Cancer Science
The official journal of the Japanese Cancer Association

Case Report

Favorable response to crizotinib in three patients with echinoderm microtubule-associated protein-like 4–anaplastic lymphoma kinase fusion-type oncogene-positive non-small cell lung cancer

Takashi Kijima,[1,3] Kengo Takeuchi,[2] Satoshi Tetsumoto,[1] Kazuki Shimada,[1] Ryo Takahashi,[1] Haruhiko Hirata,[1] Izumi Nagatomo,[1] Shigenori Hoshino,[1] Yoshito Takeda,[1] Hiroshi Kida,[1] Sho Goya,[1] Isao Tachibana[1] and Ichiro Kawase[1]

[1]Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, Osaka; [2]Pathology Project for Molecular Targets, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan

(Received March 7, 2011/Revised April 21, 2011/Accepted April 21, 2011)

The echinoderm microtubule-associated protein-like 4 (EML4)–anaplastic lymphoma kinase (ALK) is a recently identified fusion-type oncoprotein that exists in approximately 5% of non-small cell lung cancer (NSCLC). It has been demonstrated that NSCLC driven by EML4-ALK is strongly addicted to this fusion-type oncokinase. A clinical trial of crizotinib (PF-02341066) sponsored by Pfizer has proven this oncogene addiction in humans by demonstrating a high response rate to inhibition of ALK kinase activity. In the present study, we report on three cases harboring *EML4-ALK* rearrangement who were enrolled in the trial (A8081001, NCT00585195). All three patients showed favorable responses to the ALK-specific tyrosine kinase inhibitor. (*Cancer Sci* 2011; 102: 1602–1604)

Case report

Clinical findings in three patients treated with crizotinib are described below.

Case 1. A 30-year-old man who had never smoked presented with smoldering pneumonia in October 2008. A diagnosis of Stage IIIB (cT2N3M0) lung adenocarcinoma was made and the patient subsequently received two cycles of systemic chemotherapy with cisplatin and vindesine, with concurrent thoracic irradiation, followed by two cycles of consolidation with carboplatin and paclitaxel. This treatment regimen resulted in stable disease. The primary tumor did not have epidermal growth factor receptor (*EGFR*) mutations, but immunohistochemistry (IHC) was positive for anaplastic lymphoma kinase (ALK) protein using the intercalated antibody-enhanced polymer (iAEP) method with 5A4 (Abcam, Cambridge, UK) as the primary antibody (Fig. 1a).[1] Fluorescence *in situ* hybridization (FISH), using probes described elsewhere,[2] confirmed echinoderm microtubule-associated protein-like 4 (*EML4*)–*ALK* rearrangement (Fig. 1b). Multiplex RT-PCR[1,3] further showed that this patient's fusion type was variant 3 (Fig. 1c). The patient was enrolled in the trial in April 2009.[4] The patient's persistent cough disappeared within 2 weeks of initiation of oral crizotinib therapy (250 mg twice daily) and the primary tumor shrank. Although there was no change in the size of the mediastinal lymph nodes, PET CT demonstrated marked reductions in the accumulation of fluorodeoxyglucose (^{18}F) after 12 months (Fig. 1d). Transient mild nausea and diarrhea were observed for the first 4 weeks of treatment, but were well controlled over the subsequent 19 months without any other toxicity. Follow-up MRI of the brain in November 2010 showed the appearance of asymptomatic but multiple new lesions, despite favorable control outside the central nervous system (CNS). The patient received whole-brain radiotherapy in December 2010; thereafter, crizotinib was resumed after a 4-week break. The patient is currently continuing crizotinib treatment for 24 months in total.

Case 2. A 29-year-old man who was a heavy smoker presented with progressive lumbago in May 2009. Shortly thereafter, sudden onset symmetrical leg paralysis developed. Because MRI revealed tumors in multiple segments of the thoracic and lumbar spine, emergent radiotherapy to the 10th–12th thoracic vertebrae was performed. This patient's disease was diagnosed as Stage IV (cT2N3M1) lung adenocarcinoma with single brain and multiple bone metastases. Tests for *EGFR* mutations were negative and the patient was given two cycles of systemic chemotherapy with cisplatin and vinorelbine. After insertion of a cyber knife into the brain lesion, the patient was given a cycle of carboplatin and paclitaxel, resulting in stable disease. On the evidence of ALK-positive iAEP IHC (Fig. 2a) with confirmatory FISH-positive *EML4-ALK* rearrangement, oral crizotinib therapy (250 mg twice daily) was started in September 2009. All tumors except for the brain lesion shrank significantly and the patient was able to cease opioid within 1 month. Mild nausea, alternating diarrhea and constipation, and the persistence of light spectrum image bothered the patient for a few months and dysgeusia lasted throughout the duration of treatment, but these adverse events were not so severe as to diminish the patient's activities of daily living. After 6 months, a chest CT showed complete regression of the thoracic lesions and bone scintigraphy showed a marked reduction in the accumulation of ^{88m}Tc (Fig. 2b). The brain lesion remained stable and other pre-existing tumors disappeared for 9 months. However, multiple new lesions appeared in the liver and crizotinib therapy was therefore discontinued. Thereafter, the patient received four cycles of carboplatin, pemetrexed, and bevacizumab and is now being followed-up with the latter two agents being used as maintenance therapy.

Case 3. A 31-year-old man who was a light smoker presented with an asymptomatic abnormal shadow on chest X-ray found as part of the annual medical checkup in August

[3]To whom correspondence should be addressed.
E-mail: tkijima@imed3.med.osaka-u.ac.jp

doi: 10.1111/j.1349-7006.2011.01970.x

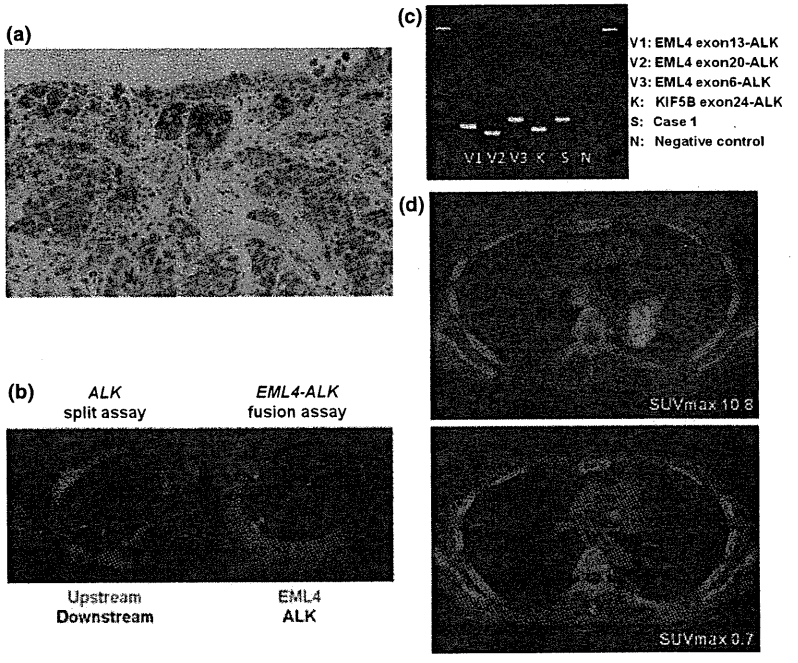

Fig. 1. (a) Intercalated antibody-enhanced polymer immunohistochemistry showing anaplastic lymphoma kinase (ALK) protein expression in acinar-type adenocarcinoma in Case 1. (b) Fluorescence *in situ* hybridization confirmed the *EML4-ALK* fusion gene. The ALK split assay was performed by labeling with probes for the upstream (green) and downstream (red) region of the *ALK* locus. The *EML4-ALK* fusion assay was performed by labeling with probes for *EML4* (green) and *ALK* (red). (c) Multiplex RT-PCR demonstrated the presence of *EML4-ALK* variant (V) 3. (d) Scans (PET CT) before (upper) and 12 months after (lower) crizotinib treatment. Accumulation of fluorodeoxyglucose (^{18}F) decreased markedly with maximum standardized uptake value (SUVmax) decreasing from 10.8 to 0.7. EML4, echinoderm microtubule-associated protein-like 4.

