

## Human Cancer Biology

See commentary p. 4909

**EML4-ALK Fusion Gene Assessment Using Metastatic Lymph Node Samples Obtained by Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration**Yuichi Sakairi<sup>1,7</sup>, Takahiro Nakajima<sup>1,2,7</sup>, Kazuhiro Yasufuku<sup>2</sup>, Dai Ikebe<sup>3</sup>, Hajime Kageyama<sup>4</sup>, Manabu Soda<sup>5</sup>, Kengo Takeuchi<sup>6</sup>, Makiko Itami<sup>3</sup>, Toshihiko Iizasa<sup>1</sup>, Ichiro Yoshino<sup>7</sup>, Hiroyuki Mano<sup>5</sup>, and Hideki Kimura<sup>1</sup>**Abstract**

**Purpose:** Anaplastic lymphoma kinase (*ALK*) fusion genes represent novel oncogenes for non-small cell lung cancers (NSCLC). Several *ALK* inhibitors have been developed, and are now being evaluated in *ALK*-positive NSCLC. The feasibility of detecting *ALK* fusion genes in samples obtained by endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) was determined. The clinicopathologic characteristics of *ALK*-positive lung cancer were also analyzed.

**Experimental Design:** From April 2008 to July 2009, NSCLC cases with hilar/mediastinal lymph node metastases detected by EBUS-TBNA were enrolled. Positive expression of *ALK* fusion protein was determined using immunohistochemistry, and *ALK* gene rearrangements were further examined to verify the translocation between *ALK* and partner genes using fluorescent *in situ* hybridization and reverse transcription-PCR. Direct sequencing of PCR products was performed to identify *ALK* fusion variants.

**Results:** One hundred and nine cases were eligible for the analysis using re-sliced samples. Screening of these specimens with immunohistochemistry revealed *ALK* positivity in seven cases (6.4%), all of which possessed echinoderm microtubule-associated protein-like 4-*ALK* fusion genes as detected by fluorescent *in situ* hybridization and reverse transcription-PCR. All *ALK*-positive cases had an adenocarcinoma histology and possessed no *EGFR* mutations. Compared with *ALK*-negative cases, *ALK*-positive cases were more likely to have smaller primary tumors ( $P < 0.05$ ), to occur at a younger age ( $<60$  years;  $P < 0.05$ ), and to occur in never/light smokers (smoking index  $< 400$ ;  $P < 0.01$ ). Mucin production was frequently observed in *ALK*-positive adenocarcinomas (29.4%;  $P < 0.01$ ).

**Conclusions:** EBUS-TBNA is a practical and feasible method for obtaining tissue from mediastinal and hilar lymph nodes that can be subjected to multimodal analysis of *ALK* fusion genes in NSCLC.

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A small inversion within the short arm of human chromosome 2 leads to the generation of a fusion gene between the anaplastic lymphoma kinase (*ALK*) gene and the echinoderm microtubule-associated protein-like 4 (*EML4*) gene, the protein product of which is reported to function as an oncokinase in non-small cell lung cancer (NSCLC); in fact, this was the first oncogenic trans-

location to be identified in lung cancer (1). The *EML4-ALK* fusion gene has been detected in ~5% of NSCLC cases, and several *ALK* fusion gene variants have been reported (2). Standard methods for the detection of *ALK* fusion genes include reverse transcription-PCR (RT-PCR) with primers flanking the fusion points, as well as fluorescent *in situ* hybridization (FISH). Previously, immunohistochemistry-based diagnosis of *ALK* fusion genes in lung cancer has proven to be difficult, most likely due to the low expression level of the fusion protein products (3). An intercalated antibody-enhanced polymer (iAEP) technique has recently been developed that enables reliable immunohistochemistry-based detection of *ALK* fusion products (4). However, this technique has only been performed in cell lines and in large, surgically resected specimens; thus, it remains unclear whether such methodology can be applied to small biopsy samples obtained from patients with advanced NSCLC.

Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is an established modality for the definitive diagnosis of mediastinal and hilar adenopathy in

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### Translational Relevance

Acquisition of proper tissue samples for molecular analysis is not always an easy task; however, information obtained from such specimens is essential for the selection of appropriately targeted cancer therapies. This study shows that endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) contributes to the resolution of this issue in lung cancer because tissue samples obtained by EBUS-TBNA can be successfully used to assess the presence of echinoderm microtubule-associated protein-like 4–anaplastic lymphoma kinase fusion genes. We have shown that EBUS-TBNA samples could be subjected to immunohistochemistry, fluorescent *in situ* hybridization, and reverse transcription-PCR analysis. EBUS-TBNA for the assessment of mediastinal and hilar adenopathy is a practical tool that can be used in the molecularly targeted treatment era for lung cancer.

patients with lung cancer (5, 6). EBUS-TBNA is generally accepted to be as safe as standard bronchoscopy, less invasive than mediastinoscopy, and of high diagnostic quality. Compared with conventional fine-needle aspiration, EBUS-TBNA is an outstanding procedure with respect to its extremely low morbidity and its repeatability; fine-needle aspiration could cause pneumothorax, and repeated sampling might be difficult to perform.

