Heterogeneity of Anaplastic Lymphoma Kinase Gene Rearrangement in Non–Small-Cell Lung Carcinomas

A Comparative Study Between Small Biopsy and Excision Samples

Hideyuki Abe, CT, Akihiko Kawahara, CT, PhD,* Koichi Azuma, MD, PhD,† Tomoki Taira, CT,* Yorihiko Takase, CT,* Chihiro Fukumitsu, CT,* Kazuya Murata, CT, PhD,* Tomohiko Yamaguchi, CT, PhD,* Jun Akiba, MD, PhD,‡ Hidenobu Ishii, MD,† Isamu Okamoto, MD, PhD,§ Tomoaki Hoshino, MD, PhD,† Shinzou Takamori MD, PhD,‖ and Masayoshi Kage, MD, PhD**

Introduction: The standard diagnostic method for echinoderm microtubule-associated protein-like 4-anaplastic lymphoma receptor tyrosine kinase translocation is fluorescence in situ hybridization (FISH). Recently, immunohistochemistry (IHC) has been reported as a potential method in screening for anaplastic lymphoma kinase (ALK)-positive non–small-cell lung carcinomas (NSCLC), whereas several authors have reported a discordance between FISH and IHC results. We investigated the heterogeneity of *ALK* gene rearrangement in excision specimens by FISH and also examined whether the FISH score of *ALK* gene rearrangement corresponded in excision and biopsy samples from the same patient.

Methods: Twenty ALK IHC-positive patients including six patients treated with crizotinib therapy were evaluated for the presence of *ALK* FISH. For evaluation of heterogeneity of *ALK* gene rearrangement in excision specimens, we defined six to 10 observation areas in each case, and the number of *ALK* FISH positive observation areas (≥15% rearrangement detected) was investigated. *ALK* FISH score in small biopsy samples was classified as positive (≥15% rearrangement detected), equivocal (5–14% rearrangement detected), or negative (<4% rearrangement detected).

Results: Of a total of 64 tumor observation areas from nine excision specimens, 50 areas were positive for *ALK* gene rearrangement (81.8%). In the comparison of excision and small biopsy samples, all excision specimens were *ALK* FISH-positive (100%; 6 of 6), whereas only three of the small biopsy samples in these patients were positive (50%; 3 of 6), two were equivocal (33%; 2 of 6), and one was negative (17%; 1 of 6). The two equivocal patients received crizotinib and showed a response.

Conclusion: *ALK* gene rearrangement heterogeneity was observed in NSCLC specimens by FISH. Our findings suggested that IHC-positive/FISH-equivocal cases should not be considered true "false-negatives" when a small biopsy sample was used for *ALK* analysis.

Key Words: Anaplastic lymphoma kinase, In situ hybridization, Immunohistochemistry, Crizotinib, Biopsy sample.

(*J Thorac Oncol.* 2015;10: 800–805)

Anaplastic lymphoma kinase (*ALK*) gene rearrangement with echinoderm microtubule-associated protein-like 4-anaplastic lymphoma receptor tyrosine kinase (*EML4-ALK*) translocation has been described in a subset of patients with non–small-cell lung carcinomas (NSCLC), along with several other translocation events, such as *TFG-ALK* and *KIF5B-ALK*.[1,2] This genetic rearrangement occurs in 2% to 7% of NSCLC patients, predominantly in younger individuals with adenocarcinoma who are never-smokers or light smokers.[3,4] *ALK* gene rearrangements in NSCLC provide the basis for targeted therapy with crizotinib and other specific *ALK* inhibitors,[5] and the clinical efficacy of the *ALK* inhibitor crizotinib has been demonstrated in *ALK* fusion-positive NSCLC.