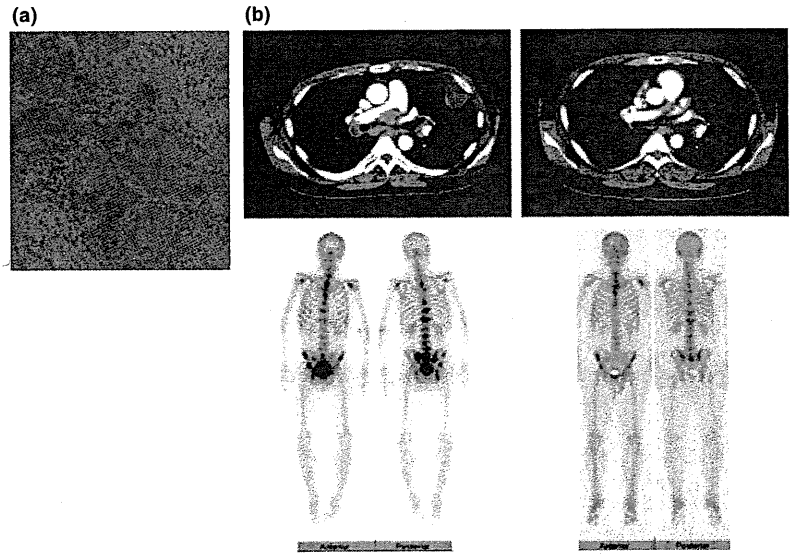

Fig. 2. (a) Intercalated antibody-enhanced polymer immunohistochemistry showing anaplastic lymphoma kinase protein expression in acinar-type adenocarcinoma in Case 2. (b) Chest CT and bone scintigraphy before (left) and 6 months after (right) crizotinib treatment. The tumors in the thorax almost disappeared and accumulation of ^{88m}Tc in multiple bones also decreased markedly.

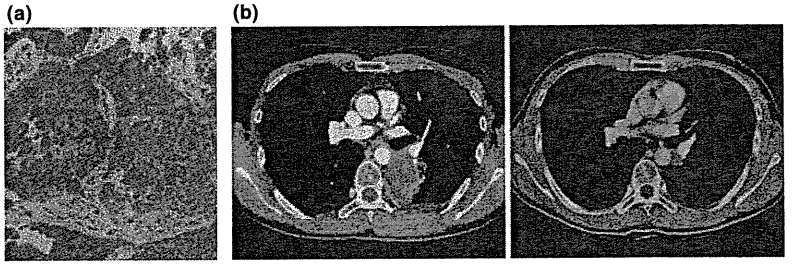

Fig. 3. (a) Intercalated antibody-enhanced polymer immunohistochemistry showing anaplastic lymphoma kinase protein expression in acinar-type adenocarcinoma in Case 3. (b) Chest CT before (left) and 12 months after (right) crizotinib treatment. Left pleural effusion disappeared and the primary tumor in the S6 segment shrank significantly.

2009. This patient's disease was diagnosed as Stage IIIB (cT4N0M0) lung adenocarcinoma with pleural dissemination. Chemoradiotherapy with cisplatin and vinorelbine failed after one cycle. Because tests for *EGFR* mutations were negative, but iAEP IHC (Fig. 3a) and FISH analyses revealed his tumor to be positive for *EML4-ALK*, the patient was started on oral crizotinib (250 mg twice daily) in December 2009. He achieved partial response shortly thereafter and the favorable

effect has lasted thus far for 17 months (Fig. 3b). The patient is currently continuing on crizotinib treatment. As for adverse effects, mild nausea and diarrhea were noted temporarily for the first 4 weeks of treatment, but persistence of vision spectrum and dysgeusia have lasted for the duration of the treatment period.

Discussion

The malignant transformation of approximately 5% of non-small cell lung cancer (NSCLC), especially adenocarcinoma, is caused by EML4-ALK and it is mutually exclusive for *EGFR* and *KRAS* mutations.[3,5–7] Basic research has shown that *EML4-ALK*-driven NSCLC is strongly addicted to this fusion-type oncogene.[5,8–10] Crizotinib is an experimental agent that targets ALK and mesenchymal-epithelial transition (MET) kinases and it is not currently approved by any regulatory agency. Pfizer's trial of crizotinib has demonstrated a high response rate (57%) in the *ALK* fusion-positive NSCLC population, indicating that these tumors have strong addiction exclusive to this oncokinase.[4] The three patients reported herein were all treated as part of the Pfizer trial after they had met the eligibility criteria and provided informed consent. All three patients showed a substantial responses to crizotinib. Although a minority of NSCLC arises from the *ALK* fusion-type oncokinase, most of ALK-positive NSCLC occur in younger people.[7] Thus, it is encouraging that ALK inhibitors will become available for use in the clinical setting. Moreover, crizotinib is also attractive as a potent "pain killer" because it was so effective against the pain produced by the bone metastases in Case 2 as to completely relieve the patient's cancer-related pain.

As for safety, temporary Grade 1 nausea and diarrhea were observed in all three patients and Grade 1 constipation coexisted in Case 2. Grade 1 dysgeusia of hot taste and visual disturbances (transient persistence of vision spectrum noted on moving from the dark to the light) occurred in Cases 2 and 3. These adverse events, except for dysgeusia, were reported in the trial[4] and all were tolerable. Crizotinib did not affect liver function, renal function, or blood cell counts in the three cases reported here.

Some responders (including two cases in the present study) develop resistance to crizotinib after a certain period, similar to most responders to EGFR tyrosine kinase inhibitors. This is a serious issue not to be overlooked. Based on the occurrence (Case 1) and unresponsiveness (Case 2) of brain metastases, crizotinib may not fully penetrate from the bloodstream into the CNS at the dose given in this trial. Costa *et al.*[11] recently reported a similar case to Case 1 in the present study, in whom the concentration of crizotinib was much lower in the cerebrospinal fluid (CSF) than in the plasma (0.0014 *vs.* 0.53 mol/L, respectively). Costa *et al.*[11] suggested that the low CSF:plasma ratio (0.0026) implied poor blood–brain barrier penetration of crizotinib, which may explain the persistent systemic disease control with crizotinib but the concurrent appearance of brain metastases.

Furthermore, two *de novo* mutations in the tyrosine kinase domain of *ALK* cDNA (G4374A and C4493A) have been identified recently from a patient who showed resistance to crizotinib.[12] These mutations result in substitutions of cysteine to tyrosine (C → Y) and leucine to methionine (L → M) at positions corresponding to amino acids 1156 and 1196, respectively. The L1196 of ALK corresponds to threonine (T) at position T315 in ABL and T790 in EGFR, the most frequently substituted position conferring resistance to inhibitors for these kinases. Conversely, C1156Y located adjacent to the αC helix on the N-terminal side has been proposed to allosterically hinder the binding of ALK inhibitors.[12] Such resistant mutations may provide another explanation for the lack of a response of brain metastases to crizotinib. Regarding the hepatic metastases in Case 2, it is unlikely that the hepatocyte growth factor/MET pathway or *MET* amplification (known resistant mechanisms for EGFR inhibitors) participated in this setting because crizotinib is a dual inhibitor for both ALK and MET kinases. We must investigate the mechanisms underlying the resistance to crizotinib to develop new treatment strategies, as well as second-generation ALK inhibitors, to overcome resistance.