In this study, we analyzed the feasibility of EBUS-TBNA for the detection of *EML4-ALK* fusion genes. We also retrospectively analyzed the clinicopathologic characteristics of *ALK*-positive lung cancer cases with mediastinal and/or hilar lymph node metastasis.

### Materials and Methods

#### Patients

From April 2008 to July 2009, 112 cases with proven hilar and/or mediastinal lymph node metastasis of NSCLC were enrolled; re-sliced specimens for histologic examination were available for 109 of these cases. Independent pathologists (D. Ikebe and M. Itami) reviewed all cases and histologically confirmed the presence of cancer cells in each specimen. Morphologic features detected with H&E staining were also recorded, and mucin production was evaluated by Alcian blue staining. First, samples were screened for *ALK* abnormalities using immunohistochemistry. Cases that were determined to be *ALK*-positive or suspicious by immunohistochemistry in our laboratory were subjected to additional evaluation by FISH and immunohistochemistry restaining by an independent pathologist (K. Takeuchi) at the Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research. Final confirmation was performed by direct sequencing of *EML4-ALK* fusion cDNAs using EBUS-TBNA

histologic cores that had been preserved at  $-80^{\circ}\text{C}$ . *EGFR* gene mutation status was also evaluated in all EBUS-TBNA samples. Associations between the presence of *ALK* fusion genes and clinicopathologic characteristics were retrospectively analyzed from medical records.

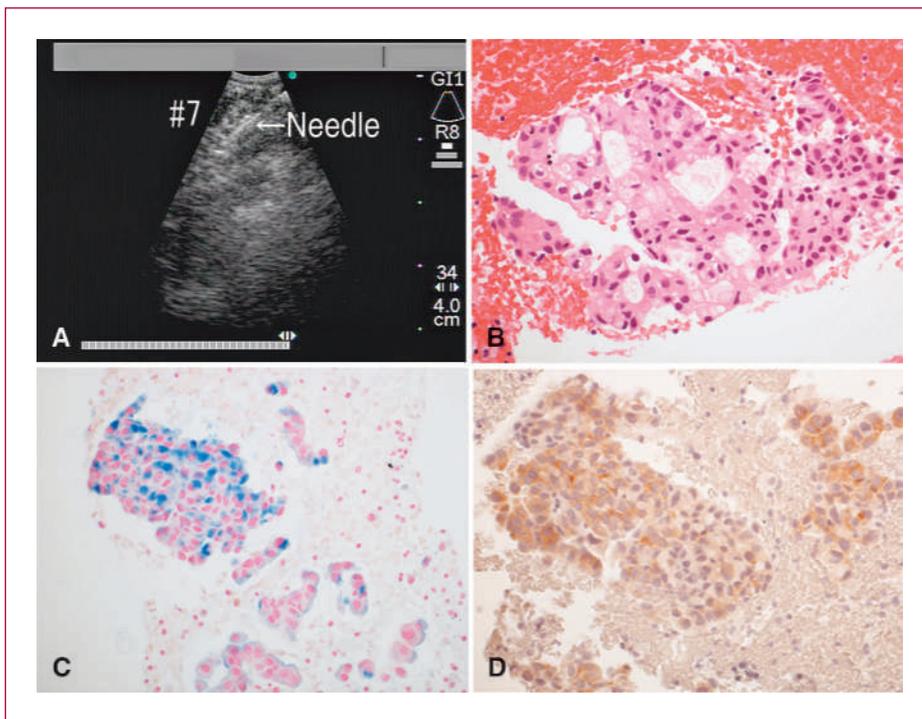
#### EBUS-TBNA

In all cases, chest computed tomography was performed prior to EBUS-TBNA. Brain magnetic resonance imaging, enhanced computed tomography, and bone scintigraphy were also performed for clinical staging of each case. EBUS-TBNA was performed for lymph nodes  $>5$  mm in short axis on chest computed tomography. To obtain a histologic core, a dedicated 22-gauge needle equipped with an internal stylet was used. After the initial puncture, the internal stylet was used to clean out the internal lumen that was clogged with bronchial tissue (Fig. 1A). The internal stylet was removed, and negative pressure was applied using a syringe. The needle was then moved back and forth inside the lymph node. Finally, the needle was retrieved, and the internal stylet was used to push out the histologic core (6). Each histologic core was divided into two samples: one was fixed with formalin and used for histologic diagnosis, and the other was mixed with Allprotect Tissue Reagent (Qiagen) following the instructions of the manufacturer, and stored at  $-80^{\circ}\text{C}$ .

