ings and those of previous studies. This may simply reflect clonal variations of individual Jurkat cell lines maintained in individual laboratories. Another possible explanation for the conflicting results is that these studies employed different gene transfer methods. Our experiment was performed using Jurkat cells stably transfected with Tec cDNA, whereas others carried out experiments with Jurkat cells transiently transfected with Tec. In most of the experimental conditions, transient transfection of cDNA results in higher levels of protein expression than those observed in the stable transformants. The differences in Tec expression levels among the experiments may have had diverse cellular effects.

In Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines from XLA patients, Fluckiger et al. [37] showed that the ectopic expression not only of Btk but also of Tec or Itk restored deficient extracellular calcium influx after BCR cross-linking in Btk-deficient cells. We, as well as Fluckiger et al. [13,37], have found that these XLA-derived Btk-deficient cell lines express endogenous Tec. The difference in the expressed amount of protein is considered the cause of the endogenous Tec's inability to compensate for Btk deficiencies. Interestingly, the overexpression of other PTK family members, such as Src (Lyn or Fyn) and Syk, failed to restore Btk-mediated signaling in XLA cells, suggesting the presence of strict kinase-substrate relationships between different PTK families regardless of the expression level [37]. These observations suggest that the expression of excess amounts of proteins may overcome the substrate specificity among individual Tec family PTKs that are present under physiological protein expression levels. This hypothesis is supported by our failure to detect any alteration of CD25 expression after TCR ligation in human primary CD4+ T-cells transiently transfected with Tec cDNA (Susaki and Kitanaka, unpublished observation). To reproduce findings obtained using the Jurkat cell line in human primary T-cells, it may be essential to establish a more sophisticated method to regulate the expression of introduced genes.

Tomlinson et al. [20] quantitated individual Tec family PTK protein levels in murine lymphoid cells. They found substantially lower Tec expression in murine primary T- and B-cells relative to Itk and Btk, respectively. They speculated that the lack of an obvious phenotype in the immune systems of Tec-deficient mice reflected the small amounts of Tec in murine lymphoid cells. Although there is not enough quantitative information on Tec expression relative to other Tec family PTKs in human lymphoid cells, our previous study revealed that EBV-transformed human B-lymphoblastoid cell lines expressed Tec levels similar to those observed in the K562 human erythroleukemia cell line [13]. In this regard, it is clear that human B-lymphoid cells express an amount of Tec comparable to the amounts in the representative human myeloid cell line. Therefore, the inability of a physiological amount of Tec to compensate for Btk in human lymphoid cells may be the reason why defective Btk function results in more severe consequences in humans than in mice [14,38]. Thus, the expression profiles and/or functional redundancies of individual Tec family PTK in lymphoid cells may differ among species. To clarify this issue, the Tec expression level should be compared against Tec's biological significance in human lymphoid cells. It is necessary to assess Tec expression in human lymphoid cells at different stages of development using quantitative methods such as flow cytometric analysis. To date, such analysis has not yet been accomplished because of the lack of a good anti-Tec antibody applicable to flow cytometric analysis (Kitanaka, unpublished observations).

In summary, we have found that the expression and activation of Tec in Jurkat cells inhibited the expression of CD25 induced by TCR cross-linking, suggesting that this PTK plays a negative regulatory role in the TCR-mediated signaling pathway. Our results imply that Tec participates in signaling that suppresses IL-2-mediated signaling by downregulating its receptor expression. Future studies should clarify the role of Tec expression and activation in the IL-2/IL-2 receptor system-mediated human T-lymphocyte activation pathway.

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References

[1] Kane LP, Lin J, Weiss A. Signal transduction by the TCR for antigen. Curr Opin Immunol 2000;12:242–9.
[2] Wang LD, Clark MR. B-cell antigen-receptor signalling in lymphocyte development. Immunology 2003;110:411–20.
[3] Kawakami Y, Yao L, Han W, Kawakami T. Tec family protein-tyrosine kinases and pleckstrin homology domains in mast cells. Immunol Lett 1996;54:113–7.
[4] Miller AT, Berg LJ. New insights into the regulation and functions of Tec family tyrosine kinases in the immune system. Curr Opin Immunol 2002;14:331–40.
[5] Khan WN, Alt FW, Gerstein RM, Malynn BA, Larsson I, Rathbun G, et al. Defective B cell development and function in Btk-deficient mice. Immunity 1995;3:283–99.
[6] Kerner JD, Appleby MW, Mohr RN, Chien S, Rawlings DJ, Maliszewski CR, et al. Impaired expansion of mouse B cell progenitors lacking Btk. Immunity 1995;3:301–12.
[7] Vetrie D, Vorechovský I, Sideras P, Holland J, Davies A, Flinter F, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature 1993;361:226–33.
[8] Tsukada S, Saffran DC, Rawlings DJ, Parolini O, Allen RC, Klisak I, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell 1993;72:279–90.
[9] Thomas JD, Sideras P, Smith CIE, Vorechovsky I, Chapman V, Paul WE. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. Science 1993;261:355–8.
[10] Rawlings DJ, Saffran DC, Tsukada S, Largaespada DA, Grimaldi JC, Cohen L, et al. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. Science 1993;261:358–61.
[11] Liao XC, Littman DR. Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. Immunity 1995;3:757–69.
[12] Liu KQ, Bunnell SC, Gurniak CB, Berg LJ. T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. J Exp Med 1998;187:1721–7.
[13] Kitanaka A, Mano H, Conley ME, Campana D. Expression and activation of the nonreceptor tyrosine kinase Tec in human B cells. Blood 1998;91:940–8.
[14] Ellmeier W, Jung S, Sunshine MJ, Hatam F, Xu Y, Baltimore D, et al. Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. J Exp Med 2000;192:1611–24.
[15] Tanaka N, Abe H, Yagita H, Okumura K, Nakamura M, Sugamura K. Itk, a T cell-specific tyrosine kinase, is required for CD2-mediated interleukin-2 promoter activation in the human T cell line Jurkat. Eur J Immunol 1997;27:834–41.
[16] Debnath J, Chamorro M, Czar MJ, Schaeffer EM, Lenardo MJ, Varmus HE, et al. rlk/TXK encodes two forms of a novel cysteine string tyrosine kinase activated by Src family kinases. Mol Cell Biol 1999;19:1498–507.
[17] Yang WC, Ghiotto M, Barbarat B, Olive D. The role of Tec protein-tyrosine kinase in T cell signaling. J Biol Chem 1999;274:607–17.
[18] August A, Gibson S, Kawakami Y, Kawakami T, Mills GB, Dupont B. CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. Proc Natl Acad Sci USA 1994;91:9347–51.
[19] Yang WC, Ching KA, Tsoukas CD, Berg LJ. Tec kinase signaling in T cells is regulated by phosphatidylinositol 3-kinase and the Tec pleckstrin homology domain. J Immunol 2001;166:387–95.
[20] Tomlinson MG, Kane LP, Su J, Kadlecek TA, Mollenauer MN, Weiss A. Expression and function of Tec, Itk, and Btk in lymphocytes: evidence for a unique role for Tec. Mol Cell Biol 2004;24:2455–66.
[21] Yamashita Y, Watanabe S, Miyazato A, Ohya K, Ikeda U, Shimada K, et al. Tec and Jak2 kinases cooperate to mediate cytokine-driven activation of c-fos transcription. Blood 1998;91:1496–507.
[22] Kitanaka A, Suzuki T, Ito C, Nishigaki H, Coustan-Smith E, Tanaka T, et al. CD38-mediated signaling events in murine pro-B cells expressing human CD38 with or without its cytoplasmic domain. J Immunol 1999;162:1952–8.
[23] Dobashi H, Sato M, Tanaka T, Tokuda M, Ishida T. Growth hormone restores glucocorticoid-induced T cell suppression. FASEB J 2001;15:1861–3.
[24] Kubota Y, Tanaka T, Kitanaka A, Ohnishi H, Okutani Y, Waki M, et al. Src transduces erythropoietin-induced differentiation signals through phosphatidylinositol 3-kinase. EMBO J 2001;20:5666–77.
[25] Sato K, Mano H, Ariyama T, Inazawa J, Yazaki Y, Hirai H. Molecular cloning and analysis of the human Tec protein-tyrosine kinase. Leukemia 1994;8:1663–72.
[26] Yang WC, Ghiotto M, Castellano R, Collette Y, Auphan N, Nunès JA, et al. Role of Tec kinase in nuclear factor of activated T cells signaling. Int Immunol 2000;12:1547–52.
[27] Yang WC, Olive D. Tec kinase is involved in transcriptional regulation of IL-2 and IL-4 in the CD28 pathway. Eur J Immunol 1999;29:1842–9.

