Detection of *ALK* Gene Rearrangement in Non-small Cell Lung Cancer

A Comparison of Fluorescence In Situ Hybridization and Chromogenic In Situ Hybridization with Correlation of ALK Protein Expression

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Introduction: Accurate determination of *ALK* rearrangement is important in lung cancer patients, especially in determining their eligibility for crizotinib therapy. Fluorescence in situ hybridization (FISH) has been regarded as the gold standard method for detecting *ALK* rearrangement. However, FISH requires a fluorescence microscope, and the signals are labile and rapidly fade over time. This study evaluates the concordance between *ALK* gene rearrangement in non-small cell lung cancer assessed by *ALK* FISH and a newly developed *ALK* chromogenic in situ hybridization (CISH) and correlates the results with ALK protein expression assessed by immunohistochemistry.

Methods: A total of 465 formalin-fixed, paraffin-embedded non-small cell lung cancer samples were analyzed by *ALK* FISH (PathVysion, Vysis, Abbott) and *ALK* CISH. For comparison, all specimens were stained by immunohistochemistry (clone 5A4, Novocastra) and interobserver reproducibility was assessed.

Results: We found that agreement between the pathologists on the CISH-determined *ALK* status was achieved in 449 patients (96.6%), and *ALK* rearrangement was identified in 18 patients (4.0%) in CISH method. Among these cases, 443 cases (95.3%) had results matching the corresponding FISH results: 17 rearranged, 425 wild types, and 1 discordant case. There was high concordance in the assessment of *ALK* gene rearrangement between FISH and CISH techniques (κ = 0.92) and between observers (κ = 0.97). In addition, there was high concordance in the *ALK* gene status and ALK protein expression between CISH and IHC tests (κ = 0.82).

Conclusions: CISH is a highly reproducible and practical method to detect *ALK* gene rearrangement and correlated well with ALK protein expression. Here, we present a diagnostic algorithm (*Chung's SNUBH ALK* protocol) to detect lung cancer with *ALK* rearrangements using IHC, FISH and CISH. Because CISH allows a concurrent analysis of histological features of the tumors and gene rearrangement, it appears to be a useful method in determining *ALK* gene rearrangement.

Key Words: Lung cancer, ALK, Fluorescence in situ hybridization, Chromogenic in situ hybridization.

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The fusion of anaplastic lymphoma kinase (*ALK*) gene with the echinoderm microtubule-associated protein-like 4 (*EML4*) gene was identified recently in non-small cell lung cancer (NSCLC).[1] ALK is a receptor tyrosine kinase described in anaplastic large cell lymphoma with t(2;5)(p23;q35) resulting in nucleophosmin (NPM)-ALK fusion protein. The ALK-signaling pathway includes several biologically important pathways involving cell proliferation, differentiation, and antiapoptosis.[2] In NSCLC, the frequency of the *EML4-ALK* fusion gene is about 3 to 6.7%.[2–7] A novel ALK tyrosine kinase inhibitor named crizotinib (PF-02341066) is currently in phase II and III clinical trials for advanced NSCLC with *ALK* rearrangement, and early reports have demonstrated dramatic clinical responses in *ALK*-rearranged NSCLC patients assessed by fluorescence in situ hybridization (FISH).[8,9] Accurate and rapid screening of *ALK* rearrangement is important in NSCLC patients, especially in determining their eligibility for crizotinib therapy. Because rearrangement of the *ALK* gene appears to be the mechanism underlying the overexpression of its gene product, immunohistochemistry (IHC) can be a screening method to detect gene rearrangement.[10]

In principle, there are two approaches to determine the *ALK* status: detection of the protein overexpression by IHC and gene rearrangement by in situ hybridization (ISH). Both

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have advantages and disadvantages; quantification of IHC is difficult, whereas ISH enables the pathologist to perform a more reliable quantification of the genomic alteration. FISH has been regarded as the gold standard method for detecting *ALK* rearrangement. However, the disadvantages of determining *ALK* rearrangement by FISH are that the fluorescent signal rapidly fades over time, and a fluorescence microscope is needed for interpretation, which is not usually done in clinical routine works. Another problem is that it is difficult to detect the overall morphology and tumor heterogeneity with FISH technology.[11] Furthermore, despite many attempts to improve accuracy of ALK testing in routine practice, several recent publications have addressed discordances between FISH and IHC assays.[5]

Chromogenic in situ hybridization (CISH) for *ALK* gene rearrangement detection is a method newly developed by two of the authors (H.N. and T.M.G.) which may overcome many of the disadvantages of FISH as it allows the quantification of the signals by conventional bright field light microscopy.[12] In addition, CISH is a fully automated bright field ISH assay and provides stable and permanent archival slides that are evaluable with conventional bright field light microscopy.[13,14] In this study, the *ALK* gene rearrangement status of 465 consecutive NSCLC cases was examined in parallel by FISH and CISH and correlated with protein expression by IHC. For validation and comparison of the genetic assays (CISH versus FISH) and to determine interobserver variability, a panel of two pathologists (K.H. and Y.S.B.) read and scored the slides independently.