#### *ALK* detection with immunohistochemistry

For detection of the *ALK* fusion gene, we applied the iAEP method, which incorporates an intercalating antibody between the primary antibody to *ALK* and dextran polymer-based detection reagents (4).

Histologic cores obtained by EBUS-TBNA were routinely fixed in 20% neutralized formalin and embedded in paraffin. Blocks were sliced at a thickness of 4  $\mu\text{m}$ , and sections were placed on silane-coated slides. Antibody preparations specific for the intracellular region of *ALK* (5A4, Abcam) were subjected to immunohistochemical staining according to standard protocols using dextran polymer reagents (anti-mouse immunoglobulin, EnVision+DAB System; Dako). The *ALK* antibody (5A4) was used at a dilution of 1:50. For antigen retrieval and deparaffinization, slides were heated for 20 minutes at  $98^{\circ}\text{C}$  in Target Retrieval Solution (low pH; Dako) with PT-link (Dako). Pretreated slides were positioned in a programmable AutoStainer instrument (EnVision System; Dako). Following the immunohistochemical program, slides were incubated at room temperature first with Peroxidase Blocking Solution (Dako) for 5 minutes and then with *ALK* antibody (5A4, 1:50; Abcam) for 30 minutes. Following application of the iAEP method, which has been described in detail elsewhere (4), we included an incubation step of 15 minutes at room temperature with intercalated immunoglobulin (Mouse-LINKER; Dako) to increase the detection sensitivity. Immune complexes were then detected using the dextran polymer reagent for 30 minutes. 5A4-positive cells were stained with 3,3'-diaminobenzidine for 5 minutes, and nuclei were then stained with hematoxylin for 2 minutes.



**Fig. 1.** Diagnosis of metastatic nodes by EBUS-TBNA. A, lymph node sampling by EBUS-TBNA. B, adenocarcinoma was revealed in the EBUS-TBNA sample. C, mucin production was observed by Alcian blue staining. D, immunohistochemistry with an anti-ALK antibody showed ALK fusion protein positivity in lung adenocarcinoma cells.

The samples obtained with EBUS-TBNA were small, paraffin-embedded biopsy specimens, which might limit the utility of immunohistochemistry. To avoid false-negative diagnosis, the first immunohistochemical procedure was used as a screening test to define three categories with which to judge the first run. Cancer cells were defined as “positive” if staining was as strongly positive as a positive control (clinical lung cancer tissues previously defined as positive by both molecular and immunohistochemistry analyses) and a fine, granular cytoplasmic staining pattern was observed. Cancer cells that showed no staining were classified as “negative.” The “suspicious” classification was defined as the presence of weakly stained cells that were considered difficult to differentiate from background staining. While using these categories, we further subdivided the suspicious category into “probably positive” and “probably negative” categories. Probably positive meant that the tumor cells stained, but not strongly, whereas probably negative indicated very weak staining that was difficult to differentiate from background staining. After the screening immunohistochemistry, suspicious cases were re-tested by immunohistochemistry in addition to FISH by a second independent pathologist (K. Takeuchi).

#### Fluorescence *in situ* hybridization

To further confirm the *ALK* genomic rearrangement, two FISH assays were performed: an *ALK* split assay and an *EML4-ALK* fusion assay. Unstained sections were processed with a Histology FISH Accessory Kit (Dako), subjected to hybridization with fluorescently-labeled bacterial artificial chromosome clone probes for *EML4* and *ALK* (self-produced

probes; *EML4* RP11-996L7, *ALK* RP11-984I21, and RP11-62B19) or for genomic regions upstream and downstream of the *ALK* breakpoint (Dako), stained with 4,6-diamidino-2-phenylindole, and examined with a fluorescence microscope (BX51; Olympus; ref. 7). FISH analysis was performed at the Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research (K. Takeuchi). The FISH positivity criteria for EBUS-TBNA samples were defined as “over 50% cancer cells.” As EBUS-TBNA samples are small biopsy samples, entire tumor cells in the paraffin-embedded section were evaluated.

#### RT-PCR and direct sequencing

Frozen histologic cores obtained by EBUS-TBNA were used to extract RNA. All immunohistochemistry-positive or suspicious cases were subjected to direct sequencing of the fusion cDNAs. RNA was extracted from frozen samples using the AllPrep DNA/RNA mini kit (Qiagen), and cDNA cloning was performed with the High Capacity RNA-to-cDNA Kit (Applied Biosystems). For RT-PCR analysis of *EML4-ALK*, we used primer sequences that have been described previously (2). After PCR amplification, PCR products were analyzed using agarose gel electrophoresis. RT-PCR products were extracted from gel slices using the QIAquick Gel Extraction Kit (Qiagen). Purified products were then sequenced with a capillary sequencer. Resultant nucleotide sequences were compared with previously reported sequences for determination of the *EML4-ALK* variant. *EGFR* mutation status was also examined using the peptide nucleic acid/locked nucleic acid PCR clamp method for samples obtained with EBUS-TBNA (8).