[28] Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. Cell 1993;73:5–8.
[29] Kim HP, Imbert J, Leonard WJ. Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. Cytokine Growth Factor Rev 2006;17:349–66.
[30] Mahajan S, Ghosh S, Sudbeck EA, Zheng Y, Downs S, Hupke M, et al. Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-N-(2,5-dibromophenyl)propenamide]. J Biol Chem 1999;274:9587–99.
[31] Yang WC, Collette Y, Nunès JA, Olive D. Tec kinases: a family with multiple roles in immunity. Immunity 2000;12:373–82.
[32] Berg LJ, Finkelstein LD, Lucas JA, Schwartzberg PL. Tec family kinases in T lymphocyte development and function. Annu Rev Immunol 2005;23:549–600.
[33] Schaeffer EM, Debnath J, Yap G, McVicar D, Liao XC, Littman DR, et al. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science 1999;284:638–41.
[34] Kurosaki T. Regulation of B-cell signal transduction by adaptor proteins. Nat Rev Immunol 2002;2:354–63.
[35] Takesono A, Finkelstein LD, Schwartzberg PL. Beyond calcium: new signaling pathways for Tec family kinases. J Cell Sci 2002;115:3039–48.
[36] Lucas JA, Felices M, Evans JW, Berg LJ. Subtle defects in pre-TCR signaling in the absence of the Tec kinase Itk. J Immunol 2007;179:7561–7.
[37] Fluckiger AC, Li Z, Kato RM, Wahl MI, Ochs HD, Longnecker R, et al. Btk/Tec kinases regulate sustained increases in intracellular Ca^{2+} following B-cell receptor activation. EMBO J 1998;17:1973–85.
[38] Conley ME, Rohrer J, Rapalus L, Boylin EC, Minegishi Y. Defects in early B-cell development: comparing the consequences of abnormalities in pre-BCR signaling in the human and the mouse. Immunol Rev 2000;178:75–90.

Human Cancer Biology
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Clinical Cancer Research

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 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 translocation 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°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°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.

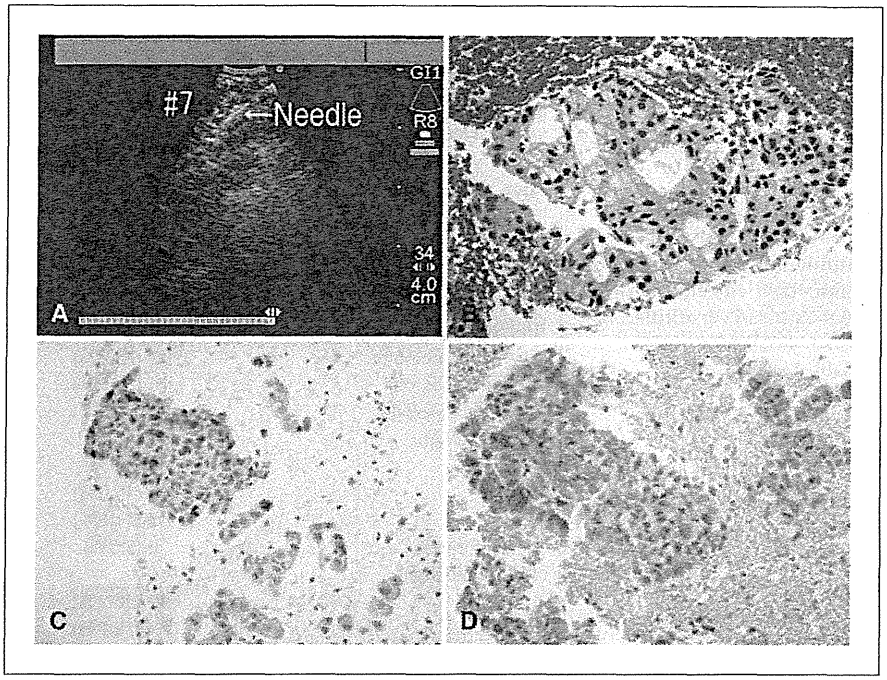

Fig. 1. Diagnosis of metastatic nodes by EBUS-TBNA. A, lymph node sampling by EBUS-TBNA. B, adenocarcinoma was revealed in the EBUS-TBNA sample. C, mucin production was observed by Alcian blue staining. D, immunohistochemistry with an anti-*ALK* antibody showed *ALK* fusion protein positivity in lung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

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 ≥ 400)	70 (64.8%)
EGFR 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.

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 (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

