A Novel, Highly Sensitive Antibody Allows for the Routine Detection of ALK-rearranged Lung Adenocarcinomas by Standard Immunohistochemistry

Mari Mino-Kenudson1,*, Lucian R. Chirieac2,*, Kenny Law2, Jason L. Hornick2, Neal Lindeman2, Eugene J. Mark1, David W. Cohen3, Bruce E. Johnson4, Pasi A. Jänne4, A. John Iafrate1,†, and Scott J. Rodig2,†

1Department of Pathology, Massachusetts General Hospital, Boston, MA
2Department of Pathology, Brigham and Women’s Hospital, Boston, MA
3Department of Pathology, Beth Israel-Deaconess Medical Center, Boston, MA
4Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA

Abstract

Purpose—Approximately 5% of lung adenocarcinomas harbor an EML4-ALK gene fusion and define a unique tumor group that may be responsive to targeted therapy. However ALK-rearranged lung adenocarcinomas are difficult to detect by either standard fluorescence in-situ hybridization (FISH) or immunohistochemical (IHC) assays. In the present study we used novel antibodies to compare ALK protein expression in genetically defined lung cancers and anaplastic large cell lymphomas (ALCL).

Experimental Design—We analyzed 174 tumors with one standard, and two novel monoclonal antibodies recognizing the ALK protein. Immunostained tissue sections were assessed for the level of tumor-specific ALK expression by objective quantitative image analysis and independently by three pathologists.

Results—ALK protein is invariably and exclusively expressed in ALK-rearranged lung adenocarcinomas but at much lower levels than in the prototypic ALK-rearranged tumor, anaplastic large cell lymphoma, and as a result, often not detected by conventional IHC. We further validate a novel IHC that shows excellent sensitivity and specificity (100% and 99%, respectively) for the detection of ALK-rearranged lung adenocarcinomas in biopsy specimens with excellent interobserver agreement between pathologists (kappa statistic, 0.94).

Conclusions—Low levels of ALK protein expression is a characteristic feature of ALK-rearranged lung adenocarcinomas. However a novel, highly sensitive IHC assay reliably detects lung adenocarcinomas with ALK rearrangements and obviates the need for FISH analysis for the majority of cases and therefore could be routinely applicable in clinical practice to detect lung cancers that may be responsive to ALK inhibitors.
Keywords
Lung adenocarcinoma; ALK; immunohistochemistry

Introduction
Lung cancer remains the leading cause of cancer death worldwide. In the United States alone there are more than 200,000 new cases of lung cancer resulting in 150,000 deaths per year. (1) Despite improvements in the detection and treatment of lung cancer, the overall 5-year survival rate remains at 15%. (2) A subset of lung cancers harbor activating mutations in the epidermal growth factor receptor (EGFR) gene. (3, 4) The majority of patients with lung cancers harboring EGFR mutations, but only a small fraction of those without EGFR mutations, can show dramatic responses to drugs that inhibit EGFR kinase activity resulting in prolonged patient survival. (5) Therefore the identification of critical tyrosine kinases, and the development of specific tyrosine kinase inhibitors (TKIs) targeting individual tumors has become a new paradigm in lung cancer treatment. (6, 7)

Recently, two groups independently discovered that rare lung adenocarcinomas harbor rearrangements of the anaplastic large cell kinase (ALK) gene that result in the pathologic expression of a fusion protein, most commonly EML4-ALK. (8, 9) EML4-ALK demonstrates constitutive kinase activity, and ALK-rearranged lung cancer cell lines are dependent upon ALK kinase activity for growth and survival. (8, 10, 11) However, standard TKIs that target EGFR are poor inhibitors of ALK kinase activity, and as such, they have shown no therapeutic benefit to patients with ALK-rearranged lung adenocarcinomas. (12) In contrast, a novel TKI that targets ALK kinase activity has shown dramatic clinical responses among the few patients with ALK-rearranged tumors treated to date. (13) These data highlight the importance of developing TKIs that specifically target ALK enzymatic activity, and in addition, a method to accurately identify patients with ALK-rearranged lung adenocarcinoma and thus the best candidates for clinical trials. (12, 13)