The aims of this study were (1) to evaluate the concordance between *ALK* CISH and FISH assay in determining the status of *ALK* rearrangement; (2) to assess the interobserver interpretative reproducibility; and (3) to correlate CISH with protein expression by IHC.

PATIENTS AND METHODS

Patient Samples

Formalin-fixed, paraffin-embedded (FFPE) NSCLC specimens from 465 patients diagnosed at Seoul National University Bundang Hospital from May 2003 to May 2008 were included in this study. All cases were diagnosed as primary lung origin on review of the medical records. The 465 cases of NSCLC were classified according to the 2004 WHO classification.[15] The cohort consisted 269 cases of adenocarcinoma (ADC), 169 cases of squamous cell carcinoma (SCC), 10 cases of adenosquamous carcinoma (ASC), 5 cases of pleomorphic carcinoma (PLC), 2 cases of large cell carcinoma (LCC), 8 cases of large cell neuroendocrine carcinoma (LCNEC), 1 case of carcinosarcoma (CS), and 1 case of lymphoepithelioma-like carcinoma (Table 1). No patients had received any therapy before surgery. This study was approved by the Institutional Review Board at Seoul National University Bundang Hospital.

Construction of Tissue Microarray

Representative core tissue sections (diameter 2 mm) were obtained from individual NSCLC tissue (donor blocks) and arranged in new recipient paraffin blocks (tissue array blocks) as previously described.[16] Three semiserial sections were cut and examined for the *ALK* status by IHC, FISH, and CISH assays.

TABLE 1. Clinicopathologic Characteristics Related CISH and FISH

	ALK CISH			*ALK* FISH		
Variables	**+**	**−**	***p***	**+**	**−**	***p***
Total	18 (4.0%)	431 (96.0%)		19 (4.2%)	434 (95.8%)	
Sex						
Male	12 (66.7%)	294 (68.2%)	NS	11 (57.9%)	298 (68.7%)	NS
Female	6 (33.3%)	137 (31.8%)		8 (42.1%)	136 (31.3%)	
Age (yr)						
>65	5 (27.8%)	220 (51.0%)	NS	6 (31.6%)	224 (51.6%)	NS
≤65	13 (72.2%)	211 (49.0%)		13 (68.4%)	210 (48.4%)	
Smoking habit						
Never	9 (50.0%)	160 (37.1%)	NS	10 (52.6%)	161 (37.1%)	NS
Smoker	9 (50.0%)	271 (62.9%)		9 (47.4%)	273 (62.9%)	
Histology						
ADC	17 (94.4%)	245 (56.8%)	<0.001	18 (94.7%)	245 (56.5%)	<0.001
SCC	0	160 (37.1%)		0	163 (37.5%)	
Others	1 (5.6%)	26 (6.1%)		1 (5.3%)	26 (6.0%)	
p-stage						
I	8 (44.4%)	190 (44.1%)	NS	8 (42.1%)	190 (43.8%)	NS
II–IV	10 (55.6%)	241 (55.9%)		11 (57.9%)	244 (56.2%)	
Total	449			453		

CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization; ADC, adenocarcinoma; SCC, squamous cell carcinoma; NS, not significant.

Fluorescence In Situ Hybridization

FISH assay was performed on FFPE tumor tissues using a break-apart *ALK* probe that hybridizes to the band 2p23 with SpectrumOrange (red) and SpectrumGreen on either side of the *ALK* gene breakpoint (Vysis LSI ALK dual-color, break-apart rearrangement probe; Abbott Molecular, Abbott Park, IL) according to the manufacturer's instructions. Briefly, 3-μm-thick sections from FFPE tissue blocks were deparaffinized, dehydrated, immersed in 0.2N HCl, and washed. The sections were immersed in 0.01M citrate buffer (Abbott Molecular), boiled in a microwave for 5 minutes, treated with pretreatment reagent (Abbott Molecular) at 80°C for 30 minutes, and reacted with protease mixed with protease buffer (Abbott Molecular). After applying the probe mixture onto the tissue sections, sealed slides were incubated in a humidified atmosphere with Hybrite (Abbott Molecular) at 75°C for 5 minutes to denature the probe and target DNA and sequentially incubated at 37°C for 16 hours to allow hybridization. Then they were immersed in 0.3% NP-40 (Abbott Molecular)/2× saline sodium citrate for washing. For nuclear counterstaining, 4,6-diamidino-2-phenylindole (DAPI) II with antifade compound *p*-phenylenediamine was applied. Signals for each probe were evaluated under a microscope equipped with a triple-pass filter (DAPI/Green/Orange; Abbott Molecular) and an oil immersion objective.