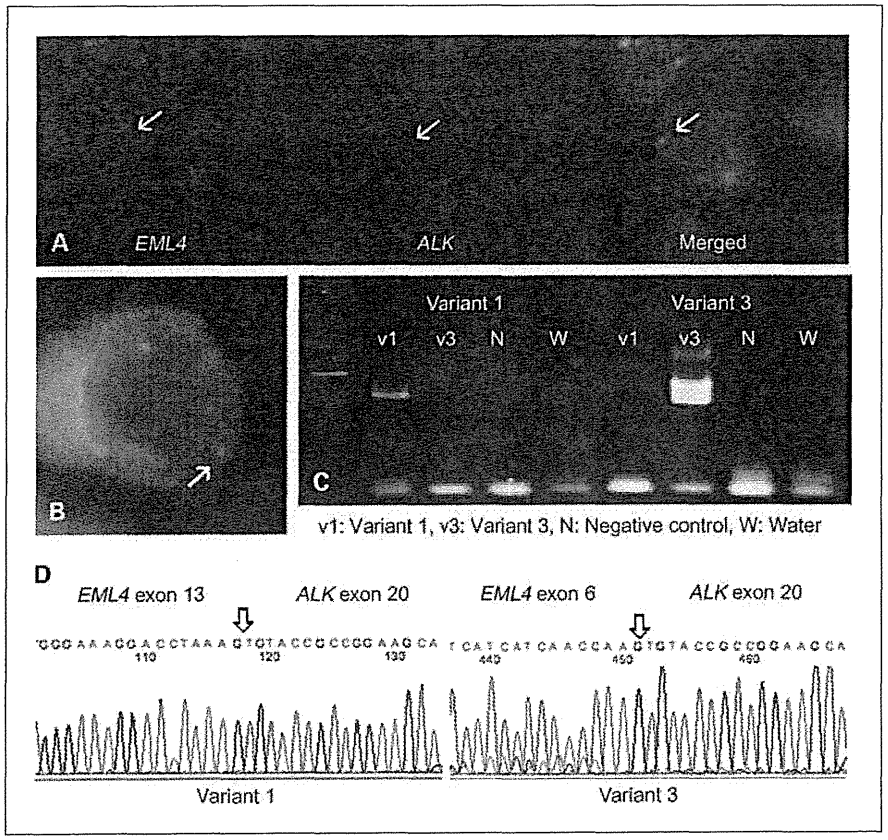

Fig. 2. Molecular analysis of *ALK* fusion genes. A, FISH *EML4-ALK* fusion assay with labeled probes for *EML4* (green, arrow) or *ALK* (red, arrow). The *EML4-ALK* fusion gene is observed (yellow, arrow). B, *EML4-ALK* split assay with labeled probes for the upstream (red) or downstream (green, arrow) 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	*EML4-ALK* 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
EGFR wild-type	84	7	77	0.3317
ALK variant 1		4		
ALK 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, recipent 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

1. Mano H. Non-solid oncogenes in solid tumors: EML4-ALK fusion genes in lung cancer. Cancer Sci 2008;99:2349–55.
2. 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.
3. 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.
4. 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.
5. 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.
6. 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.
7. 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.
8. 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.
9. 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.
10. 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.
11. 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.
12. 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.
13. 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.
14. 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.

INTERNAL MEDICINE

□ CASE REPORT □

Incidentally Proven Pulmonary "ALKoma"

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Abstract

Genetic alterations of echinoderm microtubule-associated protein-like 4 (*EML4*)-anaplastic lymphoma kinase (*ALK*) inversion were recently found in lung cancer. A 39-year-old woman with multiple brain metastases and bulky mediastinal lymph node metastases was admitted. Biopsy from her supraclavicular lymph nodes was performed to differentiate the diagnosis between lymphoma and lung cancer. Pathologically, the lymph nodes had a feature of adenocarcinoma. On the other hand, the commercially available chromosomal fluorescent in situ hybridization (FISH) analysis showed split signals of *ALK*, which was confirmed to be the *EML4-ALK* inversion. The commercial-based ALK FISH is useful for screening pulmonary ALKoma.

Key words: EML4-ALK, lung cancer, oncogene addiction

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Introduction

The echinoderm microtubule-associated protein-like 4 (*EML4*)-anaplastic lymphoma kinase (*ALK*) inversion was recently detected in 6.7% of Japanese non-small cell lung cancer (NSCLC) patients (1). The fusion gene encodes a constitutive active oncoprotein with activated ALK kinase, resulting in the aberrant activation of the downstream signaling targets including Akt, signal transducer and activator of transcription (STAT) 3, and Ras-extracellular signal-regulated kinase (ERK) 1/2 (2).

The term ALKoma, coined by Benharroch et al, originally was used to represent anaplastic large cell lymphoma (ALCL) carrying the t(2 ; 5)(p23 ; q35) chromosome translocation (3). In 1994, Morris et al found that the t(2 ; 5) translocation fuses part of the nucleophosmin (*NPM*) gene on chromosome 5q35 to a portion of the ALK receptor tyrosine kinase gene on chromosome 2p23 (4). As with other fusion proteins found in hematological malignancies, ALKoma is also thought to become addicted to the ALK signaling pathway (3). Recently oncogene addiction has mainly been recognized among non-smoking NSCLC patients (5, 6). Just as epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitors (TKIs) have become a mainstay of therapy for patients harboring *EGFR* mutation, patients with *EML4-ALK* inversion may benefit from therapy with ALK inhibitors. We herein report an incidentally proven *EML4-ALK* inversion in primary pulmonary adenocarcinoma.

Abbreviations: EML4: echinoderm microtubule-associated protein-like 4, ALK: anaplastic lymphoma kinase, NSCLC: non-small cell lung cancer, ALCL: anaplastic large cell lymphoma, NPM: nucleophosmin, EGFR: epidermal growth factor receptor

Case Report

A 39-year-old woman was admitted to hospital because of generalized seizures. An initial screening head and body CT showed multiple brain metastases and swelling lymph nodes throughout the thorax (Fig. 1A, B). Fiber optic bronchoscopy showed direct invasion of tumor to the carina (Fig. 1C). After a crisis of generalized seizures, neurological disorders were not obvious. The patient's performance status (PS) was graded as one, because of a dry cough, which had

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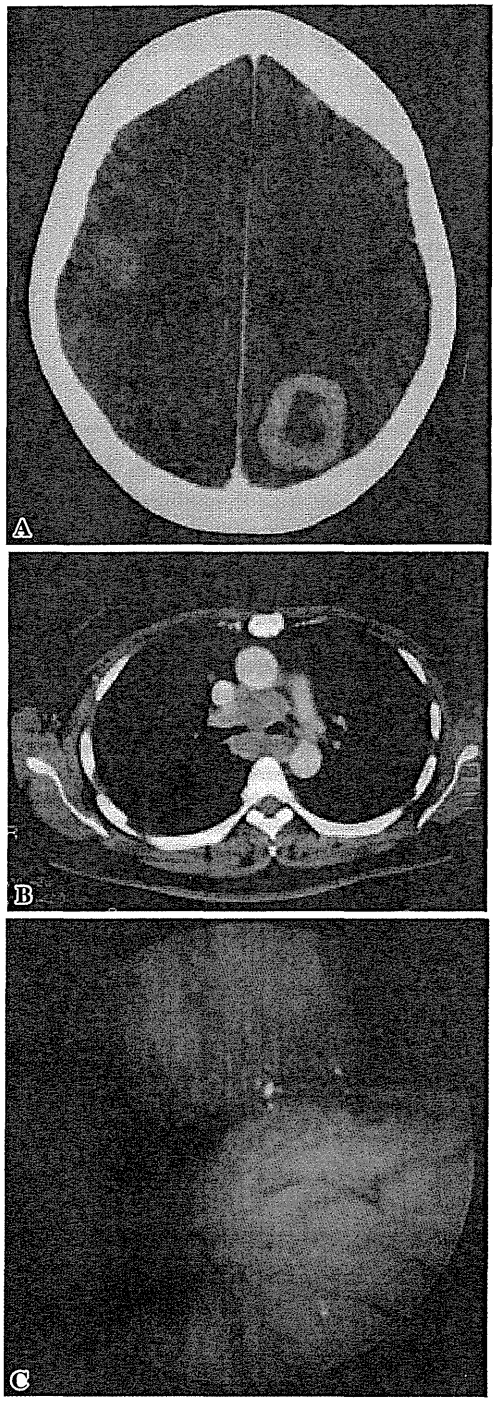

Figure 1. The imaging at presentation. Computed tomography presents multiple brain metastases (A) and mediastinal lymphadenopathy (B). Fiber optic bronchoscopy showed a direct invasion from metastatic lymph nodes to the carina (C). Both bronchi are too narrow to perform further examinations.

been apparent for the past 6 months before her admission to our hospital. Fine needle aspiration was performed from her right supraclaviclar lymph nodes, and malignancy at any origin was detected. As the patient was suspected to have malignant lymphoma or lung neoplasm, she was transferred to our institution for further examinations and therapies.