Testing for ALK-rearrangements is most frequently performed during the pathologic evaluation of anaplastic large cell lymphoma (ALCL), a tumor of T cell origin, and most generally involves one of three methodologies: karyotyping (g-banding) of tumor metaphase spreads, fluorescence in-situ hybridization (FISH) with probes flanking the ALK locus, and immunohistochemical staining of tumor biopsy tissue with antibodies specific for the ALK protein. (14) All three methodologies are highly specific for detecting ALK-rearrangements in ALCL and extensively validated. (15) However each of these methods confronts significant challenges when applied to the detection of ALK-rearrangements in lung adenocarcinoma. (16) Unlike ALCL, ALK-rearrangements in lung adenocarcinoma frequently involve an intrachromosomal deletion of genetic material 5' of the ALK locus not visible by routine karyotypic analysis. Although FISH analysis is able to resolve the defect, the altered probe hybridization patterns are subtle, and has lead to false negative results. (16) Finally, we have reported that lung adenocarcinomas known to harbor an ALK-rearrangement by FISH analysis do not always have detectable ALK protein expression when tested by conventional, or tyramide-amplified immunohistochemistry (IHC). This finding, coupled with reports that tumors can express EML4-ALK transcript expression in the absence of ALK protein, raises questions as to whether ALK protein expression is invariably expressed by tumors harboring an EML4-ALK fusion, and whether IHC is a reliable diagnostic test for the diagnosis of ALK-rearranged lung adenocarcinoma. (16, 17)

Here we compare ALK protein expression in a large cohort of genetically defined tumors by IHC using novel, highly sensitive antibodies and show that the ALK protein is universally
expressed by ALK-rearranged lung adenocarcinomas. We also show that ALK protein expression in ALK-rearranged lung adenocarcinomas is much lower than in ALK-rearranged ALCLs and, as a result, often not detected by conventional IHC. This novel immunohistochemical test shows very high sensitivity and specificity for detecting ALK-rearranged lung adenocarcinomas when evaluated by experienced pathologists and therefore will serve as a robust diagnostic tool in routine surgical pathology practice.

Materials and Methods

Case Selection

Optimization Studies—We examined 19 cases of ALCL and two cases of ALK-rearranged inflammatory myofibroblastic tumor (IMT) in order to test and optimize two newly developed monoclonal antibodies specific for the ALK protein in FFPE specimens.

Lung Adenocarcinoma Studies—We examined 153 cases of lung adenocarcinoma [22 ALK-rearranged including 8 previously analyzed(16) and 131 ALK-germline]. All cases consisting of FFPE tissue biopsies or resection specimens were evaluated using standard pathology methods as described(18), and classified according to standard pathology practice. (14,19) Cases primarily originated at the authors’ primary institutions (BWH, DFCI, BI, and MGH); 13 cases of the ALK-rearranged lung adenocarcinomas were samples from outside institutions sent as referrals. Among the lung adenocarcinomas, 116 of 153 cases were part of a tissue microarray described previously.(16)

Immunohistochemistry

Immunohistochemical staining was performed on 4 μm thick, FFPE tissue sections. Briefly, slides were deparaffinized and pretreated with 1 mM EDTA, pH 8.0 (Zymed, South San Francisco, CA) and heat-mediated antigen retrieval in a steam pressure cooker (Decloaking Chamber, BioCare Medical, Walnut Creek, CA). All further steps were performed at room temperature in a hydrated chamber. Endogenous peroxidase activity was quenched with Peroxidase Block (DAKO USA, Carpinteria, CA) for 5 minutes and slides were pre incubated in 20% normal goat serum in 50-mM Tris-HCl (pH 7.4). Mouse monoclonal anti-human CD246 (clone: ALK1, DAKO USA, Carpinteria, CA) was applied either at 1:50 in DAKO diluent (for ALCLs) or at 1:2 in DAKO diluent (for lung adenocarcinomas and IMTs) overnight. The slides were then washed in Tris-HCl, and detected with horse radish peroxidase (HRP) conjugated anti-mouse Envision+ kit (DAKO). Rabbit monoclonal anti-human CD246 (clones D5F3 and D9E4, Cell Signaling Technology, Danvers, MA, USA) was applied either at 1:500 (for ALCLs) or at 1:100 (for lung adenocarcinomas and IMTs) in DAKO diluent overnight. The slides were then washed in Tris-HCl, and detected with horse radish peroxidase (HRP) conjugated anti-rabbit Envision+ kit (DAKO). All slides were counterstained with hematoxylin.