Fluorescence in situ hybridization (FISH) is the standard method for detecting *ALK* rearrangements in NSCLC patients.[6] Although FISH is regarded as the "standard procedure" for detection of rearrangements, it is technically demanding, expensive, and requires the scrutiny of large numbers of individual cells by a highly experienced diagnostician. The use of ALK immunohistochemistry (IHC) has been proposed for the screening of patients.[7] In recent years, Cabillic et al.[8] reported that a single FISH or IHC analysis performed alone would have failed to detect approximately one-fourth of the ALK-positive cases in a large-scale parallel FISH and IHC study of ALK status. Although several authors reported that ALK IHC is a highly sensitive method with a significant correlation with *ALK* FISH,[9,10] the discordance of FISH and IHC in ALK status was also shown using samples of excision specimen, biopsy, transparietal punch, and liquid.[7]

*Department of Diagnostic Pathology, Kurume University Hospital, Kurume, Japan; †Division of Respirology, Neurology, and Rheumatology, Department of Internal Medicine, ‡Department of Pathology, Kurume University School of Medicine, Kurume, Japan; §Center for Clinical and Translational Research, Kyushu University Hospital, Fukuoka, Japan; and ‖Department of Surgery, Kurume University School of Medicine, Kurume, Japan.

Disclosure: The authors declare no conflict of interest.

Address for correspondence: Hideyuki Abe, CT, Department of Diagnostic Pathology, Kurume University Hospital, Kurume, Japan. E-mail: abe_hideyuki@kurume-u.ac.jp

DOI: 10.1097/JTO.0000000000000507

ISSN: 1556-0864/15/1005-0800

In this study, we investigated the heterogeneity of *ALK* gene rearrangement in excision specimens by FISH and also compared the FISH score of *ALK* gene rearrangement between excision and biopsy samples in the same patients.

MATERIALS AND METHODS

Clinical Samples

Diagnostic records at the Kurume University Hospital from 2001 to 2014 were reviewed to identify patients with a diagnosis of NSCLC that tested positive for ALK IHC. Paraffin-embedded blocks from a total of 20 patients (26 specimens consisting of 11 biopsies and 15 excisions) were retrieved (Table 1). Of these patients, six patients received crizotinib treatment. This study conforms to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Kurume University Hospital.

Immunohistochemistry

Paraffin-embedded tissue samples were cut at 4 μm and examined on a coated slide glass. Immunostaining with ALK antibody (×200, D5F3, Cell Signaling Technology, Inc., Danvers, MA) was performed on a fully automated Bond-Max system using onboard heat-induced antigen retrieval with ER2 for 20 minutes and a Refine polymer detection system (Leica Microsystems, Newcastle, United Kingdom). Diamino benzidine was used as the chromogen in all these immunostainings. The cell line H2228 was used as a positive control.

TABLE 1. Summary of Patient Data

Patient	Age	Sex	Biopsy	Excision	ALK IHC	TKI
1	73	F	NS	AD	Positive	
2	52	M	NS	AD	Positive	
3	71	F	NS	AD	Positive	
4	75	F	NS	AD	Positive	
5	53	M	NS	AD	Positive	
6	53	F	NS	AD	Positive	
7	55	M	NS	AD	Positive	
8	33	F	NS	AD	Positive	
9	55	M	NS	AD	Positive	
10	69	F	AD	ADSQ	Positive	
11	58	F	AD	AD	Positive	
12	74	F	AD	AD	Positive	
13	56	F	AD	AD	Positive	
14	69	F	AD	AD	Positive	
15	70	F	AD	AD	Positive	Yes
16	62	F	AD	NS	Positive	Yes
17	38	F	AD	NS	Positive	Yes
18	51	F	AD	NS	Positive	Yes
19	57	F	AD	NS	Positive	Yes
20	65	F	AD	NS	Positive	Yes

NS, no sample; AD, adenocarcinoma; ADSQ, adenosquamous carcinoma; TKI, tyrosine kinase inhibitor treatment of crizotinib; ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry.