Acknowledgments

The authors thank the three patients for consenting to the publication of their clinical details. The authors are also grateful to Dr Yung-Jue Bang and the medical staff of Seoul National University Hospital (Seoul, Korea) for their support in the treatment of these patients.

Disclosure Statement

KT has acted in an advisory role for Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). The other authors report no potential conflicts of interest. None of the authors was an investigator in the trial conducted by Pfizer.

References

1 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**: 3143–9.

2 Sakairi Y, Nakajima T, Yasufuku K *et al.* EML4-ALK fusion gene assessment using metastatic lymph node samples obtained by endobronchial ultrasound-guided transbronchial needle aspiration. *Clin Cancer Res* 2010; **16**: 4938–45.

3 Takeuchi K, Choi YL, Soda M *et al.* Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. *Clin Cancer Res* 2008; **14**: 6618–24.

4 Kwak EL, Bang YJ, Camidge DR *et al.* Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010; **363**: 1693–703.

5 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**: 561–6.

6 Inamura K, Takeuchi K, Togashi Y *et al.* EML4-ALK fusion is linked to histological characteristics in a subset of lung cancers. *J Thorac Oncol* 2008; **3**: 13–7.

7 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**: 508–15.

8 Soda M, Takada S, Takeuchi K *et al.* A mouse model for EML4-ALK-positive lung cancer. *Proc Natl Acad Sci USA* 2008; **105**: 19893–7.

9 Choi YL, Takeuchi K, Soda M *et al.* Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. *Cancer Res* 2008; **68**: 4971–6.

10 Koivunen JP, Mermel C, Zejnullahu K *et al.* EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. *Clin Cancer Res* 2008; **14**: 4275–83.

11 Costa DB, Kobayashi S, Pandya SS *et al.* CSF Concentration of the anaplastic lymphoma kinase inhibitor crizotinib. *J Clin Oncol* 2011; doi:10.1200/JCO.2010.34.1313 [Epub ahead of print].

12 Choi YL, Soda M, Yamashita Y *et al.* EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med* 2010; **363**: 1734–9.

doi: 10.1111/j.1349-7006.2011.01970.x

Published OnlineFirst March 23, 2011; DOI:10.1158/1078-0432.CCR-11-0063

Clinical Cancer Research

Pulmonary Inflammatory Myofibroblastic Tumor Expressing a Novel Fusion, PPFIBP1 –ALK: Reappraisal of Anti-ALK Immunohistochemistry as a Tool for Novel ALK Fusion Identification

Kengo Takeuchi, Manabu Soda, Yuki Togashi, et al.

Clin Cancer Res 2011;17:3341-3348. Published OnlineFirst March 23, 2011.

Published OnlineFirst March 23, 2011; DOI:10.1158/1078-0432.CCR-11-0063

Clinical Cancer Research

Imaging, Diagnosis, Prognosis

Pulmonary Inflammatory Myofibroblastic Tumor Expressing a Novel Fusion, PPFIBP1–ALK: Reappraisal of Anti-ALK Immunohistochemistry as a Tool for Novel ALK Fusion Identification

Kengo Takeuchi[1,2], Manabu Soda[4], Yuki Togashi[1,2], Emiko Sugawara[1,5], Satoko Hatano[1,2], Reimi Asaka[1,2], Sakae Okumura[3], Ken Nakagawa[3], Hiroyuki Mano[4,6], and Yuichi Ishikawa[2]

Abstract

Purpose: The anaplastic lymphoma kinase (ALK) inhibitor crizotinib has been used in patients with lung cancer or inflammatory myofibroblastic tumor (IMT), both types harboring ALK fusions. However, detection of some ALK fusions is problematic with conventional anti-ALK immunohistochemistry because of their low expression. By using sensitive immunohistochemistry, therefore, we reassessed "ALK-negative" IMT cases defined with conventional immunohistochemistry (approximately 50% of all examined cases).

Experimental Design: Two cases of ALK-negative IMT defined with conventional anti-ALK immunohistochemistry were further analyzed with sensitive immunohistochemistry [the intercalated antibody-enhanced polymer (iAEP) method].

Results: The two "ALK-negative" IMTs were found positive for anti-ALK immunohistochemistry with the iAEP method. 5′-rapid amplification of cDNA ends identified a novel partner of ALK fusion, protein-tyrosine phosphatase, receptor-type, F polypeptide-interacting protein-binding protein 1 (PPFIBP1) in one case. The presence of PPFIBP1–ALK fusion was confirmed with reverse transcriptase PCR, genomic PCR, and FISH. We confirmed the transforming activities of PPFIBP1–ALK with a focus formation assay and an *in vivo* tumorigenicity assay by using 3T3 fibroblasts infected with a recombinant retrovirus encoding PPFIBP1–ALK. Surprisingly, the fusion was also detected by FISH in the other case.

Conclusions: Sensitive immunohistochemical methods such as iAEP will broaden the potential value of immunohistochemistry. The current ALK positivity rate in IMT should be reassessed with a more highly sensitive method such as iAEP to accurately identify those patients who might benefit from ALK-inhibitor therapies. Novel ALK fusions are being identified in various tumors in addition to IMT, and thus a reassessment of other "ALK-negative" cancers may be required in the forthcoming era of ALK-inhibitor therapy. *Clin Cancer Res; 17(10); 3341–8. ©2011 AACR.*

Introduction

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that was discovered in anaplastic large cell lymphoma (ALCL) in the form of a fusion protein, NPM–ALK. (1, 2). In addition to ALCL (fused to NPM, TPM3, TPM4, ATIC, TFG, CLTC, MSN, MYH9, or ALO17; refs. 1–10), ALK has further been found to generate fusions in inflammatory myofibroblastic tumor (IMT; TPM3, TPM4, CLTC, CARS, RANBP2, ATIC, or SEC31L1; refs. 10–15), ALK-positive large B-cell lymphoma (CLTC, NPM, SEC31L1, or SQSTM1; 16–19), lung cancer (EML4 or KIF5B; refs. 20, 21), and ALK-positive histiocytosis (TPM3; ref. 22) Besides, some ALK fusions have been reported without showing histopathologic evidence: TPM4–ALK in esophageal squamous cell carcinoma (23, 24), TFG–ALK in lung adenocarcinoma (25), and EML4–ALK in colon and breast carcinomas (26). The wild-type ALK is mainly expressed in the developing nervous system, and is usually not expressed in other normal tissues (27). A fusion protein formation with a partner through chromosomal translocations is the most common mechanism of ALK overexpression and ALK kinase domain activation. These features render ALK fusion oncokinase an ideal molecular target.

Recently, the ALK inhibitor crizotinib has been used in patients with lung cancer or IMT, both types harboring ALK fusions (28, 29). The compound showed a 57% response rate in lung cancers (28), and a strong response for several months in IMT (29). Crizotinib and other ALK inhibitors

Authors' Affiliations: [1]Pathology Project for Molecular Targets; [2]Division of Pathology; and [3]Department of Thoracic Surgical Oncology, Thoracic Center, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo; [4]Division of Functional Genomics, Jichi Medical University, Tochigi; [5]Department of Comprehensive Pathology, Graduate School, Tokyo Medical and Dental University; and [6]Department of Medical Genomics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Kengo Takeuchi, Pathology Project for Molecular Targets, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo 135-8550, Japan. Phone: +8133-520-0111; Fax: +8133-570-0230; E-mail: kentakeuchi-tky@umin.net

doi: 10.1158/1078-0432.CCR-11-0063

Published OnlineFirst March 23, 2011; DOI:10.1158/1078-0432.CCR-11-0063

Translational Relevance

Anaplastic lymphoma kinase (ALK) inhibitors have become one of the most promising groups of molecularly targeted drugs. Therefore, ALK is no longer a mere research target or simply a diagnostic marker, but is directly linked to the therapeutic benefit of patients harboring the fusions.