The mouse monoclonal antibody ALK1 was raised against a peptide encoded by amino acids 1359–1460 of the human ALK protein (Pulford et al., 1997). This peptide spans the c-terminal portion of the tyrosine kinase domain of ALK and preserved in NPM-ALK, EML4-ALK, and all other known pathologic ALK fusions. The rabbit monoclonal antibodies D5F3 and D9E4 were raised against a peptide derived from the c-terminal portion of the human ALK downstream of the kinase domain and preserved in NPM-ALK, EML4-ALK, and all other known pathologic ALK fusions (H. Haack, personal communication).

FISH

FISH was performed on FFPE tumor tissues using a break-apart probe specific to the ALK locus (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular,
Abbott Park, Illinois, USA) per manufacturer’s instructions on all cases of lung adenocarcinoma. FISH positive cases were defined as >15% split signals in tumor cells as previously described.(12)

**Image Analysis**

Whole section slides stained with D5F3 antibody or ALK1 antibody (n = 56 and n = 51, respectively) were scanned at 200X magnification using an Aperio ScanScope XT workstation (Aperio Technology, Inc., Vista, CA). Images were visualized and annotated using ImageScope software (version 10.0.35.1800, Aperio Technology) and were analyzed using a standard analysis algorithm (color deconvolution v9.0, Aperio Technology). Briefly, one pathologist (SJR) selected 3 regions, minimum, consisting of tumor cells with most intense staining. These regions were then subjected to the deconvolution program which separates brown (DAB) from blue (hematoxylin) tissue staining. Based on a single baseline optical density determined as the threshold for positive staining by a pathologist (and which is applied to all analyzed slides), the software calculated a score that was defined as the average optical density of the positively stained area times the percentage of the area staining above baseline. The threshold for positive staining with objective analysis was determined as the minimum image analysis score necessary to ensure perfect specificity of the assay (to exclude any false positive cases). For both D5F3 and ALK1 antibodies, a score of ≥1.0 was considered positive in the ALCLs. For the D5F3 antibody a score ≥1.0 was considered positive and for the ALK1 antibody a score >2.7 was considered positive in the lung adenocarcinomas.

**Study of Interobserver Variation and Statistical Analysis**

Three independent pathologists (M.M.-K., L.R.C. and S.J.R.) blindly reviewed each stained slide, and semiquantitatively graded intensity of staining (0–3) and assessed the percentage of immunoreactive tumor cells in 10% increments. Prior to the analysis, each pathologist reviewed 2–3 lung adenocarcinomas of known ALK-status stained with each antibody. This allowed each pathologist to assess the level of non-specific or background staining characteristic of the individual reagents. Positive ALK protein expression as determined by any individual pathologist was defined as tumor-specific staining of any intensity over background staining in ≥10% of the tumor cells. The correlation between individual, blinded pathologist’s scoring was performed using the Fleiss’ kappa statistic. For sensitivity and specificity calculations, an individual case was deemed positive for ALK protein expression if 2 of 3 pathologists scored the case as positive. Sensitivity, specificity, positive predictive value, and negative predictive value for detecting ALK-rearranged cases were calculated using GraphPadPrism (version 5.02 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com).