FISH for *ALK* Rearrangement

Unstained 4-μm paraffin-embedded tissue samples were put through deparaffinization and protease pretreatment steps before being denatured and hybridized overnight with the *ALK* break-apart probe (Vysis LSI ALK Dual Color, break-apart rearrangement probe; Abbott Molecular, Abbott Park, IL) according to manufacturer's instructions. Tissue sections then underwent saline-sodium citrate washes and were mounted in 4′,6-diamidino-2-phenylindole for nuclei counterstaining.[11,12]

Evaluation of *ALK* Gene Rearrangement

Figure 1 shows representative examples of *ALK* FISH-positive (split or single orange) and *ALK* FISH-negative (fused or single green) patterns. Evaluation of the signal was done according to the Vysis protocol. The *ALK* probe consists of one *orange* (telomeric flank) and one *green* (centromeric flank) signals. Adjacent *orange* and *green* signals that are less than two signal diameters apart or are overlapping are considered as one whole fused signal. Splitting of the *orange* and *green* signals into two or more signal diameters apart indicates *ALK* gene rearrangement. Positive signals were considered as follows: *orange* and *green* signals are separated by more than twice the size of an isolated signal or

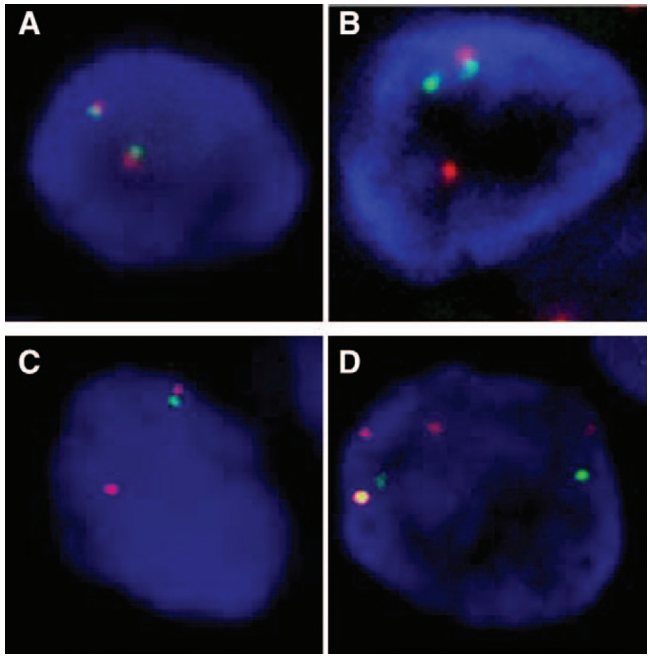

FIGURE 1. Representative signal findings are shown for the different cellular positive (split or single *orange*) and negative (fused or single green) patterns for anaplastic lymphoma kinase (ALK) by fluorescence in situ hybridization (FISH). *A,* Negative *ALK*-FISH pattern. The signals are either overlapping, adjacent, or less than two signal diameters apart. *B,* Classic *ALK*-FISH positive pattern. Classic break apart pattern shows one fused, one *orange*, and one *green* signal in a cell. *C,* *ALK*-FISH positive pattern. One fused *orange* and *green* signal and one *orange* signal only. *D, ALK*-FISH positive pattern. One fused, two broken apart signals, and one *orange* signal only.

a single *orange* signal without a corresponding *green* signal, along with a fused signal, indicates loss of the *green* fragment. Negative signals were considered follows: *orange* and *green* signals overlap or are separated by less than twice the size of an isolated signal or a single *green* signal without a corresponding *orange* signal, along with a fused signal, indicates loss of the *orange* fragment. Nuclei with one isolated signal were considered as uninformative. Signals were counted in at least 50 evaluable tumor nuclei for each examination in excision and biopsy specimens by each observer, and the number of abnormal cells was summed for determination of FISH status.

For evaluation of heterogeneity of *ALK* gene rearrangement in excision specimens, we defined six to ten observation areas in each case, and the number of *ALK* FISH-positive observation areas (≥15% rearrangement detected) was investigated in all cases (patient 1–9). In *ALK* FISH score in small biopsy samples, a patient was considered positive for *ALK* rearrangement if ≥15% of cells showed split signals and was also considered "equivocal" for *ALK* rearrangement if 5% to 14% of cells showed split signals. All FISH analyses were evaluated by two experienced observers (H.A. and Y.T.) who were unaware of the conditions of the patients.

