Bright-Field Dual-Color Chromogenic In Situ Hybridization for Diagnosing Echinoderm Microtubule-Associated Protein-Like 4-Anaplastic Lymphoma Kinase-Positive Lung Adenocarcinomas

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Introduction: A subset of lung cancers harbors an *EML4-ALK* (echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase) gene fusion, and detecting this subset may hold therapeutic implications. Many prior studies used fluorescence in situ hybridization (FISH) analysis for this detection, but FISH may have disadvantages including signal decay and dark-field examination that may obscure tissue architecture. In this study, we explored the potential of the *ALK*-break-apart chromogenic in situ hybridization (CISH) method to detect *ALK*-rearranged lung cancer.

Methods: We examined 15 lung adenocarcinomas with reverse-transcriptase polymerase chain reaction-proven *EML4-ALK* fusion transcripts and 30 ALK-negative cases. One hundred tumor cells were evaluated by CISH and FISH for each case, and a detailed signal profile was recorded and compared.

Results: CISH preserved tissue architecture and cytomorphology considerably and facilitated the signal evaluation using a routine light microscope. Positive rearrangement signals (splits or isolated 3′ signals) were identified in 13 to 78% (mean ± SD, 41% ± 19%) of tumor cells in the ALK-positive cohort and in 0 to 15% (mean ± SD, 6% ± 4%) of cells in the ALK-negative cohort. The two groups were best separated by a cutoff value of 20%, with a sensitivity of 93% and a specificity of 100%. The only false-negative tumor having only 13% CISH-positive cells displayed predominantly (76%) isolated 5′ signals unaccompanied by 3′ signals. FISH showed largely similar signal profiles, and the results were completely concordant with CISH.

Conclusions: We have successfully introduced CISH for diagnosing *EML4-ALK*-positive lung adenocarcinoma. This method allows simultaneous visualization of genetics and tumor cytomorphology and facilitates the molecular evaluation and could be applicable in clinical practice to detect lung cancer that may be responsive to ALK inhibitors.

Key Words: Lung, Adenocarcinoma, Chromogenic in situ hybridization, Fluorescence in situ hybridization, EML4-ALK, Diagnosis.

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The recent discovery of a fusion gene that joins the echinoderm microtubule-associated protein-like 4 (*EML4*) and anaplastic lymphoma kinase (*ALK*) in a subset (1–5%) of non-small cell lung carcinomas (NSCLCs) has added a novel molecular subtype to the classification scheme for pulmonary neoplasms.[1] The *EML4-ALK* fusion seems to be formed as the result of a small inversion within the short arm of chromosome 2, and the encoded protein, a chimera comprising the N-terminal portion of EML4 and the intracellular catalytic domain of ALK, is dimerized, leading to constitutive activation.[2] To date, a number of fusion variants have been identified, and *KIF5B* was discovered to be another fusion partner of *ALK*.[3] The importance of recognizing this molecular subtype was highlighted by an international phase I/II clinical trial in which the ALK inhibitor crizotinib (PF02341066) yielded encouraging overall response and disease control rates in a cohort of patients with *ALK*-rearranged NSCLCs.[4] Therefore, an accurate and practical assay is urgently needed to detect this molecular subset of lung cancer.

Currently, the methods available for detecting *ALK* rearrangement are reverse-transcriptase polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH). ALK immunohistochemistry (IHC), which previously yielded low sensitivity,[3,5] has recently been modified to yield high detection rates approaching those of RT-PCR and FISH.[3,6] RT-PCR is a single direct test to detect *EML4-ALK*; however, it generally requires good quality RNA and a multi-

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plex system[7] because of the wide variations in fusion types and the rare presence of *ALK* fusion partners other than *EML4*. Many prior studies thus favored FISH analysis with the *ALK* break-apart probe as a genetic confirmation,[4,8,9] because it is easily applied to formalin-fixed paraffin-embedded tissue, and it covers multiple *ALK* fusion variants. Nevertheless, FISH is not without limitations, including a requirement for highly specialized equipment, cumbersome manipulation of the fluorescent microscope, inevitable signal decay after storage, and dark-field examination that may obscure tissue architecture and cytomorphology.[6] The latter feature is particularly undesirable in testing for lung cancers, because they often assume complex morphologies[10] intimately admixed with nonneoplastic cells, and differentiating tumor cells from the nonneoplastic elements may be difficult without architectural/cytoplasmic information.

Chromogenic in situ hybridization (CISH), which was developed to overcome the aforementioned disadvantages of FISH, has been used in the diagnostic pathology of other organ systems with excellent concordance with FISH.[11–13] When applied to formalin-fixed paraffin-embedded materials and examined under the routine bright-field microscope, CISH enables detection of specific genetic alterations while preserving tumor architecture and cytomorphology. In this study, we explore the potential of CISH in diagnosing ALK-positive NSCLCs in correlation with the results of multiplex RT-PCR for *EML4-ALK* and *KIF5B-ALK, ALK*-break-apart FISH, and a sensitive ALK IHC newly developed at our laboratory.

