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.

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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.

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

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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

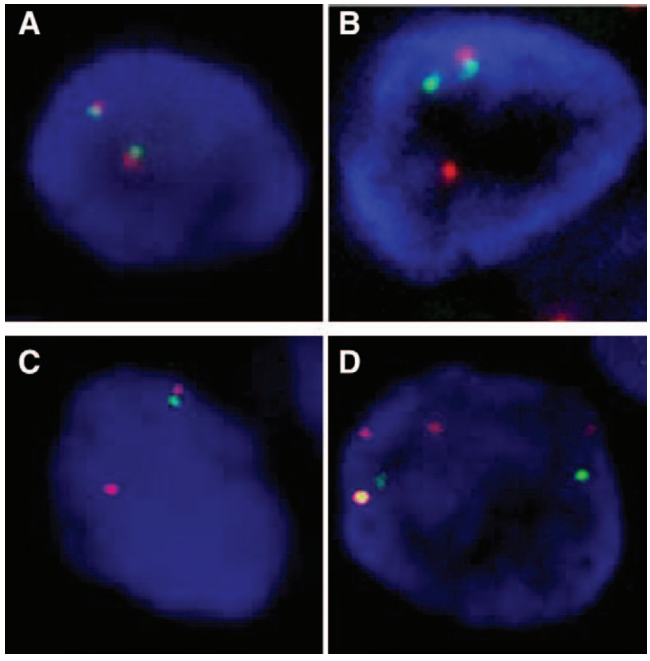

FIGURE 1. Representative signal findings are shown for the different cellular positive (split or single *orange*) and negative (fused or single green) patterns for anaplastic lymphoma kinase (ALK) by fluorescence in situ hybridization (FISH). *A*, Negative *ALK*-FISH pattern. The signals are either overlapping, 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.