### Ethics committee approval

This research was approved by the Ethics Committee of Chiba Cancer Center (nos. 20-21 and 21-10). Written consent was obtained from all patients. All samples were coded and managed independently.

### Statistical analysis

For clinical characteristics and genetic factors, frequency analysis was performed with Fisher's exact test (dichotomous factors) and  $\chi^2$  test (multinomial factors). Mann-Whitney *U* test was applied to continuous data. General data analysis was conducted with StatView 5.0 (SAS Institute, Inc.). All *P* values were based on a two-sided hypothesis, *P* < 0.05 was considered to have statistical significance.

## Results

### Patient characteristics

The clinical characteristics of all 109 patients are listed in Table 1; 82 patients (75.2%) were male. The median age was 64.4 years (range, 38–90 y). Histologic examination was performed in all cases, leading to a diagnosis of adenocarcinoma (Fig. 1B) in 82 cases (75.2%), squamous cell carcinoma in 18 cases, and "other" in 9 cases. With respect to smoking status, 22 cases (20.4%) were never-smokers, 15 (13.9%) were light smokers (defined as a smoking index score <400), and 72 were heavy smokers (smoking index score  $\geq$ 400). A total of 191 mediastinal lymph nodes and 84 hilar lymph nodes (2.52 lymph nodes/patient) were detected with EBUS, and 158 mediastinal lymph nodes and 71 hilar lymph nodes (2.10 lymph nodes/patient) were sampled. The median size of the sampled lymph nodes was 12.1 mm (range, 3.0–33.4 mm) in the short axis on ultrasound. According to criteria from the International Union Against Cancer, there were 9 stage II cases, 49 stage III cases, and 45 stage IV cases; the remaining 6 cases were defined as having recurrent lung cancer. *EGFR* gene mutations were detected in 25 cases (22.9%), which included 9 cases with in-frame deletions at exon 19, 9 cases with a point mutation at exon 21, 3 cases with a point mutation at exon 18, 2 cases with point mutations at exons 18 and 21, 1 case with a point mutation at exon 20, and 1 case with point mutations in exons 20 and 21.

### ALK fusion gene assessment

Out of 109 cases examined by immunohistochemistry using the iAEP method, 6 *ALK*-positive cases and 17 suspicious cases (1 probably positive and 16 probably negative) cases were detected. The staining of the small histologic core did not show any heterogeneity.

FISH confirmed the existence of an *ALK* fusion gene in all six *ALK*-positive cases (Figs. 1D, 2A and B), and there were no false-positive cases for immunohistochemistry. Sixteen probably negative cases were determined to be negative for the *ALK* fusion gene by re-testing with immunohistochemistry and FISH. One probably positive case had too few tumor cells to be used for FISH analysis; however, RT-PCR assessment confirmed the presence of *EML4-ALK*

**Table 1. Clinical characteristics of patients with NSCLC**

Parameter	Number of cases (%)
	109
Age	
Mean (y)	64.4 (range, 38–90)
Gender	
Male	82 (75.2%)
Female	27 (24.8%)
Pathology	
Adenocarcinoma	82 (75.2%)
Squamous cell	18 (16.5%)
Other histology	9 (8.3%)
Clinical stage	
II	9 (8.3%)
III	49 (45.0%)
IV	45 (41.3%)
Recurrence	6 (5.5%)
Bone metastasis	
Yes	22 (20.2%)
No	87 (79.8%)
Brain metastasis	
Yes	16 (14.7%)
No	93 (85.3%)
Smoking	
Never (SI = 0)	22 (20.4%)
Light (SI < 400)	15 (13.9%)
Heavy (SI $\geq$ 400)	70 (64.8%)
<i>EGFR</i> mutation status	25 (22.9%)
Exon 18	3
Exon 19	9
Exon 20	1
Exon 21	9
Exons 18 + 21	2
Exons 20 + 21	1

Abbreviation: SI, smoking index.

fusion cDNA. *EML4*, *ALK*, and fusion signals (arrows in Fig. 2A) are presented in the green, red, and merged image and a pair of split signals (arrow in Fig. 2B, downstream) shows rearrangement of *ALK*. In Fig. 2C, unique bands in each *ALK*-positive case reveal variant 1 and variant 3 *EML4-ALK* fusion genes. Thus, the *ALK* fusion gene was detected in a total of seven cases (6.4%). Direct sequencing of the PCR products revealed that four cases carried *EML4-ALK* variant 1, whereas three cases had variant 3. The fusion point of *ALK* and *EML4* is observed in the cDNA sequence (arrow in Fig. 2D).