**Results**

**D5F3 and D9E4 antibodies detect ALK protein expression in formalin fixed tumor samples**

We first sought to determine whether 2 novel rabbit, monoclonal antibodies, clones D5F3 and D9E4, are able to detect ALK protein expression in FFPE specimens. We optimized standard immunohistochemical staining using known cytogenetically defined types of tumors: an ALK-rearranged ALCL, an ALK-germline ALCL, and an ALK-rearranged inflammatory myofibroblastic tumor (IMT, Figure 1). We confirmed that both D5F3 and D9E4 antibodies showed robust tumor-specific staining in ALK-rearranged tumors and essentially no tissue staining in ALK-germline tumors using a wide range of antibody dilutions, antigen retrieval methods, and secondary antibody detection methods (data not shown). The optimal staining conditions were chosen based on specifics of ALK expression in each tumor type. Where ALK protein expression in the ALK-rearranged ALCL was relatively easy to detect (Figure 1A, B and Figure 2), lower titers of antibody were used to conserve reagent and minimize any background staining (Figure 1D, E). In contrast, in the 2 ALK-rearranged IMTs that we tested,
we found lower levels of ALK protein that necessitated the highest concentration of D5F3 and D9E4 antibodies possible without an increase in non-specific background staining (Figure 1 and data not shown). For these cases we also determined the maximum concentration of a standard IHC reagent, the monoclonal antibody ALK1, that we could use.(16) At these highest concentrations, we found that IHC using either D5F3 or D9E4 resulted in more intense staining of tumor cells (Figure 1G; H) than IHC using ALK1 antibody (Figure 1I); however, the staining intensity with D9E4 was slightly lower than D5F3. We, thus, decided to use the D5F3 antibody in the subsequent evaluation.

**Correlation between D5F3 and ALK1 antibodies in detecting ALK protein in ALCL**

IHC using the antibody ALK1 has been extensively tested against genetically defined groups of ALCLs and shown to be an effective surrogate for genetic testing.(15) We therefore compared IHC staining using one of our novel antibodies, D5F3, with that using ALK1 antibody on a group of 19 ALCLs. The staining patterns were objectively analyzed by image analysis (Figure 2A) and blindly interpreted by 3 pathologists. By image analysis, the cases were separable into 2 groups: 11 cases with staining intensity scores ≥1.0 and deemed positive for ALK protein, and 8 cases with intensity scores <1.0 and deemed negative for ALK protein. Importantly using this threshold for staining, all cases deemed positive for ALK protein using ALK1 antibody were also deemed positive using D5F3 antibody, indicating a perfect correlation. The same cases were independently and blindly read by 3 pathologists. Using both the ALK1 and D5F3 antibodies and evaluating ALK protein expression, there was a perfect agreement between individual pathologists and with the objective analysis, performed by digital scanning (data not shown). Cytogenetic/FISH analysis and IHC analysis were performed on the tumor samples in 9 of 19 cases and in 12 of 19 cases, respectively, at the time of the original diagnosis. The staining results using D5F3 or ALK1 antibody in the current analysis were in complete concordance with those prior studies (data not shown). The ALK-rearranged tumor with the lowest staining intensity was categorized positive for ALK protein expression (Case #11, Figure 2A), and the ALK-germline tumor with the highest staining intensity was categorized negative for ALK protein expression (Case #12, Figure 2A) by both standard microscopy and digital scanning and were easily distinguishable visually (Figure 2B). We conclude that IHC staining with D5F3 antibody is specific to ALK protein expression and is comparable to IHC staining with ALK1 antibody for establishing the diagnosis of ALK-positive ALCL.