The laboratory data, including the tumor markers (carcinoembrionic antigen and soluble interleukin 2 receptor) were normal. The white blood cell count was 11,770/μL, probably due to the prophylactic use of corticosteroids against seizures. As the patient was thought to be in need of immediate therapy, an open biopsy from her right supraclavicular lymph nodes under local anesthesia was performed on the day of the transfer. The frozen samples were subjected to pathological examination, to *EGFR* mutation analysis (the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PCR) clamp method (7), Mitsubishi Chemical Medience, Tokyo, Japan), and to a comprehensive analysis for malignant lymphoma. Pathologically, the lymph nodes had a feature of moderately to poorly differentiated adenocarcinoma (Fig. 2A), with positive immunohistochemical staining for thyroid transcription factor-1 and epithelial markers (CAM5.2 and AE1/AE3). Immunohistochemical staining for lymphocyte markers was negative (CD20, CD45 RO, and CD30). Finally, her clinical diagnosis was determined to be cTxN3M1(BRA), clinical stage IV, adenocarcinoma of the lung. Although she was a young, never-smoking Japanese woman (8), she was found to be negative for *EGFR* mutations.

Meanwhile, the results of a comprehensive analysis for malignant lymphoma were reported. These analyses consisted of flow cytometric analyses with CD45 gating and a chromosomal G-banding analysis. In addition, the chromosomal fluorescent in situ hybridization (FISH) analyses were performed to detect the transition of *ALK* (2p23), *BCL6* (3q27), *IGH/BCL1* t(11 ; 14)(q13 ; q32), *IGH/BCL2* t(14 ; 18)(q32 ; q21), and *IGH/CMYC* t(8 ; 14)(q24 ; q32), based on a pathologist's decision ("ML-NET", SRL, Tokyo). In this case, the FISH analyses were added because the sample was not adequate for G-banding. Surprisingly, the FISH analysis of *ALK*, using 5′-(green) and 3′-(red) sequences for hybridization probes, showed the split signals of *ALK*, in up to 96% of the cells counted (total 100 cells) (Fig. 2B). In order to analyze the counterpart of transition for *ALK*, multiplex reverse transcription PCR of the EML4-ALK fusion transcripts was performed by YLC, MS and HM, and the transition was found to be EML4-ALK inversion, variant 2 (Fig. 2C).

Abbreviations: PCR: polymerase chain reaction, FISH: fluorescent in situ hybridization

Discussion

EML4-ALK inversion was first identified by Soda et al, from a lung adenocarcinoma specimen that was surgically resected from a 62-year-old male with a history of smoking. They made a cDNA library from the specimen, inserted cDNAs into the plasmid clones, and then infected them into mouse 3T3 fibroblasts with recombinant retrovirus to assess its ability to transform the foci. *The EML4-ALK* inversion transcripts were found in one of the transformed foci (1).

ALK, as well as leukocyte tyrosine kinase (*LTK*), is a re-

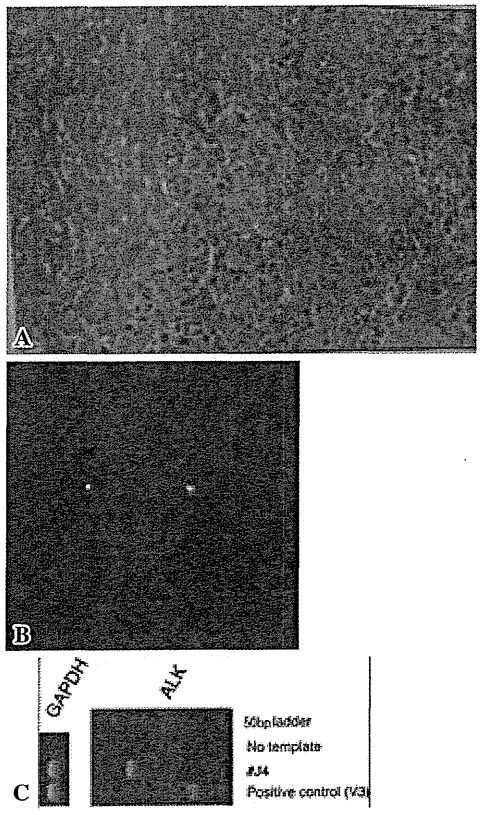

Figure 2. Histopathology and genetic analyses. Moderately to poorly differentiated adenocarcinoma is recognized with acinar patterns (A). A genomic FISH analysis showed that 96% of the cells which were analyzed had the split signal of ALK. A representative cell is shown (B). Multiplex RT-PCR to capture all in-frame fusions between *EML4* and *ALK* messages was conducted with the following primers; 5′-GTGCAGTGTTTAGCATTCTTGGGG-3′, 5′AGCTACATCACACACCTTGACTGG-3′, 5′-TACCAGTGCTGTCTCAATTGCAGG-3′, 5′-GCTTTCCCCGCAAGATGGACGG-3′, 5′-CAGCTGAGAGAGTGAAAGCTTTGG-3′, 5′-GACAGTTGGAGGAATCTGTCGATG-3′, 5′-ATCCTGCGGAACACTATTCAGTGG-3′, 5′-TCAAGCACATCTCAAGAGCAAGTG-3′ and 5′-TCTTGCCAGCAAAGCAGTAGTTGG-3′. Examination of an enlarged lymph node revealed the successful amplification for the *EML4-ALK* variant 2 transcript (indicated as #J4).

ceptor tyrosine kinase similar to the insulin receptor subfamily of kinases. *LTK* is found in murine B lymphocyte precursors and in forebrain neurons. *ALK* is usually found in the nervous system, where it serves the normal neural differentiation and construction. By transfusing its kinase domain with an activating counterpart with coiled-coil domain, like *NPM*, TRK-fused gene (*TFG*), *EML4* and so on, *ALK* gains oncogenic potential via a constitutional dimerization (2).

Since the receptor tyrosine kinases are one of the main targets of therapy in malignancy, Rikova et al performed a global survey of phosphotyrosine using lung cancer cell lines and clinical samples (9). Along with the well-known phosphorylation of EGFR and MET, the tyrosine phosphorylation of ALK was found in one cell line and in seven patients. A further analysis revealed three *EML4-ALK* inversions and one *TFG-ALK* fusion in 103 NSCLC patients, thus resulting in an overall frequency of ALK fusion of 4% in the Chinese population (9). A NSCLC cell line, H3122, which harbored an *EML4-ALK* inversion, showed massive apoptosis with an ALK kinase inhibitor, TAE-684 (10). Furthermore, transgenic mice expressing *EML4-ALK* conditionally in lung alveolar epithelial cells, which developed innumerable lung adenocarcinomas within a few weeks after birth, responded greatly with the oral administration of small-molecule inhibitors of the ALK kinase (11). Therefore, ALK is a novel therapeutic target in NSCLC. A phase I trial using PF02341066, TKI for MET and ALK, is ongoing for NPM-ALK-positive lymphoma (NCT00585195).

