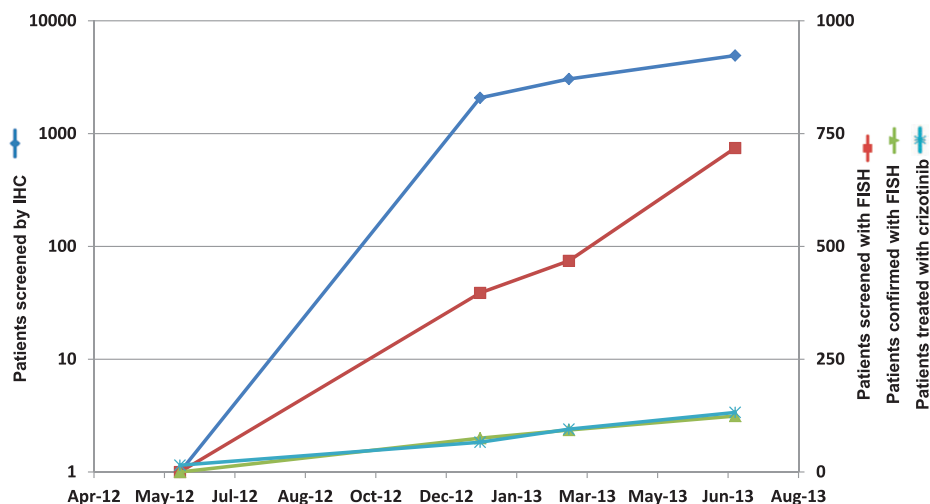


FIGURE 3. Implementation of clinical ALK testing in Canada after completion of CALK study.

DISCUSSION

In the CALK study, we have implemented a strategy for optimization and standardization of ALK IHC and FISH testing nationally across 14 centers in Canada. Aside from its practical goal for the multicenter standardization of these tests, CALK also demonstrated that high-level correlation between centers can be achieved using optimized ALK IHC protocols and standardized controls designed to enable the calibration of highly sensitive and specific assays, regardless of the antibodies or autostainer platform used. We have also demonstrated that variation in FISH scoring of aberrant ALK signals may occur in laboratories with pretrained technologists, although overall ALK FISH testing is very robust. Our concurrent IHC and FISH testing of consecutive clinical samples showed that with the optimized protocols, diffuse IHC+ staining has 100% sensitivity in detecting true FISH+ ALK tumors, whereas FISH+ results with low split-signal count could be false positive when IHC is negative. Our results showed that IHC can be deployed as a screening method to detect ALK-rearranged lung cancers, with rapid uptake of ALK testing across a country. Data also suggest that a clearly positive IHC result may be used as a diagnostic test to determine ALK+ status. With a relatively low prevalence of ALK rearrangements in lung cancer, it is challenging for individual centers to independently optimize ALK testing and gain testing expertise using a large number of positive controls that may not be available at individual institutions. Furthermore, there are very few ALK-rearranged lung cancer cell lines available for such purposes, and cell lines alone are inadequate to validate FISH cutoff values on paraffin sections. Through formation of a pathology network, the CALK multicenter collaboration addressed these challenges, allowing the screening of a large number of resection cases to identify ALK+ tumors, and facilitating standardization of results by establishment of reference ranges and test optimization through the creation of reference sets and common standardized controls.

The CALK study identified several factors that can affect the sensitivity and specificity of ALK IHC and FISH testing. With respect to IHC, laboratories may vary widely in their choice of routine IHC protocols, primary antibody and detection system selection, and immunoscore criteria. Protocols producing IHC results that best predicted ALK FISH consensus status were considered optimal. Distribution of the graded control block provides a common external standard with which to assess adjustments in individual laboratory protocols that are required during optimization and standardization of the IHC assay. Through these efforts, it was possible to obtain highly concordant IHC results between testing centers and with gene rearrangement status by FISH.