Pathologic diagnoses for ALK fusion-positive tumors have been made reliably with anti-ALK immunohistochemistry. Since the discovery of EML4–ALK, however, an unexpected problem in anti-ALK immunohistochemistry has become apparent, that is, the inability to detect a low level of EML4–ALK expression. To overcome this, we developed the intercalated antibody-enhanced polymer immunohistochemistry, which successfully detected EML4–ALK.

In other words, this indicates that unknown ALK fusions, particularly those expressed at a low level, may wait to be discovered in "ALK-negative" tumors defined with conventional immunohistochemistry. In the forthcoming era of ALK-inhibitor therapy, "ALK-negative" tumors should be reassessed with a high sensitive immunohistochemistry and, if positive, be further examined with appropriate molecular method(s).

have thus become one of the most promising groups of molecularly targeted drugs. Therefore, the sensitive and accurate identification of ALK fusion in tumors has also become clinically relevant, because it is no longer a mere research target or simply a diagnostic marker, but is directly linked to the therapeutic benefit of patients harboring the fusions.

Identification of such ALK fusions, especially within ALCL, has been prompted by the immunohistochemical staining pattern with antibodies to ALK. In ALCL, the most common ALK fusion is NPM–ALK (comprising approximately 80% of all cases), and its immunohistochemical staining pattern is both nuclear and cytoplasmic. NPM has a nuclear localization signal in the C-terminal region, and therefore the heterodimers of wild-type NPM with NPM–ALK fusion protein are transported to the nucleus whereas NPM–ALK homodimers remain within the cytoplasm (30). In contrast, other fusions do not localize in the nucleus and do not show a nuclear staining pattern in anti-ALK immunohistochemistry. Interestingly, each ALK fusion usually has its own characteristic anti-ALK immunohistochemical staining pattern, because the subcellular localization of ALK fusions is dependent on the corresponding fusion partners. Anti-ALK immunohistochemistry has thus become a highly useful tool for both research and diagnostic purposes.

Since the discovery of EML4–ALK fusion in lung cancer (20), however, an unexpected problem in anti-ALK immunohistochemistry has become apparent, that is, the inability to detect a low level of fusion expression. To overcome this, we developed the intercalated antibody-enhanced polymer (iAEP) method, which moderately raises sensitivity in the immunohistochemical detection system (21). With this very simple method, anti-ALK immunohistochemistry has become a potent weapon in the diagnosis of EML4–ALK-positive lung cancer (21, 31–33). Other researchers used an anti-ALK rabbit monoclonal antibody, which is usually more sensitive than mouse monoclonal antibody, which can stain EML4–ALK (34). However, most EML4–ALK-positive lung cancer tissues do not stain well with conventional anti-ALK immunohistochemical methods because of the low message/protein level of EML4–ALK (21, 35). The expression level of a fusion gene depends on the promoter activity of the 5′-side gene, and that of EML4 is likely to be lower than that of the other ALK fusion partner genes, which may explain why EML4–ALK had not been discovered until 12 years after the development of the first anti-ALK antibody became available for immunohistochemistry (36). In other words, a tumor that immunostains for ALK only by a sensitive immunohistochemistry method may harbor a novel ALK fusion. Interestingly, in this study, we detected 2 IMT cases positive for ALK immunohistochemistry only when stained by iAEP method (21), and successfully identified a novel fusion gene, protein-tyrosine phosphatase, receptor-type, F polypeptide-interacting protein-binding protein 1 (PPFIBP1)–ALK.

Materials and Methods

Materials

Pathologic specimens from 2 pulmonary IMT cases, originally diagnosed as fibrous histiocytoma (1988: case 1, 45-year-old male; 1998: case 2, 34-year-old female), were reassessed morphologically and immunohistochemically. Surgically removed tumor specimens were routinely fixed in 20% neutralized formalin and embedded in paraffin for conventional histopathologic examination. For case 2, total RNA was extracted from the corresponding snap-frozen specimen and purified with the use of an RNeasy Mini kit (Qiagen). The study was approved by the institutional review board of the Japanese Foundation for Cancer Research.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was sliced at a thickness of 4 μm, and the sections were placed on silane-coated slides. For antigen retrieval, the slides were heated for 40 min at 97°C in Target Retrieval Solution (pH 9.0; Dako). For the conventional staining procedure, the slides were incubated at room temperature with Protein Block Serum-free Ready-to-Use solution (Dako) for 10 minutes and then with primary antibodies against ALK (5A4), smooth muscle actin, muscle-specific actin (HHF35), CD34, cytokeratins (AE1/AE3), S100, or desmin for 30 minutes. The immune complexes were then detected with dextran polymer reagent (EnVision + DAB system; Dako) and an AutoStainer instrument (Dako). The iAEP method was also used for the sensitive detection of ALK, as described previously (21).

Published OnlineFirst March 23, 2011; DOI:10.1158/1078-0432.CCR-11-0063

Isolation of PPFIBP1–ALK fusion

To obtain cDNA fragments corresponding to a novel *ALK* fusion gene, we used a 5′-RACE method with the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions, with a minor modification: the ALK2458R primer (5′-GTAGTTGGGGTTGTAGTCGGT-CATGATGGT-3′) was used as the gene-specific reverse primer.

From the oligo(dT)-primed cDNA obtained from case 2 RNA, a 471bp cDNA fragment containing the fusion point was specifically amplified with the primers PPFIBP1-592F (5′-AGAGACACAGAGGGGCTGATT-3′) and ALK3078RR (5′-ATCCAGTTCGTCCTGTTCAGAGC-3′).

PCR analysis of genomic DNA for *PPFIBP1–ALK* in case 2 was carried out with a pair of primers flanking the putative fusion point, PPFIBP1-607F (5′-CTGATTCAGGAGATCA-ATGATTTGAGGT-3′) and Fusion-RT-AS (5′-TCTTGCCAG-CAAAGCAGTAGTTGG-3′).

From the cDNA, a full-length cDNA for *PPFIBP1–ALK* was amplified by PCR with the PA-w-cDNA-in-S primer (5′-TATCTGGGTTGGAATTTGCCCCTG-3′) and the KA-w-cDNA-in-AS primer (5′-TGAGTGTGCGACCGAGCTCAGG-3′) and PrimeSTAR HS DNA polymerase (TakaraBio).

FISH

FISH analysis of gene fusion was carried out with bacterial artificial chromosome (BAC) clone-derived DNA probes for *ALK* and *PPFIBP1*. Unstained sections (4 μm thick) were subjected to hybridization with an *ALK*-split probe set (Abbott) or BAC clone-derived probes for *ALK* (RP11–984I21, RP11–62B19) and *PPFIBP1* (RP11–1060J15). Hybridized slides were then stained with DAPI and examined with the fluorescence microscope BX51 (Olympus).

Transformation assay for ALK fusion proteins

Analysis of the transforming activity of PPFIBP1–ALK was carried out as described previously (20, 37, 38). Briefly, the pMXS-based expression plasmid for PPFIBP1–ALK, EML4–ALK variant 1, or NPM–ALK was used to generate recombinant ecotropic retrovirus, followed by individual infection of mouse 3T3 fibroblasts (39). Formation of the transformed foci was evaluated after culturing the cells for 14 days. The same set of 3T3 cells was subcutaneously injected into nu/nu mice, and tumor formation was examined after 20 days. The animal experiments were approved by the animal ethics committee of Jichi Medical University.