**ALK is universally expressed at lower levels in ALK-rearranged lung adenocarcinomas than in ALCLs**

We previously reported that among 10 cases of ALK-rearranged lung adenocarcinomas stained by conventional IHC using ALK1 antibody, we could detect ALK protein in only four cases. (16) These findings suggested that ALK protein expression may be lower in many cases of ALK-rearranged lung adenocarcinoma relative to ALCL and that conventional IHC analysis may not a useful surrogate for FISH analysis. Alternatively, ALK protein may not be expressed in all cases of ALK-rearranged lung adenocarcinomas. To determine whether IHC using D5F3 better detects ALK expression in lung adenocarcinoma, we compared 22 ALK-rearranged cases with 131 ALK-germline cases. ALK-rearranged cases included those positive for the transcription factor TTF-1 (16 cases, 89%) and negative for TTF-1 (2 cases, 11%). In all cases the ALK status of the tumor was determined by FISH analysis. Because our preliminary data indicated that low level ALK expression may be difficult to detect in lung adenocarcinoma, we increased the titer of D5F3 and ALK1 antibodies in our IHC reactions to maximize the sensitivity without sacrificing the specificity of the assay (see Materials and Methods). Furthermore, we did not find that alternative antigen retrieval methods improved the sensitivity of the assay (data not shown). In 17 of 19 ALK-rearranged lung adenocarcinomas for which a direct comparison can be made, D5F3 antibody showed more intense staining of the tumor.
cells than ALK1 antibody (Figure 3A). For the vast majority of ALK-rearranged lung adenocarcinomas the relative amount of staining with D5F3 was, objectively, much higher than found with ALK1 (6.6 ± 4.7 O.D. x % positive staining vs. 2.9 ± 2.6 O.D. x % positive staining, \( p < 0.001 \)), and in the 2 cases in which D5F3 antibody showed lower staining than ALK1 antibody, the relative difference was modest (Figure 3A). Importantly, the objective intensity score of D5F3 staining in ALK-rearranged lung adenocarcinomas was lower than that in ALK-rearranged ALCLs in 31 of 33 cases (compare Figure 2A and Figure 3A, D5F3 staining). Given that we used 5X more D5F3 antibody per IHC reaction (but the same retrieval and secondary reagents) to test the lung adenocarcinomas than we used to test the ALCLs (decreasing the dilution of antibody from 1:500 to 1:100), we conclude that ALK protein expression is always lower in ALK-rearranged lung adenocarcinomas relative to ALK-rearranged ALCLs.

D5F3 antibody, but not ALK1 antibody, is a useful surrogate to genetic testing in the diagnosis of ALK-rearranged lung adenocarcinoma

Both visual examination (Table 1) and objective quantitation of IHC staining (Figures 3B and C) confirm that IHC using D5F3 antibody is superior to ALK1 antibody in assessing the ALK expression in lung adenocarcinomas. A major difference between the antibodies is the level of non-specific staining of tumor and non-neoplastic tissues at high concentrations of ALK1 antibody. In fact, when the intensity of non-specific staining is objectively calculated, and the threshold that will ensure 100% specificity is established (Figure 3B, dotted horizontal line) only 6 of 19 (32%) ALK-rearranged lung adenocarcinomas are identified. Objective analysis of cases stained with D5F3 antibody show very little non-specific or background staining at the antibody titer necessary to detect ALK expression in lung adenocarcinoma 100% sensitivity and specificity for the 37 cases (Figure 3C, dotted horizontal line). Similarly, evaluation of 153 cases of lung adenocarcinoma (22 ALK-rearranged; 131 ALK-germline) by the three pathologists, blinded to the genetic status of the tumors at the time of examination, showed reproducibly high sensitivity and specificity for the identification of ALK-rearranged tumors in the clinical setting (sensitivity = 100%, specificity = 99%; Table 1).

Weak staining with D5F3 antibody suggests the presence of an ALK-rearranged lung adenocarcinoma

Visual inspection of select ALK-rearranged and ALK-germline adenocarcinomas stained with D5F3 reveals that the expression of the ALK protein may be faint, or detectable in only in a subset of the tumor cells. Overall, approximately one-third of ALK-rearranged lung adenocarcinomas show robust staining of the tumor cells for ALK protein with D5F3 antibody (Figure 4A, D, representing Case #1; Case#2 respectively from Figure 3C). Another one-third of ALK-rearranged lung adenocarcinomas show moderate, but distinct tumor cell staining (not shown), and the final one-third show weak and sometimes focal staining for ALK protein with D5F3 antibody (Figure 4G). Importantly, ALK-germline lung adenocarcinomas show no staining with D5F3 antibody under the conditions we describe here (Figure 4J).