There were two positive *ALK* rearrangement patterns. One was the break-apart (BA) pattern with one fusion signal (native *ALK*) and two separated orange and green signals. The distance between two separated signals was estimated using the two times of biggest signal size.[17] Another was isolated red signal (IRS) pattern with one fusion signal (native ALK) and one red signal without corresponding green signal. The positive cases were defined as more than 15% BA or IRS in 50 tumor cells as previously described.[10,17]

Chromogenic In Situ Hybridization

ALK dual-color break-apart CISH was performed as described previously.[12] Briefly, NSCLC slides were deparaffinized with EZ Prep (Ventana Medical Systems, Inc., Tucson, AZ) and Liquid Coverslip (LCS) (Ventana Medical Systems, Inc.). Then for DNA target retrieval, heat treatment with 1× Reaction Buffer (Ventana Medical Systems, Inc.) followed by protease digestion with ISH Protease 2 (Ventana Medical Systems, Inc.) for 8 minutes. Digoxigenin (DIG)-labeled 5′ ALK probe and 2,4 dinitrophenyl (DNP)-labeled 3′ ALK probe were cohybridized at 44°C for 5 hours after denaturing at 85°C for 20 minutes. Stringency wash was conducted at 72°C with 2× SCC (Ventana Medical Systems, Inc.). DIG hapten was visualized with fast blue detection after incubation with mouse anti-DIG antibody and alkaline phosphate (AP)-conjugated goat anti-mouse antibody. The AP enzyme was inactivated with a hybridization buffer for 32 minutes at 37°C. DNP hapten was visualized with fast red detection after incubation with rabbit anti-DNP antibody and AP-conjugated goat anti-rabbit antibody. After CISH detection was completed, all slides were counterstained with Hematoxylin II (Ventana Medical Systems, Inc.) and Bluing Reagent (Ventana Medical Systems, Inc.). The criteria for positivity in CISH were the same as in FISH.

Immunohistochemistry

FFPE tissues were sectioned at 4-μm thickness and stained using Ventana automated immunostainner (Ventana Medical Systems, Inc.) according to the manufacturer's protocol. Briefly, the slides were dried at 60°C for 1 hour and deparaffinized using EZ Prep (Ventana Medical Systems) at 75°C for 4 minutes. Cell conditioning (heat pretreatment) was performed using CC1 solution containing Tris/Borate/EDTA at 100°C for 20 minutes. Antibody for ALK (mouse monoclonal, clone 5A4) (Novocastra, Newcastle, UK) was diluted to 1:30, treated, and incubated at 42°C for 2 hours. Signals were detected using i-view detection kit (Ventana Medical Systems) based on the labeled streptavidin-biotin (LSAB) method. Each step of the kit included the treatment of inhibitor (1% H_2O_2) (4 minutes), biotinylated Ig (8 minutes), streptavidin-horseradish peroxidase (8 minutes), DAB (chromogen + substrate) (8 minutes), and copper (4 minutes) at 37°C. Counterstaining was performed with Mayer's hematoxylin (ScyTek, Logan, UT) for 2 minutes at room temperature.

Semiquantitative assessment was done by estimating the staining intensity and percentage of tumor cells with positive cytoplasmic staining. Each cell was first scored as 0, 1, 2, or 3, which corresponded to negative, weak, moderate, and strong staining intensities, respectively. ALK IHC scores were assigned as follows: 0 = no stained cells; 1 = faint or weak staining intensity with >5% tumor cells or any staining intensity with ≤5% tumor cells; 2 = moderate staining intensity with >5% tumor cells; 3 = strong and granular staining intensity with >5% tumor cells.[10] Although ALK IHC score 1 was hardly discernable in the low-power objective lens (under objective lens ×10), ALK IHC scores 2 and 3 were readily identifiable. Increases in the staining intensity were associated with increases in the number of positively stained cells observed: a staining intensity of 1 resulted in an average of 14.7% positively stained cells, a staining intensity of 2 resulted in 58.2% positively stained cells, and a staining intensity of 3 resulted in 97.3% positively stained cells.

Statistical Analysis

Accordance between FISH and CISH, and IHC and CISH was analyzed using kappa (κ) statistics. Analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL).

RESULTS

Clinicopathologic Characteristics

A total of 465 consecutively resected NSCLC samples were examined. *ALK* rearrangement was determined in 453 patients (97.4%) by FISH and 449 patients (96.6%) by CISH. There were 12 and 16 cases not evaluable in FISH and CISH, respectively. Reasons for failure were complete detachment of tissues or unrecognizable ISH signal. Clinicopathologic features and *ALK* CISH and FISH results are shown in Table 1. The mean age of the 465 patients was 63.8 years and ranged from 21 to 84 years. *ALK* CISH results were similar to *ALK* FISH as previously described.[10] *ALK*-positive cases had predominantly ADC histology. *ALK*-positive patients showed no statistical difference in gender, age, or smoking history compared with *ALK* wild-type patients.