### Clinicopathologic characteristics of lung cancers possessing *ALK* fusion genes

Clinicopathologic characteristics were compared between the 7 *ALK*-positive cases and the 102 *ALK*-negative

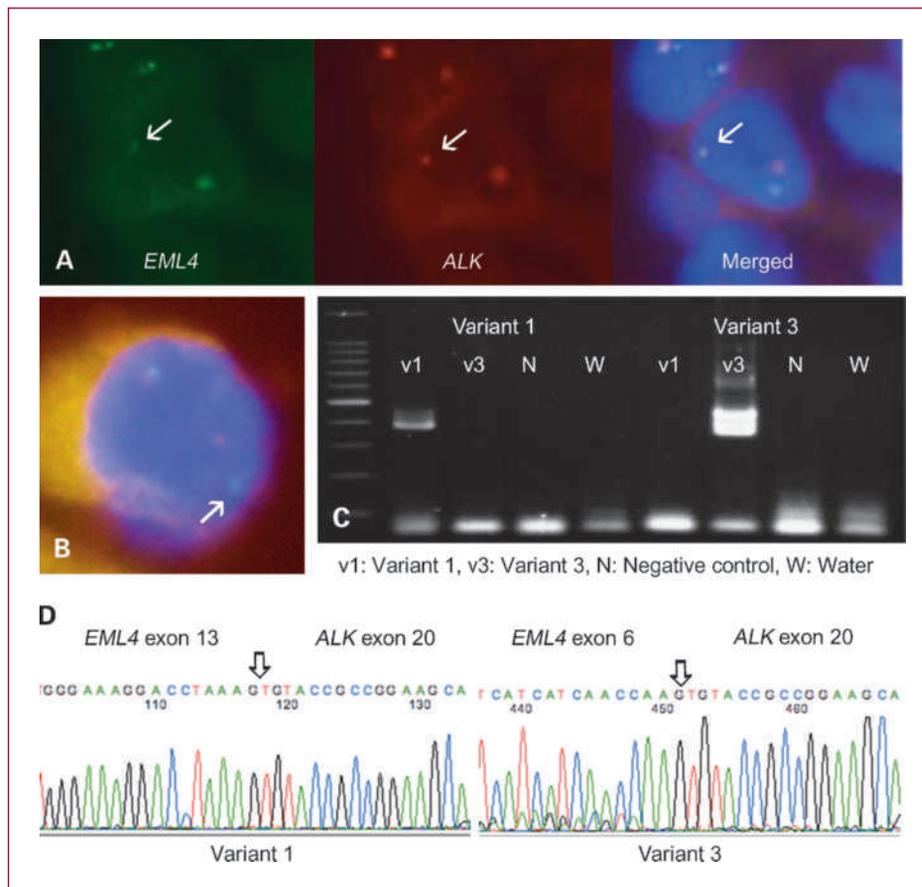
cases (Table 2). All *ALK*-positive cases had an adenocarcinoma histology and lacked *EGFR* gene mutations. With respect to smoking habits, six out of the seven *ALK*-positive cases were either never-smokers or light smokers (smoking index score <400). No significant difference in gender was observed between *ALK*-positive and *ALK*-negative patients; however, *ALK*-positive patients were significantly younger than *ALK*-negative patients (55.4 versus 65.0 years;  $P = 0.0408$ ). No significant differences in the incidence of bone metastasis (9.1% versus 5.7%;  $P = 0.64$ ) or brain metastasis (12.5% versus 5.4%;  $P = 0.30$ ) were observed. Overall, the mean primary tumor diameter was 40.4 mm; interestingly, the mean primary tumor diameter of *ALK*-positive cases was 28.6 mm, which was significantly smaller than that of *ALK*-negative cases (41.9 mm;  $P < 0.05$ ). Mucin production was significantly more frequently observed in *ALK*-positive cases as shown by Alcian blue staining (Fig. 1C;  $P < 0.01$ ). Finally, among the 84 cases expressing wild-type *EGFR*, 8.3% (7 of 84) were *ALK*-positive.