RESULTS

Heterogeneity of *ALK* Gene Rearrangement by FISH

All samples were successfully examined by ALK IHC (D5F3 clone) and *ALK* FISH (Fig. 2). We first examined the heterogeneity of *ALK* gene rearrangement using excision specimens from patients 1 to 9 (Table 2). Patients 1, 5, 7, and 9 were positive in six of six tumor observation areas (100%; 6 of 6), whereas patient 8 was positive in only 4 of 8 tumor observation areas (50%; 4 of 8). In a total of 64 observation tumor areas, positive *ALK* gene rearrangement was observed in 50 areas (81.8%).

Comparison of *ALK* Gene Rearrangement Between Biopsy and Excision Specimens

We next compared FISH scores of *ALK* gene rearrangement in biopsy and excision specimens from the same patients (patient 10–15; Table 3). All excision specimens from patients were FISH-positive (100%; 6 of 6). In biopsy, three FISH-positive patients (50%; 3 of 6) showed greater than 15% cells with *ALK* gene rearrangement. Two patients (33%; 2 of 6) were classified as equivocal with 5% to 14% cells of *ALK* gene rearrangement. One patient (17%; 1 of 6) was negative by FISH because *ALK* gene rearrangement was only 2% in cancer cells (Fig. 3).

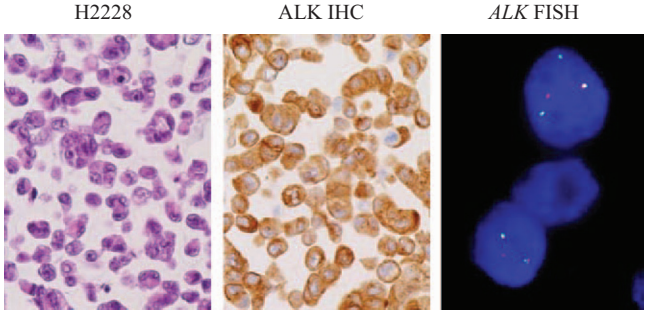

FIGURE 2. Representative images of hematoxylin and eosin stain, ALK immunohistochemistry, and *ALK* fluorescence in situ hybridization in a H2228 cell line.

TABLE 2. Number of Positive Area of *ALK* Gene Rearrangement by FISH

Patient	Number of Positive Area (≥ 15%) with *ALK* Gene Rearrangement	Observation Area	Average (%)
1	6	6	100.0
2	5	6	83.3
3	5	6	83.3
4	6	10	60.0
5	6	6	100.0
6	6	10	60.0
7	6	6	100.0
8	4	8	50.0
9	6	6	100.0
	50	64	81.8

ALK, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization.

ALK FISH in Small Biopsy Sample and Patient Response to Crizotinib

We finally evaluated advanced patients who received crizotinib. With the exception of patient 15, advanced patients (patients 16–20) were not able to undergo surgical resection of the primary lung cancer; however, ALK IHC showed strongly positive in the small biopsy samples (Table 4). In FISH, four patients were *ALK* FISH-positive, two were equivocal, and none were *ALK* FISH-negative. Two patients who were IHC-positive/FISH-equivocal received crizotinib and showed a response. In Figure 4, one NSCLC patient was treated with crizotinib and manifested a tumor response.

TABLE 3. Comparison of *ALK* Gene Rearrangement Between Biopsy and Excision Specimens

	ALK FISH	
Patient	Biopsy	Excision
10	Positive	Positive
11	Positive	Positive
12	Positive	Positive
13	Equivocal (8%)	Positive
14	Negative (2%)	Positive
15	Equivocal (9%)	Positive

ALK, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization.