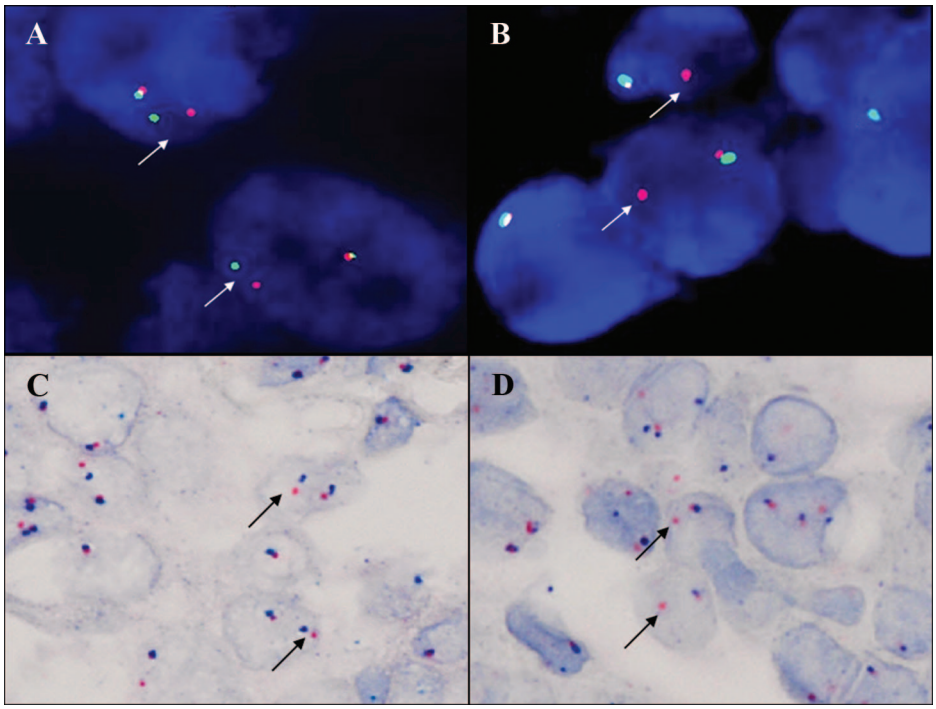

FIGURE 1. ALK gene rearrangement pattern by fluorescence in situ hybridization (FISH) (*A* and *B*, ×100) and chromogenic in situ hybridization (CISH) (*C* and *D*, ×63); two distinct red and green (break-apart) signals and one intact fusion signal in FISH (*A*); two distinct red and blue (break-apart) signals and one intact fusion signal in CISH (*C*); an isolated red signal (IRS) and one intact fusion signal in FISH and CISH, respectively (*B* and *D*).

ALK Rearrangement Assessed by CISH

ALK rearrangement was assessed using CISH in 449 patients (96.6%), and *ALK* rearrangement was identified in 18 patients (4.0%). The majority of the *ALK* CISH-negative cases showed two fusion signals (purple-colored signals) or close proximity of the red and blue signals less than two signal size. A few cells demonstrated an isolated blue signal (IBS) with loss of the corresponding red signal. An independent blue signal corresponds to no rearrangement, whereas a red signal corresponds to rearrangement associated with the tyrosine kinase domain. The clinical significance of IBS is unknown.

The *ALK* CISH-positive NSCLC cases showed two major patterns using the ALK dual-color break-apart probe as follows: (1) the break-apart (split) pattern was observed in 72.2% of cases (13/18) (Figure 1*C*) and (2) IRS pattern: the isolated (single) red signal was predominant in 27.8% of cases (5/18) (Figure 1*D*).

ALK Gene Status in the Normal Tissue by CISH

Most normal lymphocytes, stromal cells, bronchiolar epithelial cells, or alveolar pneumocytes showed one or two blue, red signals or fused purple signals. The red and blue signals were colocalized and produced overlapping purple dots or close proximity less than two signal size distance. It should be noted that there were a few cells showing IBS or IRS in the lymphocytes. As in FISH or CISH tests on tissue sections, most of the cells are partially included within a tissue section due to nuclear truncation, and a few IBS or IRS might be artifacts due to sectioning process. It was more obvious for CISH method rather than FISH method because CISH permits easier distinction of tumor cells from normal tissue. The incidence of BA or IBS in the normal tissue was rare, but exact evaluation was impossible due to paucity of normal cells in the TMA.

ALK Rearrangement Assessed by FISH[10]

ALK rearrangement was assessed using FISH in 453 patients (97.4%), and *ALK* rearrangement was identified in 19 patients (4.2%). The majority of the *ALK* FISH-negative cases showed two fusion signals or close proximity of the red and green signals. However, a few cells demonstrated an isolated green signal (IGS) with loss of the corresponding red signal. An independent green signal corresponds to no rearrangement, whereas a red signal corresponds to rearrangement associated with the tyrosine kinase domain. The clinical significance of IGS is unknown.

The *ALK* FISH-positive NSCLC cases showed two major patterns using the LSI ALK dual-color break-apart probe as follows: (1) the break-apart (split) pattern was observed in 73.7% of cases (14/19) (Figure 1*A*) and (2) IRS pattern: the isolated (single) red signal was predominant in 26.3% of cases (5/19) (Figure 1*B*). The interobserver agreement between pathologists was excellent ($\kappa = 0.94$).