The potential for false negatives in screening for ALK-rearranged lung adenocarcinomas by IHC

In our hands, the IHC test we describe here shows very high sensitivity and specificity for detecting ALK-rearranged lung adenocarcinomas and as such can act as a surrogate for genetic testing. However the results of one case illustrate that a false negative result can occur. Case #20 (Figure3C) showed positive staining for ALK protein objectively and by pathologists' interpretation of the IHC (Table 1, Figure 5A). Over the course of this patient 3 more biopsy samples were obtained, one of which showed positive staining (Figure 5B), one of which showed equivocal staining (Figure 5C) and one of which showed no staining despite repeated
testing (Figure 5D). We considered the possibility that the third biopsy sample without detectable ALK expression did not harbor an ALK-rearrangement, however FISH analysis of this specimen confirmed the presence of the genetic abnormality (Figure 5D, inset) and RT-PCR analysis also confirmed the expression of EML4-ALK transcript (Table S1). We therefore conclude that the IHC result is a false negative.

**Correlation between IHC, FISH, and RT-PCR analysis**

In addition to IHC and FISH analysis, RT-PCR analysis has been suggested as a potential tool to detect ALK-rearrangements in lung adenocarcinoma (28,30). We had sufficient tissue to perform RT-PCR analysis on 10 cases of ALK-rearranged lung adenocarcinoma in an assay designed to detect the three major EML4-ALK variant fusions: V1, V2; V3 (ref. 30). We obtained a positive RT-PCR result in 9 of the 10 cases that included 6 cases with an EML4-ALK V1 fusion and 3 cases with an EML4-ALK V3 fusion. All of these cases were considered positive for an ALK-rearrangement by FISH analysis and for ALK-protein expression by IHC using the D5F3 antibody (Table S1). We found one case to be positive for ALK-rearrangement by FISH analysis and for ALK-protein expression by IHC but negative by RT-PCR (Table S1). Our failure to detect an ALK fusion transcript in this single case might be due to the presence of an EML4-ALK fusion variant not assayed for by our RT-PCR test or an ALK fusion with a gene other than EML4 (30). Thus within this limited sample set, we have find that a sensitive IHC-based assay can show benefit over RT-PCR based analyses.

**Discussion**

Until recently, chromosomal abnormalities leading to pathologic ALK protein expression had been described only in ALCLs, IMTs, and rare diffuse large B cell lymphomas.(14,20–23) The identification of an ALK-rearrangements in ALCL has traditionally been through karyotypic analysis of metaphase spreads, or FISH analysis of mitotic or interphase nuclei using probes flanking the ALK locus.(14) However with the advent of monoclonal antibodies that recognize the ALK protein, IHC analysis of tumor tissues has become a highly sensitive and cost effective surrogate for genetic testing.(24–26)

The ALK locus is now recognized to be pathologically dysregulated in approximately 5% of lung adenocarcinomas.(8,9,16,27) Most commonly, the genetic lesion consists of an intrachromosomal deletion and inversion event resulting in an EML4-ALK fusion that cannot be detected by conventional karyotypic analysis.(8–10) Therefore, the diagnosis of ALK-rearranged lung adenocarcinoma requires IHC, FISH, or RT-PCR based analysis of biopsy tissue.