Many authorities consider FISH testing as the standard test for determining ALK status.¹⁶ Camidge *et al*¹⁷ reported previously that the mean percentage of signals positive for ALK rearrangement in ALK+ tumors was 53.8% (95% CI 22.2–86.6%), whereas in ALK– tumors was 6.0% (95% CI 3.5–9.5%). In our study, a majority of FISH+ cases also showed greater than 40% mean positive signals across 12 reporting laboratories, but occasional outlier scores occurred. This was despite involving mostly experienced cancer cytogenetic technologists who had undertaken specific pretraining for

ALK FISH testing. A common pitfall of FISH tests on tumor cells is the unintentional scoring of non-tumor cells leading to a false-negative result. This problem could be alleviated by close guidance and supervision by pathologists on the tumor areas to score. This is particularly important for NSCLC, as tumors often show significant chronic inflammatory cell infiltrate and/or stromal fibroblastic reaction that can be difficult to distinguish from tumor nuclei with the fluorescence microscope. Our experience also suggests that strict adherence to a more than or equal to two signal-width separation for break-apart signals is crucial. A pathologist should be involved by educating the technologists on histology of the sample being analyzed, and reviewing cases close to 15% cutoff (10–20%) to ensure that the counts are representative of tumor cells.^{8,18} Furthermore, there is a need for ongoing proficiency testing for ALK FISH and IHC, both centrally organized and between collaborating institutions, particularly for cases with a discordant FISH and IHC result.

Although ALK diagnosis by FISH testing is considered a standard method for ALK-rearranged lung cancers in the United States, screening by IHC has been proposed or adopted for clinical practice in many other parts of the world.^{2,9,13,19} This strategy would significantly reduce the cost of ALK testing and also expedite its turnaround time, by conducting FISH testing only to confirm IHC results. This testing paradigm has largely been adopted across Canadian health care institutions. This paradigm would work only if highly sensitive ALK IHC protocols are developed by using standard controls/calibrators confirmed to be suitable to identify FISH+ tumors. Although initial IHC equivocal diagnoses could be high at some centers (Table 1), this rate will likely decrease significantly as pathologists gain experience in interpreting IHC results and confidence

TABLE 1. Concurrent ALK IHC/FISH Analysis Study Post-validation

	Total	FISH–	FISH +	FISH
				Uninformative ^a
Center 1 (n = 99)				
IHC–	96 (97.0%)	95	0	1
IHC+	2 (2.0%)	0	2	0
IHC equivocal	1 (1.0%)	1	0	0
Center 2 (n = 132)				
IHC–	101 (76.5%)	85	0	16
IHC+	7 (5.3%)	0	7	0
IHC equivocal	24 (18.2%)	22	0	2
Center 3 (n = 180)				
IHC–	164 (91.1%)	146	0	18
IHC+	10 (5.6%)	0	9	1
IHC equivocal	6 (3.3%)	6	0	0
Combined (n = 411)				
IHC–	361 (87.8%)	326	0	35
IHC +	19 (4.6%)	0	18	1
IHC equivocal	31 (7.6%)	29	0	2

^aFISH result was not available because of inadequate residual tumor tissue or technical failure. These cases were excluded from analysis of sensitivity and specificity. FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

TABLE 2. Correlation Between ALK IHC and ALK FISH in Cases Studied by Both Techniques for the Current and Published Studies

	Antibody	Total Cases Studied	IHC Negative		IHC Positive		IHC Doubtful (Equivocal)	
			FISH–	FISH+	FISH–	FISH+	FISH–	FISH +
This study	5A4	373	326	0	0	18	29	0
Published studies								
Rodig et al. ²⁰	ALK1	243	233	2	0	8	—	—
Yi et al. ¹²	ALK1	101	69	0	22	10	—	—
Paik et al. ¹³	5A4	640	602	0	10	28	—	—
Park et al. ¹⁹	5A4	262	234	0	3	25	—	—
McLeer-Florin et al. ²	5A4	81	59	0	1	19	0	2
Zhang et al. ²¹	SP8	130	110	0	0	15	—	—
Sholl et al. ²²	5A4	176	162	1	0	13	—	—
Minca et al. ¹⁰	D5F3	249	217	0	0	32	—	—
Selinger et al. ²³	ALK1/D5F3	587	581	0	6	7	—	—
Martinez et al. ⁹	D5F3	79	73	0	1	5	—	—
Takamochi et al. ²⁴	5A4	360	347	0	3	10	—	—
Total		2908	2687	3	73	172	0	2

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

that focal/weak equivocal staining is consistently associated with FISH– results. A review of the literature has identified 11 studies involving slightly more than 2900 NSCLC cases that have been studied by both IHC and FISH (Table 2). Although a majority of these studies were retrospective or single-institution analyses, among 2690 IHC-negative cases reported, only three were found to be FISH+, giving a false-negative rate of ~0.1%.^{2,8–10,12,13,18,20–24} As IHC is relatively easy to implement clinically and mostly automated, rapid uptake of population-wide testing can be readily accelerated and achieved, as we have demonstrated in Canada after completion of the CALK study.

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