Additionally, we aim to provide a detailed description of the hybridization signal pattern in the tested cases. In most prior FISH studies, the test results were simply recorded as either positive or negative in relationship to the preset cutoff value[4–6,8,14]; however, this value varied among laboratories including 5%,[14] 15%,[5,8] and 50%,[15] as did the definition of FISH-"positive" cells.[4,5,8,14] The number and type of cells to be counted were not mentioned in many reports.[5–8,15] *ALK*-rearranged NSCLCs tend to show complex in situ signal patterns on break-apart probes; therefore, formulating practical diagnostic criteria requires more than just setting a cutoff value, and studies that evaluate actual signal profiles should make reliable interpretation possible. Recently, Camidge et al.,[9] inspired by Perner et al.,[16] specifically described FISH signal patterns in ALK-positive NSCLCs and took an important step toward formulating objective criteria for the in situ assessment. Unfortunately, their study lacked RT-PCR confirmation or IHC correlation, and the number of ALK-positive cases was understandably small (13 cases), considering the rarity of this entity in the general population. Herein, we add the detailed in situ signal profile of a further 15 cases of RT-PCR-proven ALK-positive tumors, and these data should help us better understand the spectrum of signal patterns and to develop appropriate diagnostic criteria.

MATERIALS AND METHODS

Case Selection

This study was approved by the institutional review board of the National Cancer Center, Tokyo, Japan. Two cohorts of primary lung adenocarcinomas were retrieved from the National Cancer Center archive; all the tumors were surgically resected, and the histological diagnosis was confirmed according to the latest World Health Organization Classification.[10] The first cohort (ALK-positive study group) consisted of 15 cases of lung adenocarcinoma (P1–P15), previously confirmed by multiplex RT-PCR (see method later) to harbor *EML4-ALK* chimeric transcripts. The second cohort (ALK-negative control group) consisted of 30 cases of lung adenocarcinoma (N1–N30), previously shown by multiplex RT-PCR to lack the *EML4-ALK* and *KIF5B-ALK* fusion genes. Tissue microarray was constructed using duplicate 2.0 mm tissue cores sampled from two different representative areas of each tumor (Azumaya, Tokyo).

Reverse-Transcriptase Polymerase Chain Reaction

Fresh-frozen tumor tissues from each tumor were powdered using CP02 (Covaris, Woburn, MA) and sonicated using a Covaris S2 (Covaris). Total RNA was extracted using a mirVana RNA Isolation Kit (Ambion, Foster City, CA), and complementary DNA was synthesized using MMTV reverse transcription (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Switzerland). For amplification of the *ALK* fusion genes, a mixture of primers covering potential breakpoints of fusion transcripts (*EML4-ALK* and *KIF5B-ALK*) were used as reported previously.[17] The multiplex PCR conditions were 95°C for 60 seconds, followed by 50 cycles at 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 60 seconds.

Chromogenic In Situ Hybridization

The *ALK* break-apart CISH assay was performed on a BenchMark XT (Ventana, Tucson, AZ) automated slide processing system as described previously.[18] Briefly, a custom-designed *ALK* break-apart probe set, based on a previous publication,[16] which hybridizes with the neighboring centromeric (5′ probe labeled with digoxigenin) and telomeric (3′ probe labeled with 2,4 dinitrophenyl) sequence of the *ALK* gene, was cohybridized after pretreatment. The 5′ *ALK* probe signal was visualized with alkaline phosphatase (AP)-based fast blue detection; the AP was subsequently inactivated with hybridization buffer before the second AP detection step. The 3′ *ALK* probe signal was visualized with AP-based fast red detection (Figure 1). Tissue sections were counterstained lightly with diluted hematoxylin II, and nonneoplastic lung tissue was used as a negative control. One hundred nonoverlapping tumor cells with hybridization signals were examined for each case with a light microscope (Olympus BX41, Olympus, Tokyo, Japan) under a 60× objective lens without oil immersion, and a detailed signal pattern was recorded for each cell. Cells lacking any hybridization signal were not evaluated. The signal in each cell was categorized into one of the following seven patterns: (1) fused 3′/5′ only; (2) fused 3′/5′ and both isolated 3′ and 5′ (split); (3) both isolated 3′ and 5′ (split) only; (4) fused 3′/5′ and isolated 5′; (5) fused 3′/5′ and isolated 3′; (6) isolated 5′ only; and (7) isolated 3′ only (Figure 2). A fused 3′/5′ signal looked purple or black due to colocalization of red (3′) and blue (5′) signals. A split signal was defined by 5′ and 3′ probes observed at a distance more than 1 time the signal size, and signals separated by less