The clinicopathological background in patients with *EML4-ALK* inversion has been previously well described in two series. Inamura et al described that *EML4-ALK* positive lung cancers are characterized by an acinar histology, harboring neither *EGFR* mutation nor *KRAS* mutation, a non- or light smoking background and a young onset (12). Another group reported similar findings in which patients with *EML4-ALK* inversion were younger, more likely to be never/light smokers. They have also shown mutations of *KRAS*, *EGFR* and the rearrangement of *EML4-ALK* to be mutually exclusive (13). Furthermore, the latter group focused on the higher incidence of metastatic diseases in patients with an *EML4-ALK* inversion or *EGFR* mutation compared to patients without those alterations. The present case closely matches these findings. It is therefore suggested that an ALK FISH analysis should be recommended in the case of a never/light smoker, of younger onset, who is negative for *EGFR* mutation and advanced diseases.

Although several counterparts of *ALK* transition have been reported in some kind of tumors (2), the *EML4-ALK* inversion occurs most frequently in NSCLC (14). The identification of fusion transcripts is somewhat difficult because of the variation in the breakpoints of inversion. Soda et al are in the process of establishing multiplex RT-PCR for detecting fusion transcripts (1, 15). However, taking into consideration that there are a few other types of *ALK*-fusion (*TFG* and *KIF5B* (16)), the commercially used, previously established ALK FISH analysis is more useful in the screening of *ALK* altered NSCLC.

Since the incidence of patients demonstrating NSCLC with ALK transition who may benefit from a timely diagnosis and appropriate therapy exceeds the incidence of ALCL, the commercially-based ALK FISH is therefore considered to be a promising diagnostic modality for determining NSCLC patients with *ALK* transition.

Abbreviations: TKI: tyrosine kinase inhibitor, TRK-fused gene

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References

1. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature **448**: 561-566, 2007.
2. Chiarle R, Voena C, Ambrogio C, Piva R, Inghirami G. The anaplastic lymphoma kinase in the pathogenesis of cancer. Nat Rev Cancer **8**: 11-23, 2008.
3. Benharroch D, Meguerian-Bedoyan Z, Lamant L, et al. ALK-positive lymphoma: a single disease with a broad spectrum of morphology. Blood **91**: 2076-2084, 1998.
4. Morris SW, Kirstein MN, Valentine MB, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science **263**: 1281-1284, 1994 (Erratum in: Science 267: 316-317, 1995).
5. Ding L, Getz G, Wheeler DA, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature **455**: 1069-1075, 2008.
6. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer **7**: 169-181, 2007.
7. Nagai Y, Miyazawa H, Huqun D, 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 **65**: 7276-7282, 2005.
8. Thatcher N, Chang A, Parikh P, et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). Lancet **366**: 1527-1537, 2005.
9. Rikova K, Guo A, Zeng Q, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell **131**: 1190-1203, 2007.
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 **14**: 4275-4283, 2008.
11. Soda M, Takada S, Takeuchi K, et al. A mouse model for EML4-ALK-positive lung cancer. Proc Natl Acad Sci U S A **105**: 19893-19897, 2008.
12. 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 **22**: 508-515, 2009.
13. 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 **27**: 4247-4253, 2009.
14. Shinmura K, Kageyama S, Tao H, et al. EML4-ALK fusion transcripts, but no NPM-, TPM3-, CLTC-, ATIC-, or TFG-ALK fusion transcripts, in non-small cell lung carcinomas. Lung Cancer **61**: 163-169, 2008.
15. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res **14**: 6618-6624, 2008.
16. 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 **15**: 3143-3149, 2009.

Treatment of Lung Cancer with an ALK Inhibitor After *EML4-ALK* Fusion Gene Detection Using Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration

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A 40-year-old man who had complained of bloody sputum was referred to our hospital for workup. Chest computed tomography showed a significant mediastinal lymphadenopathy (Figure 1*A*). Bronchoscopic examination revealed a tumor compressing the right mainstem bronchus (Figure 2*A*). Massive bleeding from the tumor was caused by passage of the bronchoscope. Therefore, a diagnosis of pulmonary adenocarcinoma was made by sputum cytology. The patient first received conventional chemotherapy in the form of four courses of cisplatin plus vinorelbine (CDDP + VNR), two cycles of cisplatin plus gemcitabine (CDDP + GEM), and four cycles of carboplatin plus gemcitabine (CBDCA + GEM). However, both the size of the tumor and the serum carcinoembryonic antigen level continued to increase. Fluorodeoxyglucose positron emission tomography suggested systemic metastasis in hilar and mediastinal lymph nodes and bone (Figure 1*B*).

We performed endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) to avoid bleeding from the tumor. Metastatic adenocarcinoma was revealed in an upper paratracheal lymph node (#2R) (Figures 2*B*, *C*). Because the epidermal growth factor receptor gene was wild type, we examined the presence of ALK fusion genes. Immunohistochemistry by the intercalated antibody-enhanced polymer (iAEP) method[1] showed an expression of ALK protein in the samples obtained by EBUS-TBNA (Figure 2*D*). *EML4-ALK* fusion gene was also confirmed by both fluorescence in situ hybridization (Figure 2*E*) and reverse transcriptase-polymerase chain reaction (Figure 2*F*). Direct sequencing of the PCR product revealed the presence of *EML4-ALK* variant 1. Thus, we referred the patient for enrollment in a clinical trial with crizotinib (PF-02341066).[2] Six weeks after administration of the crizotinib (250 mg twice a day, oral administration), the bloody sputum disappeared, and the tumor size decreased on chest computed tomography (Figure 1*C*). The carcinoembryonic antigen level also normalized. Five months after administration, an abnormal accumula-

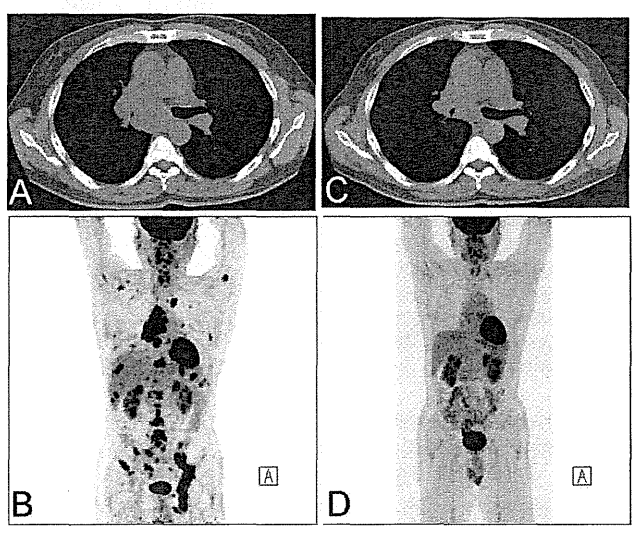

FIGURE 1. *A*, Chest computed tomography showed a narrowing of the right main bronchus due to massive lymphadenopathy. *B*, FDG-PET suggested multiple lymph node metastases and bone metastases. *C*, Six weeks after administration of the ALK inhibitor, the effect of the treatment was judged as partial response based on RECIST. *D*, Five months after administration of the ALK inhibitor, abnormal accumulation on FDG-PET had disappeared. FDG-PET, fluorodeoxyglucose positron emission tomography.