## Discussion

This is the first attempt and report about using EBUS-TBNA samples in the detection of *ALK* fusion genes, and is expected to have a major effect on the management of patients with lung cancer. EBUS-TBNA is an established

procedure for the evaluation of mediastinal and hilar adenopathy in patients with lung cancer. It is as safe, as highly diagnostic, and less invasive than other diagnostic modalities (9–11). Biopsy samples obtained with EBUS-TBNA can be subjected to histologic as well as cytologic evaluation. Nonsurgical modalities for obtaining tumor specimens are particularly critical in lung cancer because many patients have advanced disease at the time of first presentation, and are therefore not eligible for radical surgery. In addition to histologic diagnosis and stage definition, EBUS-TBNA enables molecular analysis of biopsy samples, the clinical significance of which is growing as molecularly targeted strategies for NSCLC are becoming increasingly important. We have previously reported that metastatic lymph node samples obtained by EBUS-TBNA can be applied to multidisciplinary analyses (5), and the present study is the first report of successful analysis of *ALK* fusion genes, a newly identified genetic abnormality in NSCLC, with such specimens (2). However, the small size of the paraffin-embedded biopsy samples obtained from EBUS-TBNA might limit the utility of this methodology; thus, multidirectional analysis will be critical for microsampling methods such as EBUS-TBNA.

The reliability of the newly developed immunohistochemistry (iAEP) method for the detection of *ALK* fusion



**Fig. 2.** Molecular analysis of *ALK* fusion genes. A, FISH *EML4-ALK* fusion assay with labeled probes for *EML4* (green, arrow) or *ALK* (red, arrow). The *EML4-ALK* fusion gene is observed (yellow, arrow). B, *EML4-ALK* split assay with labeled probes for the upstream (red) or downstream (green, arrow) region of the *ALK* locus. C, RT-PCR detection of the *EML4-ALK* fusion gene. D, direct cDNA sequence of *EML4-ALK* variants 1 and 3.

**Table 2.** Clinical, pathologic, and genetic analysis of *ALK*-positive NSCLC

Characteristic	<i>EML4-ALK</i> fusion			P
	NSCLC	+	-	
Female gender	27	4	23	0.062
Mean age (y)	64.4	55.4	65.0	0.0408
<60	29	5	24	0.0139
Bone metastasis	22	2	20	0.6396
Brain metastasis	16	2	14	0.2973
Mean tumor diameter (mm)	40.4	28.6	41.9	0.0478
Smoking index (n = 107)	784	161	827	0.0071
Never/light smoker	37	6	31	0.0056
Adenocarcinoma	82	7	75	0.1896
Mucin production	17	5	12	0.0009
<i>EGFR</i> wild-type	84	7	77	0.3317
<i>ALK</i> variant 1		4		
<i>ALK</i> variant 3		3		

NOTE: Two cases without primary tumors and six cases of recurrence were excluded from the tumor diameter analysis. Smoking history was recorded in 107 patients.

genes is very precise (4). This method is expected to be more practical for the detection of *ALK* fusion genes compared with FISH because FISH can sometimes be very difficult to perform for *ALK* fusion genes due to the close proximity of the two fusion gene components. We performed both fusion and split assays for FISH, and FISH was performed to confirm the immunohistochemical results. In addition, the *ALK* fusion genes are novel oncogenes in lung cancer. There is a possibility of existing unknown fusion pattern which cannot be detected by FISH or RT-PCR. Immunohistochemistry has an advantage of detecting novel unknown fusion patterns (4). In this study, we performed immunohistochemistry using the iAEP methodology and an Autostainer instrument. This technique is convenient, highly reproducible, and enables accurate diagnosis even if only a small amount of specimen is available. These features are well-suited for the screening of *ALK*-positive lung cancers using small biopsy samples. The Autostainer instrument also allows uniform immunohistochemical analysis, which may lead to consistent results among different institutions/hospitals; such uniformity is essential for the standardization of diagnostic procedures that assess the presence of *ALK* fusion genes. Recently, a highly sensitive antibody directed against *ALK* fusion products that can possibly be used for immunohistochemistry has been reported, therefore representing a novel candidate for *ALK* fusion detection (12).

The median age of *ALK*-positive cases in the present study was 55.4 years. Patients <60 years represent approximately 10% of all lung cancer deaths (6,655 of 63,255 deaths) according to the Japanese National Cancer Center Cancer

Information Service Statistics published in 2008 (13). In the present study, a significant number of *ALK*-positive cases were <60 years of age (17.2%, 5 of 29;  $P < 0.05$ ). *ALK*-positive cancer may therefore be more common in patients with early-onset NSCLC. However, it should be noted that two *ALK*-positive cases were >70 years of age (71 and 73 years); therefore, although patient age may become a predictor of *ALK* fusion gene positivity, *ALK* screening must also be performed in elderly individuals. The median diameter of primary lung tumors was significantly smaller in *ALK*-positive cases (28.6 versus 41.9 mm;  $P < 0.05$ ), further emphasizing the importance of EBUS-TBNA because this technique does not require a large primary lesion. An additional advantage of EBUS-TBNA is that it can be used for lymph node sampling, which is relevant to the majority of advanced lung cancer cases. Although lung cancer is generally more common in smokers, most of the *ALK*-positive cases in this study (37 cases; 34.3%) were never-smokers or light smokers. The smoking index scores in the *ALK*-positive cohort were significantly lower than that of *ALK*-negative patients (161 versus 827;  $P < 0.01$ ). Hence, being a never-smoker or light smoker seems to be a strong predictor of *ALK* positivity ( $P < 0.01$ ).