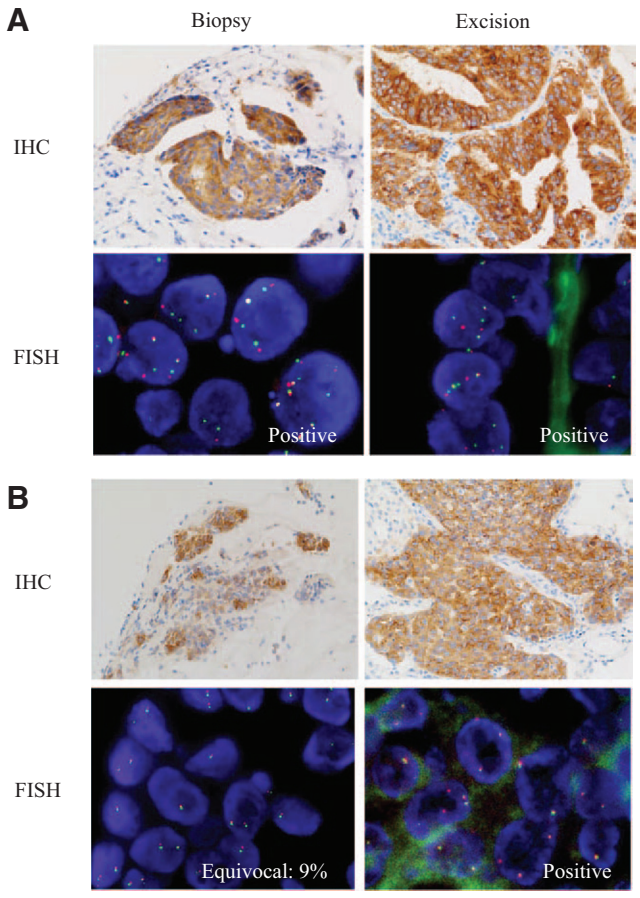

FIGURE 3. *A,* Patient 10 was positive for ALK immunohistochemistry and *ALK* fluorescence in situ hybridization (FISH) assay in both small biopsy and excision specimens. *B,* Patient 15 was positive for ALK immunohistochemistry in both small biopsy and excision specimens. The excision specimen of this patient was positive (≥15% cells with *ALK* rearrangement), whereas the *ALK* rearrangement score of biopsy was 9% for *ALK* FISH.

DISCUSSION

Approximately 70% of patients with NSCLC are diagnosed at a late stage of disease and are not candidates for surgical resection of the primary lung cancer. Indeed, EGFR mutation status and/or ALK status testing is required with increasing frequency on small biopsy samples including cytology samples. Although ALK lung cancers have distinctive morphologic features, with signet ring cells showing a significant association with *ALK* gene rearrangement, morphologic screening alone could not detect a minority of ALK lung cancers.[11,13] The standard procedure for testing for the EML4-ALK translocation is FISH.[6] In the clinical setting, we sometimes experience "false-positive" ALK lung cancer patients who were positive for IHC analysis and negative for FISH analysis in small samples, but not in surgically resected samples, suggesting that heterogeneity of *ALK* gene rearrangement in ALK lung cancer might exist in ALK-positive lung cancer. Therefore, we investigated the heterogeneity of *ALK* gene rearrangement in excision specimens by FISH and also compared the FISH score of *ALK* gene rearrangement between excision and biopsy samples from the same patient. In this study, we found intratumoral heterogeneity of *ALK* gene rearrangement in ALK lung cancer and a discordance of FISH score of *ALK* gene rearrangement between biopsy and excision samples. Indeed, our NSCLC patients who had lower scores (equivocal) for *ALK* gene rearrangement showed a tumor response after treatment with crizotinib, an ALK tyrosine kinase inhibitor.