Comparison between CISH and FISH

ALK rearrangement was identified in 19 and 18 patients by FISH and CISH, respectively. There was high concordance in the assessment of *ALK* gene rearrangement between CISH and FISH results ($\kappa = 0.92$) (Table 2). When FISH was chosen as the gold standard, the sensitivity of CISH was 94.4% and specificity 100% (positive predictive value 100%, negative predictive value 99.8%). There was only one discordant case in results between FISH and CISH. The discrepant case showed break-apart signals at a distance of the two times of biggest signal size in FISH. However, the distance of

TABLE 2. Comparison of *ALK* Status between FISH and CISH

	FISH			
CISH	**Positive**	**Negative**	**Not Applicable**	**Total**
Positive	17 (89.5%)	0	1 (8.3%)	18
Negative	1 (5.25%)	425 (97.9%)	5 (41.7%)	431
Not applicable	1 (5.25%)	9 (2.1%)	6 (50.0%)	16
Total	19	434	12	465

$\kappa = 0.92$ (>0.75, excellent; 0.4–0.7, good; <0.4, poor agreement).
CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization.

TABLE 3. Comparison of *ALK* Rearrangement Pattern between FISH and CISH

	FISH Positive		
CISH Positive	**BA**	**IRS**	**Total**
BA	12 (100%)	0	12
IRS	0	5 (100%)	5
Total	12	5	17

$\kappa = 1.00$ (>0.75, excellent; 0.4–0.7, good; <0.4, poor agreement).
CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization; BA, break-apart; IRS, isolated red signal.

TABLE 4. Comparison of *ALK* Status Between IHC and CISH

	IHC			
CISH	**3+**	**2+**	**0/1+**	**Total**
Positive	14 (87.5%)	3 (30.0%)	0	17
Negative	1 (6.25%)	7 (70.0%)	423 (100%)	431
NA	1 (6.25%)	0	0	1
Total	16	10	423	449

$\kappa = 0.82$ (>0.75, excellent; 0.4–0.7, good; <0.4, poor agreement).
CISH, chromogenic in situ hybridization; IHC, immunohistochemistry; NA, not applicable.

split signal was closer and estimated less than two signal size in CISH. Furthermore, tumor portion showing gene rearrangement (16% by FISH and 14% by CISH) was borderline to interpret as positive. So, we interpreted this case as negative in CISH.

The *ALK* rearrangement cases showed two major patterns including BA and IRS. Regarding the rearrangement pattern, there was perfect concordance in BA and IRS patterns between the two methods ($\kappa = 1.00$, Table 3).

Concordance between CISH and IHC

ALK protein expression was detected in 8.6% (40/465) of the NSCLC cases, which included scores of 0 ($n = 425$), 1 ($n = 14$), 2 ($n = 10$), and 3 ($n = 16$) as previously described.[10] To evaluate the correlation of IHC assessment to predict gene rearrangements, we compared the ALK results using IHC and CISH (Table 4). The average percentage of cells positively stained using IHC was higher in the CISH-positive tumors than in the CISH-negative tumors (92.4% versus 19.3%). All the cases with CISH-positive tumors were IHC score 3 or 2, and all the cases with IHC scores 0 or 1 were CISH negative (Figure 2). For cases with scores of 2, 30% (3/10) were CISH positive and 70% (7/10) were CISH negative.

IHC-positive (3+) cases showed CISH positive in 89.5% (14/16) and CISH negative in 5.25% (1/16). The one case with IHC score 3 (5.25%) was not evaluable by CISH test due to diffusion of signal. When IHC scores of 2+ and 3+ were regarded as positive and scores of 0 and 1+ were regarded as negative, there was high agreement between CISH and IHC results ($\kappa = 0.82$).

There was one case with IHC score 3, which were discordant with CISH results. This case was considered as positive by FISH but the BA signal percentage was borderline and considered as CISH negative (16% by FISH and 14% by CISH). Furthermore, the BA signal distance was insufficient, and interobserver variability was present.

Assuming that ALK IHC scores of 0 and 1 were *ALK* rearrangement-negative, an ALK IHC score of 3 was *ALK* rearrangement-positive, and an ALK IHC score of 2 was equivocal, the results of the ALK IHC assay using the tiered scoring method and CISH were strongly correlated ($p < 0.001$) (Figure 3).

Interobserver and Intraobserver Agreement of CISH

Tables 5 and 6 showed the results of CISH evaluation between each observer and intraobserver comparison. Two pathologists (H.K. and S.B.Y.) evaluated 449 cases excluding inadequate cases independently. The overall interobserver agreement was 99.7% ($\kappa = 0.97$) (Table 5). Discordant results were observed in 1 of the 449 cases. In that case, the distance between two separate signals was insufficient and borderline positivity of tumor proportion. One pathologist (H.K.) interpreted it as negative, whereas the other (S.B.Y.) interpreted it as positive for *ALK* rearrangement (positive in 16% of tumor cells) and the latter corresponded with the FISH result. To make a consensus, repeated interpretation was performed ($\kappa = 0.97$) (Table 6). The complete agreement was made for negative cases, but the disagreement was found in one case that was interpreted as negative at first observation, however, as positive at second observation due to borderline tumor portion showing *ALK* rearrangement.