Results

Morphology and immunophenotype of PPFIBP1–ALK-positive IMT

Histopathologic analysis of the 2 IMT cases revealed a marked proliferation of cells composed of somewhat histiocytoid spindle cells showing a fascicular or storiform pattern. The tumor cells were uniform and had pale eosinophilic cytoplasm and an oval vesicular nucleus, within which a small nucleolus was centrally located. Mild inflammatory infiltrate containing lymphocytes, plasma cells, foamy histiocytes, and multinucleated giant cells was observed (Fig. 1A and 1D). The immunophenotype of the 2 cases was negative for smooth muscle actin,

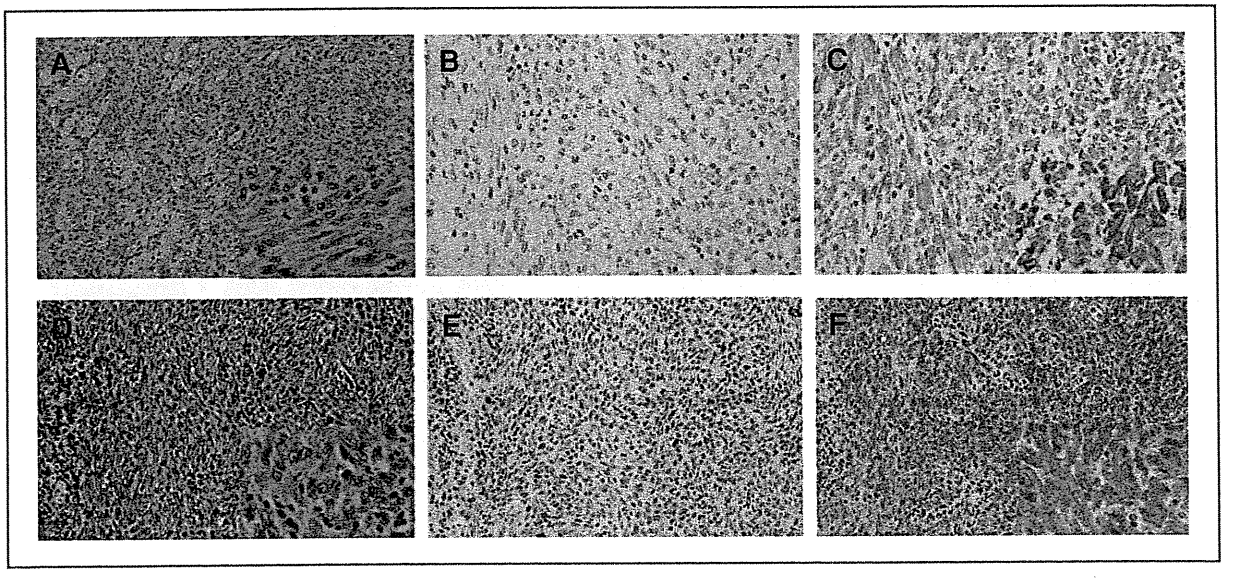

Figure 1. Histopathology of PPFIBP1–ALK-positive IMT. Diffuse proliferation of histiocytoid spindle cells showing a fascicular or storiform pattern. The tumor cells were uniform and had pale eosinophilic cytoplasm and an oval vesicular nucleus, within which a small nucleolus was centrally located. Mild inflammatory infiltrate containing lymphocytes, plasma cells, and foamy histiocytes is observed (A and D). The tumor cells were negative for ALK with conventional anti-ALK immunohistochemistry (B and E), but were clearly positive for ALK when the iAEP method was used. The staining pattern is diffuse cytoplasmic (C and F). Case 1 (A–C), Case 2 (D–F).

Published OnlineFirst March 23, 2011; DOI:10.1158/1078-0432.CCR-11-0063

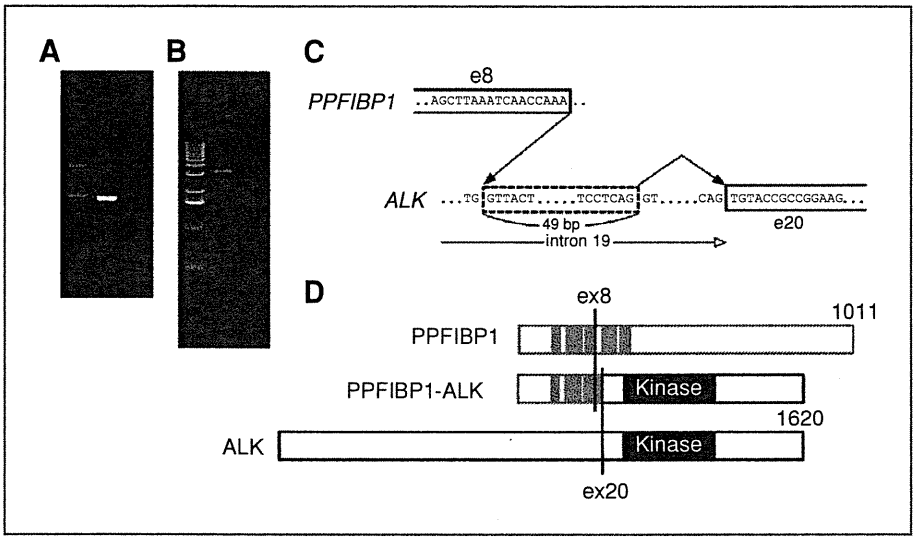

Figure 2. Identification of PPFIBP1–ALK: a PCR product of 471 bp covering the fusion point of *PPFIBP1–ALK* cDNA was specifically amplified from the tumor cells of case 2. The left lane contains DNA size standards (100 bp ladder). The right lane represents no template control (A). A PCR product of approximately 3 kbp covering the genomic fusion point of *PPFIBP1–ALK* was specifically amplified from the tumor cells of case 2. The left lane contains DNA size standards (1 kbp ladder). The right lane represents no template control (B). In our 5′-RACE products, exon 8 of *PPFIBP1* cDNA was fused to a 49 bp sequence in intron 19 of *ALK*, followed by exon 20 of *ALK* (C). PPFIBP1 contains 5 coiled-coil domains. A chromosome translocation, t(2;10)(p23;p11), generates a fusion protein in which the top 3 coiled-coil domains of PPFIBP1 and the intracellular region of ALK (containing the tyrosine kinase domain) are conserved. Numbers indicate amino acid positions of each protein (D).

HHF35, CD34, AE1/AE3, and S100. Desmin was focally positive in case 1, but was negative in case 2.

Identification of PPFIBP1–ALK as a novel ALK fusion gene

We conducted anti-ALK immunohistochemistry on 2 morphologically typical pulmonary IMT cases, originally diagnosed as fibrous histiocytoma. Immunostaining for ALK with the conventional polymer method led to the revised diagnosis of "ALK-negative" IMT (Fig. 1B and E). In the present study, anti-ALK immunohistochemistry with the iAEP method, however, showed a diffuse positive cytoplasmic staining (Fig. 1C and F), indicating the possibility of ALK fusion to a novel partner gene, the expression level of which is modest. To address this issue, in case 2 we conducted 5′-RACE assay for the isolation of an upstream cDNA to the ALK kinase domain cDNA, for which snap-frozen material was available.