Currently several laboratories rely on FISH analysis of mitotic or interphase tumor nuclei and identify a “split” hybridization signal to establish the presence of an ALK-rearrangement.(12) However FISH is unlikely to be a preferred method for screening lung adenocarcinomas in routine surgical pathology practice because 1) the “break-apart” signal pattern resulting from the intrachromosomal deletion and inversion event in the setting of polysomy typical of lung cancer is subtle and easily missed (i.e. compare Figure 4C, F; I with Figure 4L), 2) morphologic indicators of tumor versus non-neoplastic stromal or normal epithelial tissue is lost with FISH analysis, and 3) FISH remains a specialized test not routinely performed by many pathology laboratories. We previously highlighted the potential difficulty of this test when we reported a case of lung adenocarcinoma that was originally classified as ALK-germline due to misinterpretation of the FISH analysis (16). The error was recognized upon finding positive staining for ALK-protein expression by IHC, and the original FISH analysis was re-reviewed and found to be in error.
Alternatively, some laboratories have used a multiplexed RT-PCR based assay to detect EML4-ALK or alternative fusion transcripts. However this technique is also specialized, not performed in many routine pathology laboratories, and the vast majority of specimens submitted for histologic diagnosis are stored as FFPE tissue in which the RNA may be substantially degraded. Furthermore, this technique may not be able to detect all the translocations involving the ALK gene, and most importantly, it may not be entirely specific. Never the less, we had sufficient tissue to perform RT-PCR analysis on 10 cases determined to have an ALK rearrangement by FISH. Each of these cases was positive for ALK-protein by IHC using the D5F3 antibody, however in only 9 cases were we able to detect an ALK fusion transcript by RT-PCR (Table S1). Our failure to detect the ALK fusion in one case may have been due to the presence of an alternative EML4-ALK fusion variant or an ALK fusion with a gene other than EML4.

IHC remains a preferred technique for screening and diagnosis in routine surgical pathology practice and could prove to be a fast and cost-effective method to identify NSCLC patients for clinical studies of ALK inhibitors. We, and others, have shown that IHC-based screening can be used to identify lung adenocarcinomas harboring known or novel ALK-rearrangements. In an analysis of 10 cases of ALK-rearranged lung adenocarcinoma, we previously reported that a conventional, diagnostic IHC test to detect ALK-rearrangements in ALCL detected ALK protein expression in only 4 of 10 cases of ALK-rearranged lung adenocarcinoma. We were able to increase the sensitivity of the test and provide evidence of ALK protein expression in 4 of the remaining 8 cases by including a non-traditional tyramide-based amplification step in our protocol. Others have published similar results using a different, non-traditional amplification technique. However it remained unresolved whether those cases with an ALK-rearrangement detected by FISH but negative for ALK expression by IHC express the mutant protein at all.

Using 22 ALK-rearranged lung adenocarcinomas, among the largest collections reported to date, and 19 ALCLs, we compared ALK protein expression by the quantitative IHC analysis using one well-described antibody and two novel antibodies. All three antibodies recognize epitopes within the ALK protein that is preserved in all known ALK fusions. We find that tumor specific ALK protein expression in ALK-rearranged lung adenocarcinomas is much lower than that in ALK-rearranged ALCLs, and that this low level of protein expression necessitates higher titers of antibody for IHC-based detection. Furthermore we found that IHC using the novel antibody D5F3 shows much greater sensitivity than the antibody ALK1 in detecting ALK-rearranged lung adenocarcinoma by either objective image analysis or by 3 pathologists’ individual interpretations. Most importantly, the interpretation of this novel IHC test is highly reproducible among pathologists and shows complete concordance with genetic data. We acknowledge that in addition to ALK1, D5F3, and D9E4, there are other commercially available antibodies that can detect ALK protein expression in ALCL. However, due to our limited tissue available from ALK-rearranged lung adenocarcinomas, we could not extend our study with additional antibodies. Similarly, we were unable to perform a correlative study between EML4-ALK transcript expression and ALK protein expression as detected with our novel antibodies. As ALK-rearranged lung adenocarcinomas become more easily identified and larger collections are assembled, these additional comparisons can be made.

It has become clear that ALK-rearranged lung adenocarcinomas are a rare tumor with unique clinico-pathologic characteristics. Patients with ALK-rearranged lung adenocarcinomas are unresponsive to TKIs that target EGFR, however a novel TKI that targets ALK have shown dramatic clinical responses in an ongoing clinical trial. Therefore the accurate and timely identification of patients with ALK-rearranged lung adenocarcinomas is likely to be of therapeutic importance. We believe that IHC using the novel rabbit monoclonal antibody D5F3
is a preferred method for identifying ALK-rearranged lung adenocarcinomas in routine clinical practice.