DISCUSSION

In this study, the authors introduced newly developed method for the detection of *ALK* rearrangement by dual-color CISH, and the results showed an excellent concordance between CISH and FISH. We also established optimal condition of IHC for ALK protein expression, which corresponded well with the gene rearrangement results evaluated by CISH and FISH (Figure 3).[10] To the best of our knowledge, this is the first report to compare the accuracy of CISH with FISH results for *ALK* gene rearrangement and to correlate CISH results with ALK protein expression by IHC. Previous studies have shown good correlation between CISH and FISH assays to determine EGFR status in NSCLC, although the CISH tests used were single-colored probe.[11,18] However, *ALK* gene rearrangement status has not been ex-

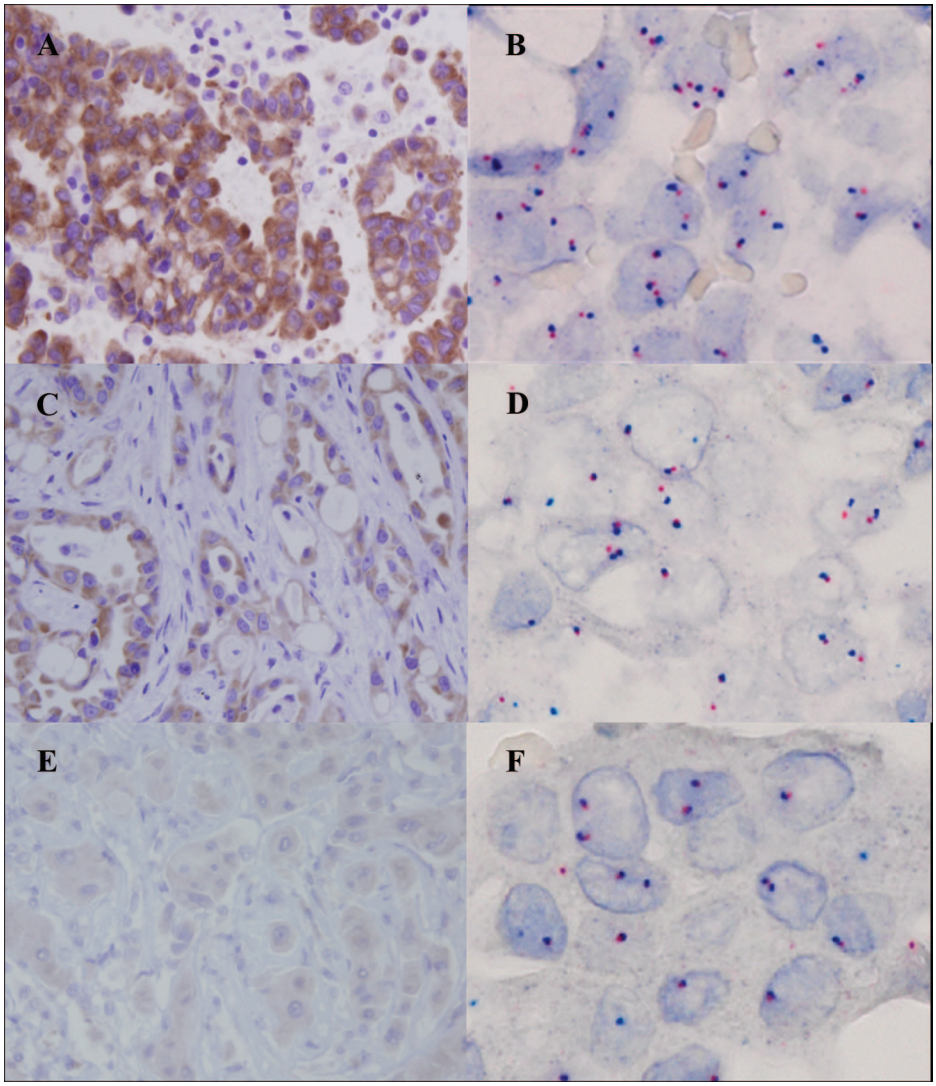

FIGURE 2. Comparison between immunohistochemical (IHC) staining patterns (×40) and chromogenic in situ hybridization (CISH) patterns (×63). ALK IHC 3+ tumor cells (*A*) showed ALK gene rearrangement in CISH (*B*); ALK IHC 2+ tumor cells (*C*) also showed ALK gene rearrangement in CISH (*D*); ALK IHC 1+ tumor cells (*E*) also showed native ALK gene status (no ALK gene rearrangement) (*F*).

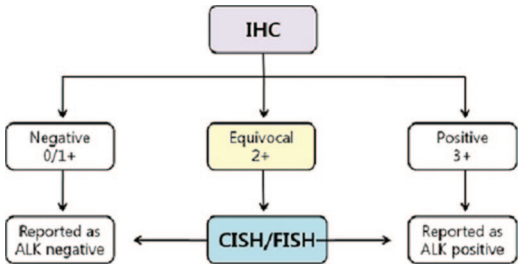

FIGURE 3. Chung's SNUBH ALK protocol presents schematic diagram to predict *ALK* gene rearrangement by IHC. IHC, immunohistochemistry, CISH, chromogenic in situ hybridization, FISH, fluorescence in situ hybridization.

amined in parallel by FISH and CISH. Our results showed an excellent concordance between CISH and FISH. We also observed a significant correlation of ALK protein expression and gene status between CISH/FISH and IHC. Our findings suggest that CISH is a reliable and appropriate method for detecting *ALK* gene status in NSCLC.