Interestingly, we isolated a cDNA fragment containing exon 8 of *PPFIBP1* followed by a 49 bp-sequence within intron 19 of *ALK* and coupled to exon 20 of *ALK* (Fig. 2), suggesting the presence of a novel fusion between *PPFIBP1* and *ALK* genes. Because insertion of the intronic 49 bp allows an in-frame fusion between the 2 genes, this rearrangement likely produces a novel fusion-type tyrosine kinase. To confirm the genomic rearrangement responsible for the *PPFIBP1–ALK* fusion, a genomic PCR assay (Fig. 2B) and both *ALK* split and *PPFIBP1–ALK* fusion FISH assays (Fig. 3) were carried out. All results were consistent with the presence of t(2;12)(p23;p11) leading to the generation of *PPFIBP1–ALK*. Owing to the limited material available in case 1, only the FISH analyses were carried out. Surprisingly, these results also indicate the presence of *PPFIBP1–ALK* (Fig. 3, Supplementary Fig. 2A–C).

Transforming activities of PPFIBP1–ALK

To prove that the t(2;12)(p23;p11) rearrangement leads to the production of PPFIBP1–ALK kinase, in case 2 we attempted to amplify from the cDNA a full-length cDNA encoding the protein. By using a sense primer at the 5′-untranslated region of *PPFIBP1* mRNA (GenBank accession no. NM_003622) and an antisense primer at

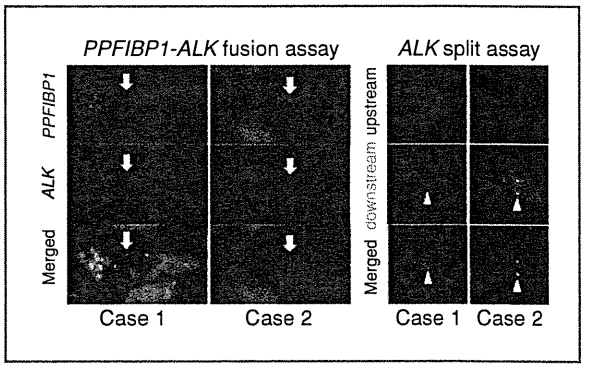

Figure 3. FISH analyses for *PPFIBP1–ALK*: sections of tumors positive for PPFIBP1–ALK were subjected to FISH analyses. In *PPFIBP1–ALK* fusion assays (left) the fusion genes are indicated by arrows. In *ALK* split assays (right) the 3′-sides of *ALK* are indicated by arrowheads. The color of fluorescence for the BAC clones and the case numbers in each hybridization are indicated. Nuclei are stained blue with DAPI.

Published OnlineFirst March 23, 2011; DOI:10.1158/1078-0432.CCR-11-0063

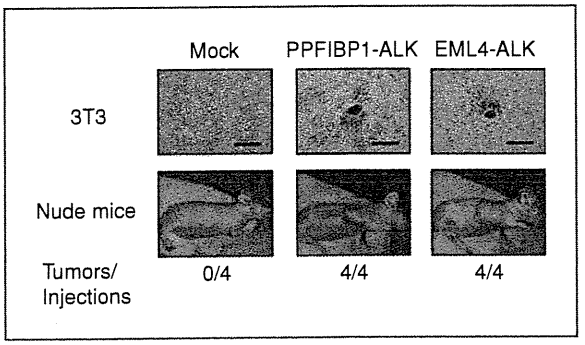

Figure 4. Transforming potential of PPFIBP1–ALK. Top, mouse 3T3 fibroblasts were infected with retroviruses encoding PPFIBP1–ALK or EML4–ALK or with the corresponding empty virus (Mock). The cells were photographed after 14 days of culture. Scale bars, 400 μm. Bottom, Nude mice were injected subcutaneously with the corresponding 3T3 cells, and tumor formation was examined after 14 days. The number of tumors formed per 4 injections is indicated at the bottom.

the 3′-untranslated region of *ALK* mRNA (GenBank accession no. NM_004304), a full-length *PPFIBP1–ALK* cDNA of 2488 bp was successfully amplified, which should have produced a fusion kinase of 811 amino acids with a predicted molecular weight of 90,740 Da (Supplementary Fig. 1).

To examine the transforming potential of PPFIBP1–ALK, a recombinant ecotropic retrovirus was generated to express PPFIBP1–ALK, which was used to infect mouse 3T3 fibroblasts. As shown in Figure 4, PPFIBP1–ALK produced hundreds of transformed foci over 14 days of culture, which was comparable with the observation with EML4–ALK. Furthermore, subcutaneous injection of the infected 3T3 cells into the shoulder of nude mice revealed that those expressing either PPFIBP1–ALK or EML4–ALK formed large tumors *in vivo*.

Discussion

Since their discovery in 1994, appropriate diagnosis of ALK fusion-positive tumors with conventional anti-ALK immunohistochemistry methods has been accepted. However, EML4–ALK in lung adenocarcinoma, identified in 2007, did not stain positive for ALK with conventional immunohistochemistry methods (21, 35). We developed a sensitive immunohistochemistry method, the iAEP method, and successfully stained EML4–ALK with ordinary anti-ALK mouse monoclonal antibodies (21, 31–33). Such observation further indicates a possibility that staining cancer specimens with sensitive immunohistochemical methods (such as iAEP) may detect novel ALK fusions in the "ALK-negative" tumors defined by conventional anti-ALK immunohistochemistry methods. On the basis of this hypothesis, we have identified a novel ALK fusion in "ALK-negative" IMT.

Caution is needed in practical settings. For example, rhabdomyosarcoma, especially of the alveolar type, often expresses wild-type ALK at a detectable level with conventional anti-ALK immunohistochemistry (40). Moreover, in our experience, a small portion of small cell carcinoma and large cell endocrine carcinoma of the lung, and some sarcomas, may be positive for ALK by iAEP immunohistochemistry, expressing wild-type ALK. Therefore, in order to specifically detect ALK fusions with sensitive anti-ALK immunohistochemistry, a confirmatory test by using FISH, RT-PCR, or similar is usually required. If a tumor is positive for a confirmatory test and the suspected partner gene is not a reported one, 5′-RACE or inverse reverse transcriptase PCR methods can be used for the identification of the suspected partner. Even if overexpressed, wild-type ALK may not be oncogenic (20, 21, 37, 38), although some investigators have suggested that wild-type ALK overexpression above a certain threshold level drives the growth of neuroblastoma (41). Further investigation will be required to clarify if wild-type ALK overexpression is a target for ALK inhibitor therapy.

IMT is a rare mesenchymal tumor that has been referred to as inflammatory pseudotumor, plasma cell granuloma, fibroxanthoma, fibrous histiocytoma, pseudosarcomatous myofibroblastic tumor, and invasive fibrous tumor of the tracheobronchial tree (42). It occurs in the soft tissues as well as in the viscera and the lung, and is more likely to occur in children and young adults. Histologically, IMT is composed of a variable admixture of bland, spindle-shaped myofibroblast-like cells and an inflammatory component of lymphocytes, eosinophils, plasma cells, and macrophages. Recent genetic studies have elucidated clonal chromosomal abnormality involving 2p23, at which ALK is located, in a subset of IMT. The expression of ALK fusion proteins is detected by anti-ALK immunohistochemistry in approximately 50% of IMT cases (42), in which various ALK fusion genes have been reported (Table 1). Collectively, these lines of evidence support ALK-positive IMT being a distinct neoplastic entity. However, the other 50% of IMT cases are negative for anti-ALK immunohistochemistry, and thus in terms of pathogenesis it remains unknown whether these ALK-negative IMTs should be included in the same entity or not. In fact, 1 ALK-negative IMT case did not respond to crizotinib therapy (29). However, we have detected a novel ALK-fusion in "ALK-negative" IMT that subsequently proved positive for ALK with the iAEP immunohistochemistry method. Therefore, unexpectedly lowly expressed ALK fusions may explain the pathogenesis of a portion of "ALK-negative" IMT cases. PPFIBP1–ALK represents such an ALK fusion, although we do not yet know what proportion of "ALK-negative" IMTs can be attributed to this novel subtype. "ALK-negative" IMT warrants screening with the iAEP method to detect this fusion or other, unrecognized, ALK fusions.

