EML4-ALK Fusion Gene Assessment Using Metastatic Lymph Node Samples Obtained by Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration

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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 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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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.

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 retesting 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°C. EGR 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°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 μm, 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°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.
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 ≥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 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.
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 genes is shown in Fig. 2. The EML4-ALK fusion gene is observed (yellow, arrow). 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.

![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.](image-url)
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 IAE9 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

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>EML4-ALK fusion</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NSCLC +</td>
<td>-</td>
</tr>
<tr>
<td>Female gender</td>
<td>27</td>
<td>4</td>
</tr>
<tr>
<td>Mean age ((y))</td>
<td>64.4</td>
<td>55.4</td>
</tr>
<tr>
<td>&lt;60</td>
<td>29</td>
<td>5</td>
</tr>
<tr>
<td>Bone metastasis</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>Brain metastasis</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Mean tumor diameter ((mm))</td>
<td>40.4</td>
<td>28.6</td>
</tr>
<tr>
<td>Smoking index ((n = 107))</td>
<td>784</td>
<td>161</td>
</tr>
<tr>
<td>Never/light smoker</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>82</td>
<td>7</td>
</tr>
<tr>
<td>Mucin production</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>EGFR wild-type</td>
<td>84</td>
<td>7</td>
</tr>
<tr>
<td>ALK variant 1</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>ALK variant 3</td>
<td>3</td>
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</tr>
</tbody>
</table>

**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.
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

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