### Statement of Translational Relevance

Approximately 5% of lung adenocarcinomas harbor the EML4-ALK gene fusion and emerging clinical data indicate that these tumors may be responsive to inhibitors that target ALK. However, it remains unclear whether all lung adenocarcinomas harboring an ALK rearrangement express mutant ALK protein. We demonstrate that ALK-rearranged lung adenocarcinomas invariably express ALK protein, but at much lower levels than in anaplastic large cell lymphoma. As a result, more than 30% of ALK-rearranged lung adenocarcinomas are not identified by standard immunohistochemistry (IHC) assays. Using a novel monoclonal antibody with increased sensitivity for ALK, we have developed an IHC assay that accurately identifies ALK-rearranged lung adenocarcinoma with high reproducibility, sensitivity, and specificity. This assay will facilitate the routine identification of ALK-rearranged lung adenocarcinomas in clinical practice and detect lung cancers that may be responsive to ALK inhibitors.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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### References


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Figure 1.
Immunohistochemical staining of FFPE tissues with D5F3 or D9E4 antibodies detect ALK protein expression in ALK-rearranged tumors. Cases of cytogenetically confirmed, ALK-rearranged anaplastic large cell lymphoma (A-C), ALK-germline anaplastic large cell lymphoma (D-F), and ALK-rearranged inflammatory myofibroblastic tumor (G-I) stained with antibodies D5F3 (A, D, G), D9E4 (B, E, H), and ALK1 (C, F, I).
Figure 2. Correlation in staining between D5F3 and ALK1 antibodies for anaplastic large cell lymphoma.

(A) Quantitative assessment of staining intensities for 19 tumors previously determined as ALK-positive or ALK-negative (by genetic testing and/or IHC) at the time of diagnosis (black bars = D5F3 antibody, grey bars = ALK1 antibody).

(B) Photomicrographs of Case #11 (i; ii) and Case #12 (iii; iv) stained with D5F3 antibody (i; iii) and ALK1 antibody (ii; iv). N.D. = not determined.
Figure 3.
Correlation in staining between D5F3 and ALK1 antibodies in lung adenocarcinoma. Quantitative assessment of staining intensities for 37 tumors determined to be ALK-rearranged (Cases 1–22) or ALK-germline (Cases 23–37) by FISH analysis. (A) Comparison of staining intensities using D5F3 antibody (black bars) or ALK1 antibody (grey bars). (B) Staining intensities using ALK1 antibody with the maximal level of background staining observed among the ALK-germline tumors indicated (horizontal dotted line) and the cases of ALK-rearranged tumors that fail to show staining over this background level (asterisks). (C) Staining intensities using D5F3 antibody with the maximal level of background staining observed among the ALK-germline tumors indicated (horizontal dotted line).
Figure 4.
Photomicrographs of lung adenocarcinomas stained with D5F3 and ALK1 antibodies and analyzed by FISH. (A, B, C) Case #1, (D, E, F) Case #2, (G, H, I) Case #22, and (J, K, L) Case #24 stained with D5F3 antibody (A, D, G, J), ALK1 antibody (B, E, H, K), or analyzed by FISH (C, F, I, L; red arrow= split red-green signals indicative of ALK-rearrangement, yellow arrow= touching red-green signals not indicative of ALK-rearrangement).
Figure 5.
Multiple biopsy samples stained with D5F3 antibody from a patient with an ALK-rearranged lung adenocarcinoma. Two biopsies (A, B) show faint, but definitive cytoplasmic staining, one biopsy (C) shows equivocal staining, and one biopsy (D) shows no staining for ALK protein. The inset in D demonstrates split red-green signals indicative of ALK-rearrangement by FISH.
**Table 1**

Interpretation of IHC staining on lung adenocarcinomas by three pathologists

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* Of the pathologists' IHC interpretation as positive staining in predicting an ALK-rearrangement