PPFIBP1 codes liprin beta 1 (also called PTPRF-interacting protein-binding protein 1). This 114 kDa protein is a member of the leukocyte common antigen–related (LAR) transmembrane tyrosine phosphatase-interacting protein family that may regulate LAR protein properties via interaction with another member of the family, liprin alpha1 (43). Liprin beta 1 expresses in intestinal lymphatic endothelial cells *in vitro* and lymphatic vasculature *in vivo*,

Published OnlineFirst March 23, 2011; DOI:10.1158/1078-0432.CCR-11-0063

Table 1. ALK fusion partners in well-documented IMT cases

Partner	Locus	Age	Sex	Site	Year, First author
TPM3	1p23	30	F	Lung	2000, Lawrence
		23	F	Abdomen	2000, Lawrence
		4	M	Lung	2006, Yamamoto
		29	F	Ileum	2006, Milne
		4	M	Lung	2007, Kinoshita
TPM4	19p13	1	M	Abdomen	2000, Lawrence
		6	M	Mesentery	2003, Hisaoka
		25	M	Prostate	2003, Hisaoka
		5	M	Mesentery	2006, Yamamoto
		5	F	Urinary bladder	2006, Yamamoto
CLTC	17q23	3	F	Neck	2001, Bridge
		37	M	Pelvis	2001, Bridge
		2	M	Thoracic cavity	2006, Yamamoto
		6	M	Mesentery	2006, Yamamoto
		0	F	Mediastinum	2007, Patel
CARS	11p15	0	M	Abdomen	2002, Cools
		10	M	Neck	2003, Debelenko
RANBP2	2q13	7	M	Abdomen	2003, Ma
		0	M	Abdomen	2003, Ma
		2	M	Abdomen	2007, Patel
		34	M	Liver	2008, Chen
		44	M	Abdomen	2010, Butrynski
ATIC	2q35	46	M	Urinary bladder	2003, Debiec-Rychter
SEC31L1	4q21	23	M	Abdomen	2006, Panagopoulos
PPFIBP1	12p11	45	M	Lung	Present case 1
		34	F	Lung	Present case 2

and plays an important role in the maintenance of lymphatic vessel integrity in *Xenopus* tadpoles (44). PPFIBP1 has 5 coiled-coil domains in exons 5 through 12, and the upper 3 domains are conserved in fusion form with ALK (Fig. 2D). The coiled-coil domain is shared in all ALK fusion partners (except for NPM, MSN, and SQSTM1), with which the ALK fusion proteins homodimerize leading to constitutive activation of ALK kinase domains (8, 19). As expected, in the present study, the oncogenicity of PPFIBP1–ALK was clearly confirmed with an *in vitro* focus formation assay and an *in vivo* tumorigenicity assay.

The difference in subcellular localization has contributed to the discovery/identification of various ALK fusions. Likewise, the difference in the expression level found is here proved important in the accurate detection of fusion proteins. Sensitive immunohistochemical methods such as iAEP will broaden the potential value of immunohistochemistry, which is a simple and long-established histopathologic technique in the fields of research and diagnosis. The ALK positivity rate (approximately 50%) in IMT should be reassessed with these more sensitive methods, possibly leading to the identification of novel ALK fusions and more candidates for ALK inhibitor therapy. A novel ALK fusion, VCL–ALK, has recently been identified in renal cancers (45, 46). In addition to IMT, therefore, a reassessment of diverse "ALK-negative" human cancers may be required in the forthcoming era of ALK inhibitor therapy.

Disclosure of Potential Conflicts of Interest

K. Takeuchi, scientific advisor for developing an anti-ALK iAEP immunohistochemistry kit (ALK Detection Kit, Nichirei Bioscience, Japan) and in charge of pathology screening for ALK fusions using the immunohistochemistry kit and an original probe set for ALK split FISH assay in a clinical trial of an ALK inhibitor (AF802, Chugai, Japan). The other authors disclosed no potential conflicts of interest.

Grant Support

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan as well as by grants from the Japan Society for the Promotion of Science; the Ministry of Health, Labor, and Welfare of Japan; the Vehicle Racing Commemorative Foundation of Japan; Princess Takamatsu Cancer Research Fund; and the Uehara Memorial Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 9, 2011; revised March 7, 2011; accepted March 15, 2011; published OnlineFirst March 23, 2011.