TABLE 5. Interobserver Variation of CISH Result

	Observer 1		
Observer 2	**Positive**	**Negative**	**Total**
Positive	18 (100%)	1 (0.2%)	19
Negative	0	430 (99.8%)	430
Total	18	431	449

κ = 0.97 (>0.75, excellent; 0.4–0.7, good; <0.4, poor agreement).
CISH, chromogenic in situ hybridization.

There was one discrepant case between CISH and FISH results that showed BA pattern *ALK* rearrangement in FISH but negative in CISH (Table 2). This case was interpreted positive by one pathologist, which showed disagreement in interobserver comparison (Table 5). The discrepancy may result from uncertain criteria of *ALK* rearrangement: (1) insufficient distance between separate signal, (2) different signal size between FISH and CISH probes, and (3) 15% criteria showing *ALK* gene rearrangement. First, the distance

TABLE 6. Intraobserver Variation of CISH Result

	First		
Second	**Positive**	**Negative**	**Total**
Positive	17 (94.4%)	0	17
Negative	1 (5.6%)	431 (100%)	432
Total	18	431	449

κ = 0.97 (>0.75, excellent; 0.4–0.7, good; <0.4, poor agreement).
CISH, chromogenic in situ hybridization.

between separate signals is estimated using the signal size as a reference, and the probes separated by at least 8 MB can be readily detected with high sensitivity and specificity in paraffin-embedded tissues.[17] This is the reason that analyses must be performed by experienced person. Although the use of bright field microscopy in automated CISH assay is more helpful to recognize the distance of separate two signals, there was still discordance related to the insufficient separate distance using break-apart probe. Second, because the signal size of CISH is bigger than that of FISH, two signal size distances in CISH may be separated far away compared with FISH. Because *ALK* gene translocation analyses using CISH is a newly developed method, further studies are required for setting up an appropriate cutoff value for break-apart distance. Finally, although previous studies including the ongoing crizotinib trials used the 15% cutoff value for *ALK* rearrangement-positive reading criterion,[8,9] it appeared to be arbitrary. It is important to consider a negative control, such as normal nonneoplastic lung tissues, for establishing a cutoff value for accurate FISH or CISH interpretation. We observed that most of the FISH-positive cases showed abnormal split or isolated red signal in more than 50% of tumor cells. Although there was a discrepant case showing borderline positivity around 15% in both FISH and CISH test, it would be necessary to confirm the *ALK* status using another method such as reverse transcription-polymerase chain reaction (RT-PCR).

An accurate, reliable, and reproducible method for determining *ALK* rearrangement is essential for identifying NSCLC patients who are candidates for treatment with an ALK inhibitor named crizotinib, which has shown significant response in NSCLC patients with *ALK* rearrangement.[3,4,8,9,17] FISH has been universally accepted as a reference standard in the assessment of *ALK* rearrangement.[8] However, FISH requires a fluorescence microscope, and the signals are labile and rapidly fade overtime. For these reasons, several bright field ISH methods have been developed as a potential alternative to FISH.[12–14] CISH allows detection of gene copy status using a conventional peroxidase-base reaction and standard bright field light microscope.[18–20] However, the characteristics of the CISH method are not absolutely ideal: the signals appeared somewhat diffuse and bigger than those of FISH, which can make quantification difficult, and signals can be difficult to distinguish from nuclear counterstains.

The introduction of an effective and widely applicable screening method using IHC to detect NSCLC with *ALK* rearrangement is essential because *ALK* rearrangement status has been rarely determined at the time of excision of the primary lesion. Nonetheless, ALK IHC testing in lung cancer remains challenging because of the relatively low level of *ALK* transcription, few data about the sensitivity or specificity of the test, and the absence of a universally accepted and evidence-based IHC scoring algorithm to predict *ALK* rearrangement using FISH.[4,7–9,11,14] We established ALK IHC interpretation guideline for screening *ALK* rearranged NSCLC patients, named *Chung's SNUBH ALK protocol* (Figure 3),[10] and we semiquantitatively evaluated ALK rearrangement using IHC and correlated the results with results obtained using CISH and FISH in this study.

We also found a high concordance between CISH and IHC (Table 4). In particular, a different *ALK* gene arrangement status was observed in tumor cells showing different IHC score. It is possible because that CISH permits easier identification of invasive tumor cells and histologic pattern and correlation with IHC pattern.