Evaluation of the clinicopathologic characteristics of patients in our cohort indicated that *ALK*-positive lung cancer tends to have an adenocarcinoma histology, expresses wild-type *EGFR*, has an early age of onset (<60 y), manifests as a relatively small primary lesion, more frequently occurs in never-smokers or light smokers (smoking index score <400), and has a mucin-producing histology. However, as EBUS-TBNA samples are obtained from metastatic lymph nodes rather than the primary tumor, these clinical features are nearly compatible with previously reported features (14). Patients harboring one or more of these predictive factors may therefore derive the most benefit from *ALK* fusion gene screening.

Recently, *ALK*-positive NSCLC was reported to be a signet ring cell type adenocarcinoma (15, 16). We assume that this description also includes mucin production, i.e., mucin-producing tumors or tumors with >10% Alcian blue staining in the cytoplasm. Herein, we performed Alcian blue staining on suspected mucin-producing tumors as part of the histologic diagnosis. By this classification, 17 (15.6%) NSCLC cases were determined to be mucin-producing cancers. These cases were all adenocarcinomas and included five *ALK*-positive cases; thus, approximately 30% of the mucin-producing adenocarcinomas showed *ALK* positivity. This is a significantly high frequency compared with that of other NSCLCs ( $P < 0.01$ ). This histologic feature, which can be assessed in cytologic samples, therefore seems to be useful for the prediction of *ALK* positivity.

The standard therapy for patients with advanced lung cancer at the time of presentation is chemotherapy and/or radiotherapy. However, standard platinum-based combined chemotherapy is not sufficient for disease eradication (17). Recently, lung cancer treatment strategies have become refined through the development of molecular markers and molecularly targeted agents. *ALK* inhibitors

have a high potential to become a definitive treatment for *ALK*-positive lung cancer, in a manner parallel to the exceptional therapeutic response of *EGFR*-positive lung cancers to *EGFR* tyrosine kinase inhibitors (18, 19). The efficacy of *ALK* inhibitors has been confirmed in cell lines (20, 21), and phase I clinical development of an oral *ALK* inhibitor for patients with lung cancer is currently under way (PF-02341066); two of the seven *ALK*-positive NSCLC cases from the present series have been enrolled in this trial (22, 23). As the background of *ALK*-positive lung cancer is similar to that of *EGFR*-positive lung cancer, and *ALK* tyrosine kinase inhibition is fundamentally similar to *EGFR* tyrosine kinase inhibition, *ALK* inhibitors might experience a similar progression of drug development and clinical and pathologic prediction of *ALK* positivity in lung cancer patients as *EGFR* tyrosine kinase inhibitors have for patients with *EGFR*-positive lung cancer. In this study, all *ALK*-positive lung cancers possessed wild-type *EGFR* and were therefore ineligible for *EGFR* tyrosine kinase inhibitor therapy (24). Therefore, *ALK* fusion gene assessment and administration of *ALK* inhibitors may become important for patients with *EGFR*-negative lung cancers.

Although some *ALK* inhibitors have already been developed and are currently being evaluated in clinical trials, it is important to establish a method for determining the existence of *ALK* fusion genes prior to the administration of *ALK* inhibitors. Both the presence of *ALK* fusion genes as well as *EGFR* gene mutations were successfully evaluated using histologic samples obtained by EBUS-TBNA of lung cancer regional lymph nodes. This diagnostic strategy allowed both pretreatment staging and evaluation of critical molecular markers to be definitively determined in a less invasive manner. There are some publications related with the genomic difference between primary tumor and metastatic site (25–29). EBUS-TBNA is a minimally invasive modality that allows the sampling of tumor cells from metastatic

lymph node with a very low morbidity. The possibility of genetic differences should be considered whenever the biomarker information is used for the selection of patients for molecular target therapies. EBUS-TBNA is an ideal approach in this aspect.

In conclusion, EBUS-TBNA sampling is feasible for *ALK* fusion gene assessment by immunohistochemistry, FISH, and RT-PCR, as well as for pathologic diagnosis. The development of a safe and highly precise modality that enables the acquisition of a sufficient amount of high-quality tissue without surgery will become increasingly important in the molecularly targeted therapy era. EBUS-TBNA is one of the best candidates for such a methodology.