Published OnlineFirst March 23, 2011; DOI:10.1158/1078-0432.CCR-11-0063

References

1. Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science 1994; 263:1281–4.
2. Shiota M, Fujimoto J, Semba T, Satoh H, Yamamoto T, Mori S. Hyperphosphorylation of a novel 80 kDa protein-tyrosine kinase similar to Ltk in a human Ki-1 lymphoma cell line, AMS3. Oncogene 1994;9:1567–74.
3. Lamant L, Dastugue N, Pulford K, Delsol G, Mariame B. A new fusion gene TPM3-ALK in anaplastic large cell lymphoma created by a (1;2)(q25;p23) translocation. Blood 1999;93:3088–95.
4. Meech SJ, McGavran L, Odom LF, Liang X, Meltesen L, Gump J, et al. Unusual childhood extramedullary hematologic malignancy with natural killer cell properties that contains tropomyosin 4–anaplastic lymphoma kinase gene fusion. Blood 2001;98:1209–16.
5. Colleoni GW, Bridge JA, Garicochea B, Liu J, Filippa DA, Ladanyi M. ATIC-ALK: A novel variant ALK gene fusion in anaplastic large cell lymphoma resulting from the recurrent cryptic chromosomal inversion, inv(2)(p23q35). Am J Pathol 2000;156:781–9.
6. Hernández L, Pinyol M, Hernández S, Beà S, Pulford K, Rosenwald A, et al. TRK-fused gene (TFG) is a new partner of ALK in anaplastic large cell lymphoma producing two structurally different TFG-ALK translocations. Blood 1999;94:3265–8.
7. Touriol C, Greenland C, Lamant L, Pulford K, Bernard F, Rousset T, et al. Further demonstration of the diversity of chromosomal changes involving 2p23 in ALK-positive lymphoma: 2 cases expressing ALK kinase fused to CLTCL (clathrin chain polypeptide-like). Blood 2000;95:3204–7.
8. Tort F, Pinyol M, Pulford K, Roncador G, Hernandez L, Nayach I, et al. Molecular characterization of a new ALK translocation involving moesin (MSN-ALK) in anaplastic large cell lymphoma. Lab Invest 2001;81:419–26.
9. Lamant L, Gascoyne RD, Duplantier MM, Armstrong F, Raghab A, Chhanabhai M, et al. Non-muscle myosin heavy chain (MYH9): a new partner fused to ALK in anaplastic large cell lymphoma. Genes Chromosomes Cancer 2003;37:427–32.
10. Cools J, Wlodarska I, Somers R, Mentens N, Pedeutour F, Maes B, et al. Identification of novel fusion partners of ALK, the anaplastic lymphoma kinase, in anaplastic large-cell lymphoma and inflammatory myofibroblastic tumor. Genes Chromosomes Cancer 2002;34: 354–62.
11. Lawrence B, Perez-Atayde A, Hibbard MK, Rubin BP, Dal Cin P, Pinkus JL, et al. TPM3-ALK and TPM4-ALK oncogenes in inflammatory myofibroblastic tumors. Am J Pathol 2000;157:377–84.
12. Bridge JA, Kanamori M, Ma Z, Pickering D, Hill DA, Lydiatt W, et al. Fusion of the ALK gene to the clathrin heavy chain gene, CLTC, in inflammatory myofibroblastic tumor. Am J Pathol 2001;159:411–5.
13. Ma Z, Hill DA, Collins MH, Morris SW, Sumegi J, Zhou M, et al. Fusion of ALK to the Ran-binding protein 2 (RANBP2) gene in inflammatory myofibroblastic tumor. Genes Chromosomes Cancer 2003;37:98–105.
14. Debiec-Rychter M, Marynen P, Hagemeijer A, Pauwels P. ALK-ATIC fusion in urinary bladder inflammatory myofibroblastic tumor. Genes Chromosomes Cancer 2003;38:187–90.
15. Panagopoulos I, Nilsson T, Domanski HA, Isaksson M, Lindblom P, Mertens F, et al. Fusion of the SEC31L1 and ALK genes in an inflammatory myofibroblastic tumor. Int J Cancer 2006;118:1181–6.
16. Delsol G, Lamant L, Mariamé B, Pulford K, Dastugue N, Brousset P, et al. A new subtype of large B-cell lymphoma expressing the ALK kinase and lacking the 2; 5 translocation. Blood 1997;89:1483–90.
17. Gascoyne RD, Lamant L, Martin-Subero JI, Lestou VS, Harris NL, Müller-Hermelink HK, et al. ALK-positive diffuse large B-cell lymphoma is associated with Clathrin-ALK rearrangements: report of 6 cases. Blood 2003;102:2568–73.
18. Van Roosbroeck K, Cools J, Dierickx D, Thomas J, Vandenberghe P, Stul M, et al. ALK-positive large B-cell lymphomas with cryptic SEC31A-ALK and NPM1-ALK fusions. Haematologica 2010;95: 509–13.
19. Takeuchi K, Soda M, Togashi Y, Ota Y, Sekiguchi Y, Hatano S, et al. Identification of a novel fusion, SQSTM1-ALK, in ALK-positive large B-cell lymphoma. Haematologica 2010.
20. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007;448:561–6.
21. Takeuchi K, Choi YL, Togashi Y, Soda M, Hatano S, Inamura K, et al. KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res 2009;15:3143–9.
22. Chan JK, Lamant L, Algar E, Delsol G, Tsang WY, Lee KC, et al. ALK+ histiocytosis: a novel type of systemic histiocytic proliferative disorder of early infancy. Blood 2008;112:2965–8.
23. Du XL, Hu H, Lin DC, Xia SH, Shen XM, Zhang Y, et al. Proteomic profiling of proteins dysregulted in Chinese esophageal squamous cell carcinoma. J Mol Med 2007;85:863–75.
24. Jazii FR, Najafi Z, Malekzadeh R, Conrads TP, Ziaee AA, Abnet C, et al. Identification of squamous cell carcinoma associated proteins by proteomics and loss of beta tropomyosin expression in esophageal cancer. World J Gastroenterol 2006;12:7104–12.
25. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 2007;131:1190–203.
26. Lin E, Li L, Guan Y, Soriano R, Rivers CS, Mohan S, et al. Exon array profiling detects EML4-ALK fusion in breast, colorectal, and non-small cell lung cancers. Mol Cancer Res 2009;7:1466–76.
27. Chiarle R, Voena C, Ambrogio C, Piva R, Inghirami G. The anaplastic lymphoma kinase in the pathogenesis of cancer. Nat Rev Cancer 2008;8:11–23.
28. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med 2010;363:1693–703.
29. Butrynski JE, D'Adamo DR, Hornick JL, Dal Cin P, Antonescu CR, Jhanwar SC, et al. Crizotinib in ALK-rearranged inflammatory myofibroblastic tumor. N Engl J Med 2010;363:1727–33.
30. Falini B, Bigerna B, Fizzotti M, Pulford K, Pileri SA, Delsol G, et al. ALK expression defines a distinct group of T/null lymphomas ("ALK lymphomas") with a wide morphological spectrum. Am J Pathol 1998;153:875–86.
31. Sakairi Y, Nakajima T, Yasufuku K, Ikebe D, Kageyama H, Soda M, et al. EML4-ALK fusion gene assessment using metastatic lymph node samples obtained by endobronchial ultrasound-guided transbronchial needle aspiration. Clin Cancer Res 2010;16:4938–45.
32. Nakajima T, Kimura H, Takeuchi K, Soda M, Mano H, Yasufuku K, et al. Treatment of lung cancer with an ALK inhibitor after EML4-ALK fusion gene detection using endobronchial ultrasound-guided transbronchial needle aspiration. J Thorac Oncol 2010;5:2041–3.
33. Jokoji R, Yamasaki T, Minami S, Komuta K, Sakamaki Y, Takeuchi K, et al. Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. J Clin Pathol 2010;63:1066–70.
34. Mino-Kenudson M, Chirieac LR, Law K, Hornick JL, Lindeman N, Mark EJ, et al. A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry. Clin Cancer Res 2010;16:1561–71.
35. Martelli MP, Sozzi G, Hernandez L, Pettirossi V, Navarro A, Conte D, et al. EML4-ALK rearrangement in non-small cell lung cancer and non-tumor lung tissues. Am J Pathol 2009;174:661–70.
36. Shiota M, Nakamura S, Ichinohasama R, Abe M, Akagi T, Takeshita M, et al. Anaplastic large cell lymphomas expressing the novel chimeric protein p80NPM/ALK: a distinct clinicopathologic entity. Blood 1995;86:1954–60.
37. Takeuchi K, Choi YL, Soda M, Inamura K, Togashi Y, Hatano S, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 2008;14:6618–24.
38. Choi YL, Takeuchi K, Soda M, Inamura K, Togashi Y, Hatano S, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res 2008;68:4971–6.

Published OnlineFirst March 23, 2011; DOI:10.1158/1078-0432.CCR-11-0063

39. Onishi M, Kinoshita S, Morikawa Y, Shibuya A, Phillips J, Lanier LL, et al. Applications of retrovirus-mediated expression cloning. Exp Hematol 1996;24:324–9.
40. Pillay K, Govender D, Chetty R. ALK protein expression in rhabdomyosarcomas. Histopathology 2002;41:461–7.
41. Passoni L, Longo L, Collini P, Coluccia AM, Bozzi F, Podda M, et al. Mutation-independent anaplastic lymphoma kinase overexpression in poor prognosis neuroblastoma patients. Cancer Res 2009;69:7338–46.
42. Travis WD, Elisabeth B, Muller-Hermelink HK, Harris CC, editors. Pathology and Genetics of Tumours of the Lung, Pleural, Thymus and Heart. Lyon: IARC Press; 2004.
43. Kriajevska M, Fischer-Larsen M, Moertz E, Vorm O, Tulchinsky E, Grigorian M, et al. Liprin beta 1, a member of the family of LAR transmembrane tyrosine phosphatase-interacting proteins, is a new target for the metastasis-associated protein S100A4 (Mts1). J Biol Chem 2002;277:5229–35.
44. Norrmén C, Vandevelde W, Ny A, Saharinen P, Gentile M, Haraldsen G, et al. Liprin (beta)1 is highly expressed in lymphatic vasculature and is important for lymphatic vessel integrity. Blood 2010;115:906–9.
45. Debelenko LV, Raimondi SC, Daw N, Shivakumar BR, Huang D, Nelson M, et al. Renal cell carcinoma with novel VCL-ALK fusion: new representative of ALK-associated tumor spectrum. Mod Pathol 2010.
46. Marino-Enriquez A, Ou WB, Weldon CB, Fletcher JA, Perez-Atayde AR. ALK rearrangement in sickle cell trait-associated renal medullary carcinoma. Genes Chromosomes Cancer 2011;50:146–53.