IHC to detect protein overexpression on the tumor cytoplasm and FISH to detect *ALK* gene rearrangement are the most frequently used methods in the laboratory. Because the incidence of *ALK* rearrangement in NSCLC is relatively low, it is difficult to use CISH or FISH on all the biopsied or archived NSCLC samples. Therefore, the development of a screening method to identify *ALK*-rearranged tumors is imperative, and the diagnostic test needs to be applicable to archived, formalin-fixed tissues that have been removed several years earlier. Because IHC is readily available in pathology laboratories, it is important to optimize the condition of ALK IHC assay as a screening method and to establish an interpretation guideline.

In this study, we observed a good correlation between results obtained using IHC and CISH/FISH in a large-scale, single-institution study using a semiquantitative IHC scoring assessment. We also presented a diagnostic algorithm to screen for NSCLC with *ALK* rearrangements using IHC, modified *Chung's SNUBH ALK protocol* (Figure 3).[10] IHC detection of ALK protein can be affected by a number of factors including variations in antigen retrieval and tissue fixatives and fixation methods, varying sensitivities of reagents, false-positive and false-negative results, and subjectivity in evaluation of staining intensity. The FISH assay is relatively expensive compared with IHC and requires expert interpreter and rapid fadeout of signals, making it somewhat impractical for routine workflow. These limitations of IHC and FISH can be overcome by using automated dual-color CISH with high concordance. We conclude that the CISH can be used for alternative detection method for *ALK* gene rearrangement and it is a reliable method that has additional practical benefits suitable for routine diagnostic practice.

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REFERENCES

1. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* 2007; 448:561–566.
2. Palmer RH, Vernersson E, Grabbe C, et al. Anaplastic lymphoma kinase: signalling in development and disease. *Biochem J* 2009;420: 345–361.
3. Wong DW, Leung EL, So KK, et al. The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. *Cancer* 2009;115:1723–1733.
4. Inamura K, Takeuchi K, Togashi Y, et al. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. *Mod Pathol* 2009;22:508–515.
5. Boland JM, Erdogan S, Vasmatzis G, et al. Anaplastic lymphoma kinase immunoreactivity correlates with ALK gene rearrangement and transcriptional up-regulation in non-small cell lung carcinomas. *Hum Pathol* 2009;40:1152–1158.
6. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. *Clin Cancer Res* 2008;14:6618–6624.
7. Solomon B, Varella-Garcia M, Camidge DR. ALK gene rearrangements: a new therapeutic target in a molecularly defined subset of non-small cell lung cancer. *J Thorac Oncol* 2009;4:1450–1454.
8. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693–1703.
9. Yoshida A, Tsuta K, Watanabe SI, et al. Frequent ALK rearrangement and TTF-1/p63 co-expression in lung adenocarcinoma with signet-ring cell component. *Lung Cancer*. 2010, doi:10.1016/j.lungcan.2010.09.013.
10. Paik JH, Choe G, Kim H, et al. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in non-small cell lung cancer: correlation with fluorescence in situ hybridization. *J Thorac Oncol* 2011;6:466–472.
11. Yoo SB, Lee HJ, Park JO, et al. Reliability of chromogenic in situ hybridization for epidermal growth factor receptor gene copy number detection in non-small-cell lung carcinomas: a comparison with fluorescence in situ hybridization study. *Lung Cancer* 2010;67:301–305.
12. Nitta H, Zhang W, Kelly BD, et al. Automated brightfield break-apart in situ hybridization (ba-ISH) application: ALK and MALT1 genes as models. *Methods* 2010;52:352–358.
13. Bartlett JM, Campbell FM, Ibrahim M, et al. Chromogenic in situ hybridization: a multicenter study comparing silver in situ hybridization with FISH. *Am J Clin Pathol* 2009;132:514–520.
14. Penault-Llorca F, Bilous M, Dowsett M, et al. Emerging technologies for assessing HER2 amplification. *Am J Clin Pathol* 2009;132:539–548.
15. Beasley MB, Brambilla E, Travis WD. The 2004 World Health Organization classification of lung tumors. *Semin Roentgenol* 2005;40:90–97.
16. Lee HJ, Xu X, Choe G, et al. Protein overexpression and gene amplification of epidermal growth factor receptor in nonsmall cell lung carcinomas: comparison of four commercially available antibodies by immunohistochemistry and fluorescence in situ hybridization study. *Lung Cancer* 2010;68:375–382.
17. Shaw AT, Yeap BY, Mino-Kenudson M, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. *J Clin Oncol* 2009;27:4247–4253.
18. Gallegos Ruiz MI, Floor K, Vos W, et al. Epidermal growth factor receptor (EGFR) gene copy number detection in non-small-cell lung cancer; a comparison of fluorescence in situ hybridization and chromogenic in situ hybridization. *Histopathology* 2007;51:631–637.
19. Lambros MB, Natrajan R, Reis-Filho JS. Chromogenic and fluorescent in situ hybridization in breast cancer. *Hum Pathol* 2007;38:1105–1122.
20. Garcia-Caballero T, Grabau D, Green AR, et al. Determination of HER2 amplification in primary breast cancer using dual-colour chromogenic in situ hybridization is comparable to fluorescence in situ hybridization: a European multicentre study involving 168 specimens. *Histopathology* 2010;56:472–480.