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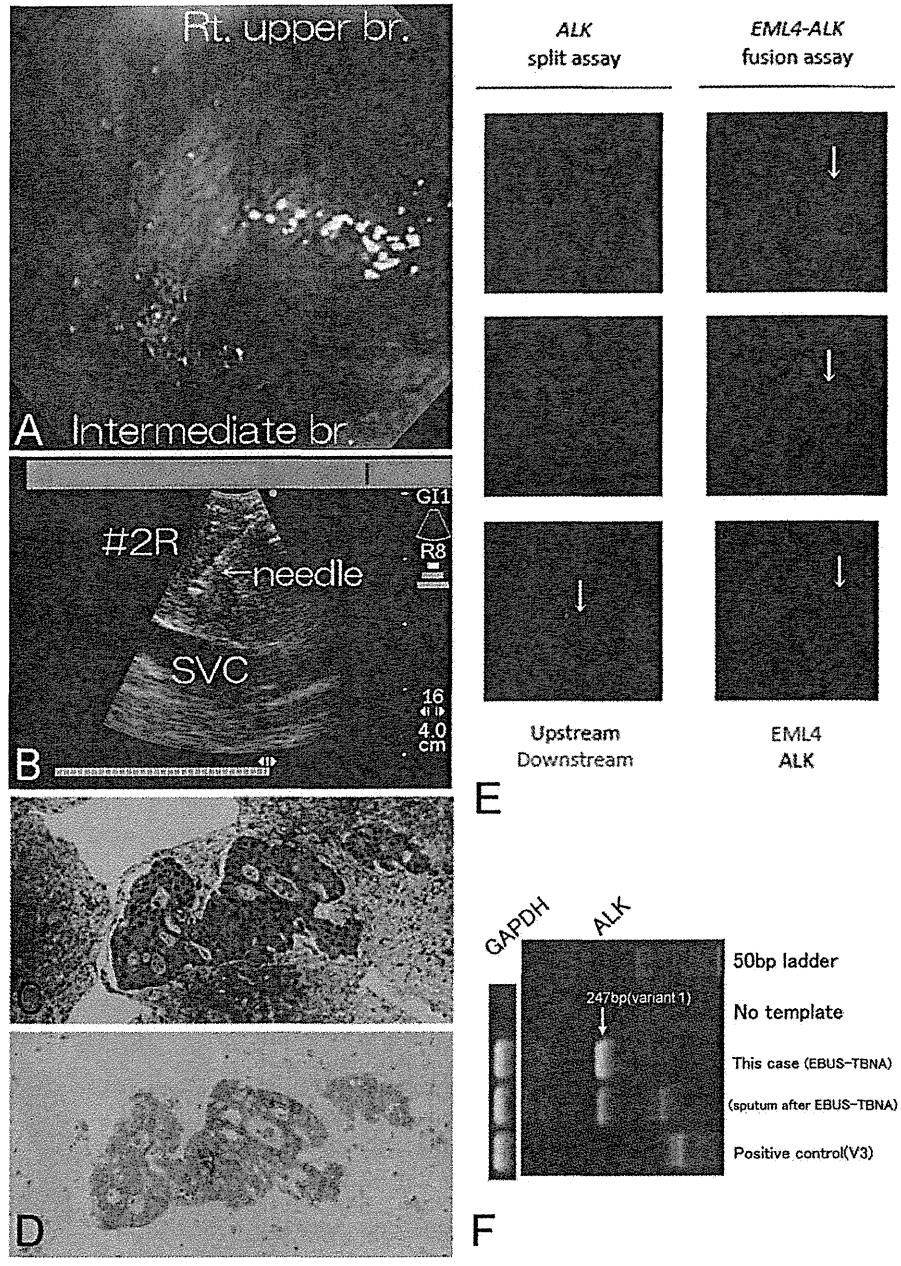

FIGURE 2. *A,* Bronchoscopic examination showed tumor compression of the right main bronchus, and the tumor had hyperplastic vessels on its surface. *B,* EBUS-TBNA was performed for a pretracheal lymph node (#2R). *C,* Histologic core revealed metastatic adenocarcinoma in #2R node. *D,* Immunohistochemistry was positive for ALK protein using the iAEP method. *E,* FISH revealed the EML4-ALK fusion gene. EML4-ALK split assay with labeled probes for the upstream (red) and downstream (green, arrow) region of the ALK locus. EML4-ALK fusion assay with labeled probes for EML4 (green, arrow) or ALK (red, arrow). Fusion gene showed EML4-ALK (arrow). *F,* RT-PCR using specific primer set for each variant also confirmed the presence of EML4-ALK variant 1 (274bp). The presence of variant 1 type fusion was also confirmed by direct sequence of the RT-PCR product (data not shown). RT-PCR, reverse transcriptase-polymerase chain reaction; FISH, fluorescence in situ hybridization.

tion almost disappeared on fluorodeoxyglucose positron emission tomography scan (Figure 1*D*). The observed side effects were only slight nausea during the early period of administration. The patient remains in good condition without tumor relapse for 10 months. The patient suddenly complained bilateral lower extremities paralysis, and the spinal cord metastasis was revealed. The patient was discontinued treatment during the trial in April 2010 because of disease progression.

DISCUSSION

Fusion of *ALK* with *EML4* gives rise to a highly potent oncogene in non-small cell lung cancer,[3] being detected in ~5% of all non-small cell lung cancer cases.[1,3,4] Presence of the ALK fusions can be detected by immunohistochemical screening[4] and can be also confirmed by fluorescence in situ hybridization and reverse transcriptase-polymerase chain reaction.[4] Recently, with progress in chemotherapeutic research, molecular targeted therapeutic agents have been developed, including ALK kinase inhibitors that are now being clinically tested.[2] Ideally, ALK fusion gene assessment should be performed using minimally invasive means to obtain biopsy samples sufficient for genetic analysis for subsequent targeted molecular therapy. Histologic as well as cytologic samples can be obtained by EBUS-TBNA, and we have previously reported that high-quality cores are adequate for molecular analyses for biomarkers.[5] The dramatic

effect of the ALK inhibitor in this patient demonstrates that adequate biomarker assessment contributes to the optimum selection of reagents in targeted molecular therapy and in individualized treatment.

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REFERENCES

1. 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;22:508–515.
2. Bang Y, Kwak EL, Shaw AT, et al. Clinical activity of the oral ALK inhibitor PF-02341066 in ALK-positive patients with non-small cell lung cancer (NSCLC). *J Clin Oncol* 2010;28(18s):Abstract 3.
3. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* 2007; 448:561–566.
4. 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;15:3143–3149.
5. 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.

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EML4-ALK Fusion in Lung

To the Editor-in-Chief:

The recent article by Martelli and colleagues[1] reports (i) the detection of *EML4-ALK* fusion cDNA[2] not only in non-small cell lung cancer (NSCLC) specimens but in non-tumor lung tissues, (ii) a very low proportion of FISH-positive cells for *ALK* rearrangements among *EML4-ALK*–positive specimens, and (iii) the failure to detect EML4-ALK protein by immunohistochemistry (IHC) and Western blotting. Based on these lines of observation, the authors questioned the clinical relevance of *EML4-ALK* in the carcinogenesis of NSCLC.

Although detection of fusion kinases in normal tissues is a potentially interesting observation, caution is warranted in the interpretation of their results.[1,3] They replicated thrice the reverse transcription-polymerase chain reaction (RT-PCR) for *EML4-ALK* and noted that "In half of the (positive) cases, one replicate experiment did not confirm the fusion transcript was present." They then suggested that the fusion gene was "expressed at very low level." It is, however, also quite possible that such unstable PCR results may simply represent contaminated experiments. If this is the case, a discussion on FISH and protein analyses would become irrelevant. In their report, the presence of the *EML4-ALK* fusion gene was only evidenced by unstable RT-PCR results and a small proportion of FISH-positive cells among specimens.