Camidge et al.[14,15] reported that intratumoral ALK gene rearrangement heterogeneity is a reflection of FISH assay technique, not biological factors. The heterogeneity of ALK gene rearrangement may be associated with the three-dimensional distribution of tumor cells in histology sections in which the semivertical orientation of tumor cells and/or nuclear truncation may hamper identification of break apart signals because FISH on conventional cytology, in which tumor cells are arranged in a single layer, has been reported to have much higher success rates for FISH than histology specimens.[16] Furthermore, it is known that both sensitivity and specificity of ALK FISH assay increase according to the increase in the number of fields and cells.[14] ALK FISH assay using a small biopsy section cannot examine as many fields and cells as is the case with excision samples. We suggest that the main cause of a discordance of FISH score of ALK gene rearrangement between biopsy and excision samples may be due to the FISH assay technique because ALK protein was diffusely positive for ALK IHC in all samples.

ALK IHC for lung cancer requires the use of enhanced detection systems and selection of an antibody rather than the detection system used in the diagnosis of anaplastic large-cell lymphoma. The evaluation of several primary antibodies, including clone of ALK1, 5A4, or D5F3, has been performed for IHC,[10,17] and a variety of signal amplification technologies, such as enhanced detection systems or antibody-enhanced polymer, have been developed to maximize IHC sensitivity.[2,18] Many studies reported that ALK IHC is a highly sensitive method with a significant correlation with *ALK* FISH. A strong intensity (2+ and 3+ score) seemed to be correlated with FISH-positive results (IHC-positive/FISH-positive), and weak intensity (1+ score) was mainly found to be FISH-negative.[12] Therefore, IHC has demonstrated potential in screening for ALK-positive NSCLC. In recent years, Cabillic et al.[8] reported that only 80 specimens (53.3%; 80 of 150) were classified as

TABLE 4. *ALK* Gene Rearrangement in Small Biopsy and Patient Response to Crizotinib

Patient	*ALK* FISH in Biopsy	Clinical Response
15	Equivocal (9%)	Yes
16	Positive	Yes
17	Positive	Yes
18	Equivocal (6%)	Yes
19	Positive	Yes
20	Positive	Yes

ALK, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization.

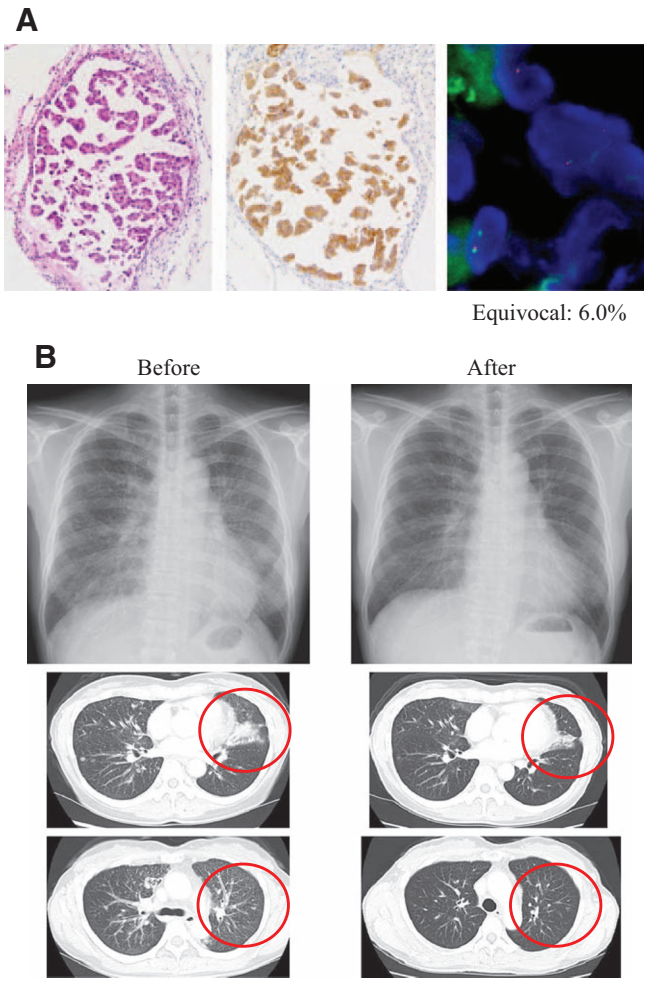

FIGURE 4. *A* Patient 18, in whom surgical resection of primary lung cancer was not possible, was positive for ALK only by immunohistochemistry. *B*, She received crizotinib and showed a partial response.