### Disclosure of Potential Conflicts of Interest

K. Yasufuku, recipient of an unrestricted grant from Olympus Medical Corporation for Continuing Medical Education; H. Mano, member of the scientific advisory board, Pfizer Inc.

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### References

- Mano H. Non-solid oncogenes in solid tumors: EML4-*ALK* fusion genes in lung cancer. *Cancer Sci* 2008;99:2349–55.
- 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.
- Martelli MP, Sozzi G, Hernandez L, et al. EML4-*ALK* rearrangement in non-small cell lung cancer and non-tumor lung tissues. *Am J Pathol* 2009;174:661–70.
- Takeuchi K, Choi YL, Togashi Y, et al. KIF5B-*ALK*, a novel fusion oncokine identified by an immunohistochemistry-based diagnostic system for *ALK*-positive lung cancer. *Clin Cancer Res* 2009;15:3143–9.
- Nakajima T, Yasufuku K, Suzuki M, et al. Assessment of epidermal growth factor receptor mutation by endobronchial ultrasound-guided transbronchial needle aspiration. *Chest* 2007;132:597–602.
- Yasufuku K, Chiyo M, Koh E, et al. Endobronchial ultrasound guided transbronchial needle aspiration for staging of lung cancer. *Lung Cancer* 2005;50:347–54.
- 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.
- Nagai Y, Miyazawa H, Huqun, et al. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. *Cancer Res* 2005;65:7276–82.
- Yasufuku K, Chiyo M, Sekine Y, et al. Real-time endobronchial ultrasound-guided transbronchial needle aspiration of mediastinal and hilar lymph nodes. *Chest* 2004;126:122–8.
- Herth FJ, Ernst A, Eberhardt R, Vilmann P, Dienemann H, Krasnik M. Endobronchial ultrasound-guided transbronchial needle aspiration of lymph nodes in the radiologically normal mediastinum. *Eur Respir J* 2006;28:910–4.
- Herth FJ, Eberhardt R, Vilmann P, Krasnik M, Ernst A. Real-time endobronchial ultrasound guided transbronchial needle aspiration for sampling mediastinal lymph nodes. *Thorax* 2006;61:795–8.
- Mino-Kenudson M, Chirieac LR, Law K, 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.
- National Cancer Center Cancer Information Service. Available from: <http://ganjoho.ncc.go.jp/public/statistics/backnumber/odjrh3000000vdf1-att/date02.pdf>. Accessed on August 28, 2009.
- Takahashi T, Sonobe M, Kobayashi M, et al. Clinicopathologic features of non-small-cell lung cancer with EML4-*ALK* fusion gene. *Ann Surg Oncol* 2010;17:889–97.

15. 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.
16. 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.
17. Wakelee H, Belani CP. Optimizing first-line treatment options for patients with advanced NSCLC. *Oncologist* 2005;10:1–10.
18. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–39.
19. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–500.
20. 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.
21. Piva R, Chiarle R, Manazza AD, et al. Ablation of oncogenic ALK is a viable therapeutic approach for anaplastic large-cell lymphomas. *Blood* 2006;107:689–97.
22. Kwak EL, Camidge DR, Clark J, et al. Clinical activity observed in a phase I dose escalation trial of an oral c-met and ALK inhibitor, PF-02341066. *J Clin Oncol* 2009;27:15s.
23. Christensen JG, Zou HY, Arango ME, et al. Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. *Mol Cancer Ther* 2007;6:3314–22.
24. Shaw AT, Yeap BY, Mino-Kenudson M, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. *J Clin Oncol* 2009;27:4247–53.
25. Kalikaki A, Koutsopoulos A, Trypaki M, et al. Comparison of EGFR and K-RAS gene status between primary tumours and corresponding metastases in NSCLC. *Br J Cancer* 2008;99:923–9.
26. Gow CH, Chang YL, Hsu YC, et al. Comparison of epidermal growth factor receptor mutations between primary and corresponding metastatic tumors in tyrosine kinase inhibitor-naive non-small-cell lung cancer. *Ann Oncol* 2009;20:696–702.
27. Daniele L, Cassoni P, Bacillo E, et al. Epidermal growth factor receptor gene in primary tumor and metastatic sites from non-small cell lung cancer. *J Thorac Oncol* 2009;4:684–8.
28. Park S, Holmes-Tisch AJ, Cho EY, et al. Discordance of molecular biomarkers associated with epidermal growth factor receptor pathway between primary tumors and lymph node metastasis in non-small cell lung cancer. *J Thorac Oncol* 2009;4:809–15.
29. Schmid K, Oehl N, Wrba F, et al. EGFR/KRAS/BRAF mutations in primary lung adenocarcinomas and corresponding locoregional lymph node metastases. *Clin Cancer Res* 2009;15:4554–60.

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