In this regard, it was surprising that the authors had not tried genomic PCR to exclude the possibility of PCR contamination.[1,3] In most of their fusion-positive cases, they found the *EML4-ALK* variant 1 cDNA, in which exon 13 of *EML4* cDNA is connected to exon 20 of *ALK* cDNA. Because the length of intron 14 of *EML4* gene and intron 19 of *ALK* gene is 5724 bp and 1932 bp, respectively, the maximum size of the genomic PCR to detect the gene fusion should be ≈7.7 kbp, which is within the scope of current long-range PCR systems. Indeed, we have been able to detect genomic PCR products among >50% of the fusion cDNA-positive cases. Interestingly, the break/fusion points in the genome vary substantially among NSCLC specimens,[2,4,5] and we have not obtained, to date, any pairs of NSCLC specimens carrying identical break/fusion points in their genome (even among those positive for the same *EML4-ALK* variants).

We speculate, therefore, that (i) if none of the fusion cDNA-positive cases reported by Martelli et al[1,3] produce specific genomic PCR products, then the fusion cDNA products likely arose from cDNA-contamination, (ii) if the fusion cDNA–positive cases yield identical genomic PCR products, then the fusion cDNAs likely arose from specimen-contamination, and (iii) if the fusion cDNA-positive cases display distinct genomic fusion points, then each specimen was truly positive for the *EML4-ALK* fusion gene. Without such careful examination, we have to conclude that their claims in the article have not as yet been clearly demonstrated.

As described previously,[6] immunohistochemical detection of the EML4-ALK protein is highly difficult, probably owing to the weak activity of the *EML4* promoter that drives the expression of *EML4-ALK* messages. We have thus examined the suitability of commercially available antibodies to ALK for IHC and successfully developed the intercalated antibody–enhanced polymer (iAEP) method, which enables reliable detection of EML4-ALK among formalin-fixed and paraffin-embedded specimens.[6] The same specimen positive for *EML4-ALK* RT-PCR can be, for instance, readily stained to be positive with iAEP, but negative with conventional IHC methods (see Supplemental Figure S1 in ref. 6). We thus agree with Martelli et al that screening of NSCLC specimens with conventional IHC methods will not detect EML4-ALK protein, but strongly argue that such failure does not simply indicate the absence of EML4-ALK. For such screening, we recommend iAEP or other sensitive techniques.[7]

It should be further noted that, in both our[6] and other researchers' IHC analyses,[7] almost all tumor cells in a given *EML4-ALK*–positive specimen were positively immunostained with anti-ALK antibodies, suggesting a homogenous presence of *EML4-ALK* within a tumor. Such observation is, however, in contrast to the FISH data by Martelli et al, which show that the *ALK* rearrangement was only positive in ≈2% of tumor cells in a given *EML4-ALK*–positive specimen. On the contrary, FISH analyses of our *EML4-ALK*–positive samples clearly demonstrate that most of the tumor cells harbor rearranged *ALK* alleles, implying that the generation of the *EML4-ALK* fusion gene is an early event in NSCLC carcinogenesis. The homogenous presence of EML4-ALK in our fusion-positive tumors, as demonstrated by both FISH and IHC, further raises a concern about the "EML4-ALK–positive tumors" as defined by Martelli et al.

Specific inhibitors to ALK enzymatic activity are already in clinical trial, as reported at the 2009 annual meeting of America Society of Clinical Oncology and the European Cancer Organization and Congress of the European Soci-

ety for Medical Oncology.[8] Such reports reveal only modest and transient side effects (nausea, vomiting, and diarrhea) with their ALK inhibitor, but without severe damage in hematopoiesis or renal function. On the other hand, the marked therapeutic efficacy of their compound against EML4-ALK–positive NSCLC makes it one of the rare, highly successful molecular targeted therapies against human cancer, in line with imatinib mesylate and gefitinib/erlotinib. These data further reinforce the essential role of EML4-ALK in the carcinogenesis of NSCLC, and question the validity of the conclusions led by Martelli et al.[1,3]

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References

1. Martelli MP, Sozzi G, Hernandez L, Pettirossi V, Navarro A, Conte D, Gasparini P, Perrone F, Modena P, Pastorino U, Carbone A, Fabbri A, Sidoni A, Nakamura S, Gambacorta M, Fernandez PL, Ramirez J, Chan JK, Grigioni WF, Campo E, Pileri SA, Falini B: EML4-ALK rearrangement in non-small cell lung cancer and non-tumor lung tissues. Am J Pathol 2009, 174:661–670
2. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y, Mano H: Identification of the transforming *EML4-ALK* fusion gene in non-small-cell lung cancer. Nature 2007, 448:561–566
3. Sozzi G, Martelli MP, Conte D, Modena P, Pettirossi V, Pileri SA, Falini B: The EML4-ALK transcript but not the fusion protein can be expressed in reactive and neoplastic lymphoid tissues. Haematologica 2009, 94:1307–1311
4. Choi YL, Takeuchi K, Soda M, Inamura K, Togashi Y, Hatano S, Enomoto M, Hamada T, Haruta H, Watanabe H, Kurashina K, Hatanaka H, Ueno T, Takada S, Yamashita Y, Sugiyama Y, Ishikawa Y, Mano H: Identification of novel isoforms of the *EML4-ALK* transforming gene in non-small cell lung cancer. Cancer Res 2008, 68:4971–4976
5. Takeuchi K, Choi YL, Soda M, Inamura K, Togashi Y, Hatano S, Enomoto M, Takada S, Yamashita Y, Satoh Y, Okumura S, Nakagawa K, Ishikawa Y, Mano H: Multiplex reverse transcription-PCR screening for *EML4-ALK* fusion transcripts. Clin Cancer Res 2008, 14:6618–6624
6. Takeuchi K, Choi YL, Togashi Y, Soda M, Hatano S, Inamura K, Takada S, Ueno T, Yamashita Y, Satoh Y, Okumura S, Nakagawa K, Ishikawa Y, Mano H: KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res 2009, 15:3143–3149
7. Rodig SJ, Mino-Kenudson M, Dacic S, Yeap BY, Shaw A, Barletta JA, Stubbs H, Law K, Lindeman N, Mark E, Janne PA, Lynch T, Johnson BE, Iafrate AJ, Chirieac LR: Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. Clin Cancer Res 2009, 15:5216–5223
8. Kwak EL, Camidge DR, Clark J, Shapiro GI, Maki RG, Ratain MJ, Solomon B, Bang Y, Ou S, Salgia R: 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, (suppl, abstr 3509)

Authors' reply:

In their letter, Mano and Takeuchi claim that our unstable PCR results in normal and cancerous lung tissues could be attribtuable to contamination. However, as clearly illustrated in our article,[1] serial dilution experiments in the H2228 cell line demonstrate the specificity and sensitivity of our RT-PCR assay. Furthermore, the identification in our EML4-ALK fusion positive tissues of alternative isoforms of variant 3, rather than the described two isoforms coexpressed in the H2228 cell line, is indicative of exclusive events in tumors, making contamination unlikely. Lastly, our experiments were confirmed independently in two laboratories (Milan and Barcelona) and always contained appropriate negative PCR controls.