IHC-positive/FISH-positive in 150 ALK lung cancers analyzed from 3244 NSCLC, and the specimens with discordant IHC/FISH analyses were IHC-negative/FISH-positive (24.0%; 36 of 150) or IHC-positive/FISH-negative (12.6%; 19 of 150). Together, detection of ALK protein and *ALK* gene rearrangement causes "false-negative" cases for each method.

In our experience, most ALK IHC cases displayed relatively homogenous staining, whereas some cases show definite heterogeneous staining. It is well known that adenocarcinoma often shows a heterogeneous feature even in the same nodule. Although various histologic patterns were observed, mucinous cribriform pattern, solid signet ring cell pattern, and papillary pattern with mucin production were predominant in our series. These histologic patterns are known to be frequently observed in adenocarcinoma with ALK rearrangement. When we examined the correlation between histologic pattern and ALK rearrangement of FISH, no apparent correlations were observed. Indeed, even areas showing the same histologic feature did not always show the same result of distribution of ALK rearrangement by FISH. It is suggested that the correlation between histologic subtypes and distribution of ALK rearrangement by FISH may be low or none. The cause of the IHC-negative/FISH-positive discordance might be fixation artifacts. It is already known that fixation method is important for accurate IHC analysis. In breast cancer, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) issued guidelines to standardize fixation for increased human epidermal growth factor receptor type 2 (HER2) testing accuracy.[19] Yamashita-Kashima et al.[20] reported that delay before formalin fixation after tissue collection and extended fixation time affects IHC analysis.[20] Nonbuffered formalin or high concentrations of neutral buffered formalin may also affect IHC. In FISH, they also described that prolongation of fixation time did not affect FISH result; however, delay before fixation strongly reduced the FISH score. Therefore, 10% neutral buffered formalin is recommended to optimize sample preparation conditions for ALK IHC and FISH in lung cancer. The cause of IHC-positive/FISH-negative discordance in this study might be tumor heterogeneity of *ALK* rearrangement. To et al. [17] reported that fusion transcriptions were detected by reverse transcription polymerase chain reaction in ALK IHC-positive cases without *ALK* rearrangement by FISH and confirmed to be EML4-ALK variant 1 by direct sequencing. Surprisingly, *ALK* FISH scores of these cases were 3.5% and 2.1%, which is lower than the 5.0% level, which we considered as "equivocal" and equivalent to "negative" in our study (patient 14). Le Quesne et al.[21] also presented data of biopsy or cytology samples with 10% to 15% positive *ALK* rearrangement by FISH, indicating that *ALK* rearrangement may definitely uneven in lung cancer tissue. Camidge et al.[14] reported that maximum sensitivity and specificity occur when four or more fields (~60 cells) were counted, whereas this method might be difficult in small biopsy samples. Therefore, IHC is essential to identify some ALK lung cancer cases that would be underestimated by FISH.

In conclusion, we found a discordance in *ALK* gene rearrangement FISH between small biopsy and excision samples in ALK lung cancer. Although our study is retrospective in nature and has a relatively small sample size, our results have a clinical application for NSCLC in which the tumors are IHC-positive/FISH-equivocal for ALK rearrangement.

REFERENCES

1. Soda M, Takada S, Takeuchi K, et al. A mouse model for EML4-ALK-positive lung cancer. *Proc Natl Acad Sci U S A* 2008;105:19893–19897.
2. Takeuchi K, Choi YL, Togashi Y, et al. KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. *Clin Cancer Res* 2009;15:3143–3149.
3. 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.
4. Rodig SJ, Mino-Kenudson M, Dacic S, et al. Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. *Clin Cancer Res* 2009;15:5216–5223.
5. Peters S, Taron M, Bubendorf L, Blackhall F, Stahel R. Treatment and detection of ALK-rearranged NSCLC. *Lung Cancer* 2013;81:145–154.
6. Lindeman NI, Cagle PT, Beasley MB, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. *J Thorac Oncol* 2013;8:823–859.

7. Zhou J, Zhao J, Sun K, et al. Accurate and economical detection of ALK positive lung adenocarcinoma with semiquantitative immunohistochemical screening. *PLoS One* 2014;9:e92828.
8. Cabillic F, Gros A, Dugay F, et al. Parallel FISH and immunohistochemical studies of ALK status in 3244 non-small-cell lung cancers reveal major discordances. *J Thorac Oncol* 2014;9:295–306.
9. Yi ES, Boland JM, Maleszewski JJ, et al. Correlation of IHC and FISH for ALK gene rearrangement in non-small cell lung carcinoma: IHC score algorithm for FISH. *J Thorac Oncol* 2011;6:459–465.
10. Selinger CI, Rogers TM, Russell PA, et al. Testing for ALK rearrangement in lung adenocarcinoma: a multicenter comparison of immunohistochemistry and fluorescent in situ hybridization. *Mod Pathol* 2013;26:1545–1553.
11. Kawahara A, Akiba J, Abe H, et al. Eml4-alk-positive lung adenocarcinoma with signet-ring cells. *Diagn Cytopathol* 2014;42:460–463.
12. Akiba J, Kawahara A, Abe H, et al. Evaluation of immunohistochemistry using two different antibodies and procedures for primary lung adenocarcinoma harboring anaplastic lymphoma kinase rearrangement. *Oncol Lett* 2014;8:2155–2159.
13. Nishino M, Klepeis VE, Yeap BY, et al. Histologic and cytomorphologic features of ALK-rearranged lung adenocarcinomas. *Mod Pathol* 2012;25:1462–1472.
14. Camidge DR, Kono SA, Flacco A, et al. Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. *Clin Cancer Res* 2010;16:5581–5590.
15. Camidge DR, Theodoro M, Maxson DA, et al. Correlations between the percentage of tumor cells showing an anaplastic lymphoma kinase (ALK) gene rearrangement, ALK signal copy number, and response to crizotinib therapy in ALK fluorescence in situ hybridization-positive nonsmall cell lung cancer. *Cancer* 2012;118:4486–4494.
16. Proietti A, Alì G, Pelliccioni S, et al. Anaplastic lymphoma kinase gene rearrangements in cytological samples of non-small cell lung cancer: comparison with histological assessment. *Cancer Cytopathol* 2014;122:445–453.
17. To KF, Tong JH, Yeung KS, et al. Detection of ALK rearrangement by immunohistochemistry in lung adenocarcinoma and the identification of a novel EML4-ALK variant. *J Thorac Oncol* 2013;8:883–891.
18. McLeer-Florin A, Moro-Sibilot D, Melis A, et al. Dual IHC and FISH testing for ALK gene rearrangement in lung adenocarcinomas in a routine practice: a French study. *J Thorac Oncol* 2012;7:348–354.
19. Rakha EA, Starczynski J, Lee AH, Ellis IO. The updated ASCO/CAP guideline recommendations for HER2 testing in the management of invasive breast cancer: a critical review of their implications for routine practice. *Histopathology* 2014;64:609–615.
20. Yamashita-Kashima Y, Shu S, Yorozu K, et al. Importance of formalin fixing conditions for HER2 testing in gastric cancer: immunohistochemical staining and fluorescence in situ hybridization. *Gastric Cancer* 2014;17:638–647.
21. Le Quesne J, Maurya M, Yancheva SG, et al. A comparison of immunohistochemical assays and FISH in detecting the ALK translocation in diagnostic histological and cytological lung tumor material. *J Thorac Oncol* 2014;9:769–774.