We disagree with Mano et al's claim that the results of genomic PCR could be used to prove a possible RT-PCR contamination in our samples, which can only be excluded by the use of appropriate controls and procedures, as outlined above. However, we used genomic PCR to amplify the sequence flanking the *EML4-ALK* variant 1 breakpoint in four positive NSCLC samples. Even though a strong amplification product had been obtained from the same DNA templates using primer sets amplifying a control genomic locus of similar size to that of the cases so far reported in literature, no amplification of the *EML4-ALK* variant 1 fusion product was identified, suggesting only a minority of cells carried the *EML4-ALK* gene. These findings concur with Maes et al[2] who reported that, in lymphoid tissues, high level detection of *NPM-ALK* and *ATIC-ALK* fusion transcripts coincided with *ALK* gene rearrangements (as detected by cytogenetics and FISH), whereas low-level detection was not supported by genomic evidence of rearrangements.

In our article,[1] we clearly stated that, unlike observations in ALK+ lymphomas, tumor cells from NSCLC specimens expressed such a low amount of the EML4-ALK fusion protein that immunoprecipitation and immunohistochemistry performed with the commercially available antibodies are unable to detect it. This is in keeping with the observation that the EML4-ALK fusion protein is detectable only using highly sensitive methods, such as mass spectrometry[3] or the intercalated antibody-enhanced polymer (iAEP) method[4] which, unfortunately, are not available in all pathology laboratories and are difficult to standardize. Therefore, the question of how best to detect the EML4-ALK fusion protein remains unanswered.

Issues concerning the frequency, heterogeneity, and tissue specificity of the *EML4-ALK* rearrangement must also be addressed carefully.

Frequency

We recently extended our FISH analysis to 173 surgically resected lung cancer specimens (mainly adenocarcinoma) from an unselected group of Caucasian patients. The incidence of truly positive cases (>50% FISH positive, fusion transcript, and protein positive) was only 0.6% (1/173 cases), which reinforces the results in our artile and is in keeping with Rodig et al's[5] recent report of 1/227 (0.45%) ALK rearranged case in a series of surgically treated Western adenocarcinoma.

Heterogeneity

The heterogeneity of the *EML4-ALK* rearrangement we detected by FISH was confirmed by others in primary tumors

and cell lines[6,7] and is supported by functional studies showing that the magnitude of growth inhibition by siRNA-mediated silencing did not correlate with the number of cells harboring the rearrangement and the lack of growth inhibition in 50% of *EML4-ALK*–positive cell lines. These observations suggest that additional signaling mechanisms independent of ALK may regulate growth and cell proliferation.

Specificity

Claims from Mano's group that the *EML4-ALK* product is specific for NSCLC is contradicted by our findings in normal tissues[1,8] and by a recent study from Lin E. et al,[6] who found *EML4-ALK* fusions in breast (2.4%) and colorectal (2.4%) cancer, in addition to NSCLC.

Finally, we wonder whether it is really appropriate to compare treatments such as ALK inhibitors in NSCLC with imatinib mesylate and gefinitinib/erlotinib in other human neoplasms. In fact: i) the role of EML4-ALK in NSCLC is not as well established as that of BCR/ABL in chronic myeloid leukemia (CML); ii) NSCLC responses to ALK inhibitors[9] are not as remarkable as the CML response to imatinib mesylate; and iii) patients with NSCLC were treated with a multikinase, c-MET and ALK, inhibitor.[9] Considering that about 20% of NSCLC have MET amplification and overexpression and that MET rearrangements are homogeneous in lung cancer,[10] it may be possible that responses to the multikinase inhibitor may be related to other coexisting oncogenic events, independently of ALK.

In conclusion, although we fully acknowledge the importance of Soda et al's discovery,[11] we believe that additional studies are required to elucidate the concurrent genetic events and cellular settings necessary for *EML4-ALK* to exert an oncogenic function and to better define the role of *EML4-ALK* in diagnosis and targeted therapy of NSCLC.

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References

1. Martelli MP, Sozzi G, Hernandez L, Pettirossi V, Navarro A, Conte D, Gasparini P, Perrone F, Modena P, Pastorino U, Carbone A, Fabbri A, Sidoni A, Nakamura S, Gambacorta M, Fernandez PL, Ramirez J, Chan JK, Grigioni WF, Campo E, Pileri SA, Falini B: EML4-ALK rearrangement in non-small cell lung cancer and non-tumor lung tissues. Am J Pathol 2009, 174:661–670
2. Maes B, Vanhentenrijk V, Wlodarska I, Cools J, Peeters B, Marynen P, de Wolf-Peeters C: The NPM-ALK and the ATIC-ALK fusion genes can be detected in non-neoplastic cells. Am J Pathol 2001, 158: 2185–2193
3. Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H, Nardone J, Lee K, Reeves C, Li Y, Hu Y, Tan Z, Stokes M, Sullivan L, Mitchell J, Wetzel R, Macneill J, Ren JM, Yuan J, Bakalarski CE, Villen J, Kornhauser JM, Smith B, Li D, Zhou X, Gygi SP, Gu TL, Polakiewicz RD, Rush J, Comb MJ: Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 2007, 131:1190–1203
4. Takeuchi K, Choi YL, Togashi Y, Soda M, Hatano S, Inamura K, Takada S, Ueno T, Yamashita Y, Satoh Y, Okumura S, Nakagawa K, Ishikawa Y, Mano H: KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res 2009, 15:3143–3149
5. Rodig SJ, Mino-Kenudson M, Dacic S, Yeap BY, Shaw A, Barletta JA, Stubbs H, Law K, Lindeman N, Mark E, Janne PA, Lynch T, Johnson BE, Iafrate AJ, Chirieac LR: Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. Clin Cancer Res 2009, 15:5216–5223
6. Lin E, Li L, Guan Y, Soriano R, Rivers CS, Mohan S, Pandita A, Tang J, Modrusan Z: Exon array profiling detects EML4-ALK fusion in breast, colorectal, and non-small cell lung cancers. Mol Cancer Res 2009, 7:1466–1476
7. Perner S, Wagner PL, Demichelis F, Mehra R, Lafargue CJ, Moss BJ, Arbogast S, Soltermann A, Weder W, Giordano TJ, Beer DG, Rickman DS, Chinnaiyan AM, Moch H, Rubin MA: EML4-ALK fusion lung cancer: a rare acquired event. Neoplasia 2008, 10:298–302
8. Sozzi G, Martelli MP, Conte D, Modena P, Pettirossi V, Pileri SA, Falini B: The EML4-ALK transcript but not the fusion protein can be expressed in reactive and neoplastic lymphoid tissues. Haematologica 2009, 94:1307–1311
9. Kwak E, Camidge D, Clark J, Shapiro G, Maki R, Ratain M, Solomon B, Bang Y, Ou S, Salgia R: Clinical acitvity observed in a phase I dose escalation trial of an oral c-met and ALK inhibitor, PF-02341066. J Clin Oncol 2009, 27:15s (Suppl, Abstr 3509)
10. Beau-Faller M, Ruppert AM, Voegeli AC, Neuville A, Meyer N, Guerin E, Legrain M, Mennecier B, Wihlm JM, Massard G, Quoix E, Oudet P, Gaub MP: MET gene copy number in non-small cell lung cancer: molecular analysis in a targeted tyrosine kinase inhibitor naive cohort. J Thorac Oncol 2008, 3:331–339
11. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, Hatanaka H, Bando M, Ohno S, Ishikawa Y, Aburatani H, Niki T, Sohara Y, Sugiyama Y, Mano H: Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007, 448:561–566