Imaging, Diagnosis, Prognosis

A Prospective PCR-Based Screening for the EML4-ALK Oncogene in Non–Small Cell Lung Cancer

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Introduction

An oncogenic fusion between the echinoderm microtubule-associated protein–like 4 gene (EML4) and the anaplastic lymphoma kinase gene (ALK) was discovered by functional screening with a non–small cell lung cancer (NSCLC) specimen (1). EML4 and ALK are located within a short distance (~12 Mbp) of each other on the short arm of human chromosome 2, and a small inversion involving the 2 loci is responsible for generation of the EML4-ALK fusion in lung cancer. The EML4-ALK tyrosine kinase undergoes constitutive dimerization through a coiled-coil domain within EML4, resulting in kinase activation and conferring potent transforming ability (2, 3). Transgenic mice expressing EML4-ALK in lung alveolar cells develop multiple adenocarcinoma nodules soon after birth, but treatment with an ALK inhibitor results in the rapid clearance of such nodules, confirming the addiction of EML4-ALK–positive tumors to the kinase activity of the fusion protein (4). The therapeutic efficacy of ALK inhibitors has been confirmed in other transgenic mice expressing EML4-ALK (5).

Several ALK inhibitors have already entered clinical trials or are under preclinical development (6–10). Marked therapeutic efficacy of one such compound, crizotinib, has been described in patients with NSCLCs positive for EML4-ALK, with an overall response rate of 57% (7), and crizotinib was recently approved as a therapeutic drug by the U.S. Food and Drug Administration. Two other ALK inhibitors, lorlatinib (8) and ceritinib (9), have also shown promise in clinical trials.

The nucleotide sequence of the novel EML4-ALK variant cDNA from patient J-#189 has been deposited in the DDBJ/EMBL/GenBank databases under the accession number AB663645.

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Abstract

Purpose: EML4-ALK is a lung cancer oncogene, and ALK inhibitors show marked therapeutic efficacy for tumors harboring this fusion gene. It remains unsettled, however, how the fusion gene should be detected in specimens other than formalin-fixed, paraffin-embedded tissue. We here tested whether reverse transcription PCR (RT-PCR)-based detection of EML4-ALK is a sensitive and reliable approach.

Experimental Design: We developed a multiplex RT-PCR system to capture ALK fusion transcripts and applied this technique to our prospective, nationwide cohort of non–small cell lung cancer (NSCLC) in Japan.

Results: During February to December 2009, we collected 916 specimens from 853 patients, quality filtering of which yielded 808 specimens of primary NSCLC from 754 individuals. Screening for EML4-ALK and KIF5B-ALK with our RT-PCR system identified EML4-ALK transcripts in 36 samples (4.46%) from 32 individuals (4.24%). The RT-PCR products were detected in specimens including bronchial washing fluid (n = 11), tumor biopsy (n = 8), resected tumor (n = 7), pleural effusion (n = 5), sputum (n = 4), and metastatic lymph node (n = 1). The results of RT-PCR were concordant with those of sensitive immunohistochemistry with ALK antibodies.

Conclusions: Multiplex RT-PCR was confirmed to be a reliable technique for detection of ALK fusion transcripts. We propose that diagnostic tools for EML4-ALK should be selected in a manner dependent on the available specimen types. FISH and sensitive immunohistochemistry should be applied to formalin-fixed, paraffin-embedded tissue, but multiplex RT-PCR is appropriate for other specimen types. Clin Cancer Res; 18(20); 5682–9. ©2012 AACR.
Translational Relevance

The recent approval of an ALK inhibitor by the U.S. Food and Drug Administration has rendered urgent the development of a diagnostic scheme for tumors harboring ALK fusion genes. Whereas FISH is effective for analysis of formalin-fixed, paraffin-embedded (FFPE) tissue, how to test other types of speciemen remains unsettled. We conducted a prospective, nationwide screening for EML4-ALK- or KIF5B-ALK-positive lung carcinomas in Japan with the use of a newly developed multiplex reverse transcription (RT)-PCR system. Various subtypes of EML4-ALK cDNA were identified in 36 of 808 specimens with adequate RNA quality. The RT-PCR results were concordant with those of immunohistochemistry, and EML4-ALK PCR products were detected in independent specimens from the same individuals. As far as we are aware, our study represents the first prospective RT-PCR–based screening for EML4-ALK, and it shows that multiplex RT-PCR is reliable for detection of the fusion gene in non-FFPE specimens.

Materials and Methods

Prospective collection of NSCLC specimens

During February to December of 2009, we collected a total of 916 lung cancer specimens from 853 independent patients through our multicenter, nationwide networks in Japan. All specimens but resected tumors were mixed with RLT buffer (Qiagen) immediately after sampling, a step that markedly inhibits RNA degradation for up to 3 days at room temperature (data not shown). Resected tumor samples were snap-frozen and stored at −80 °C until extraction of RNA and DNA. Portions of the samples were sent to Jichi Medical University (Tochigi, Japan) for multiplex RT-PCR analysis of EML4-ALK and KIF5B-ALK fusions and to Saitama Medical University (Saitama, Japan) for peptide nucleic acid–locked nucleic acid (PNA-LNA) PCR clamp analysis of EGFR mutations (16). All specimens were confirmed by pathologic analysis to contain malignant cells. More than half of the specimens were collected through the North-East Japan Study Group network according to the NEJ004 protocol. The study was approved by the Institutional Review Board of each participating center, and written informed consent was obtained from each study subject. All statistical analysis was conducted with 2-sided tests, and a P < 0.05 was considered statistically significant.

Clinicopathologic features of EML4-ALK–positive NSCLC

The clinicopathologic features of patients with EML4-ALK–positive or -negative tumors in our cohort are summarized in Table 1 and Supplementary Table S1. Consistent with previous observations, EML4-ALK–positive patients were significantly younger than those without EML4-ALK (P < 0.001, Student’ t test) and were enriched in never or light smokers (P < 0.001, Fisher exact test). Our data also indicated that EML4-ALK–positive tumors are more likely to occur in women than in men (P < 0.001, Fisher exact test). In the present cohort, EML4-ALK was detected only in lung adenocarcinoma (P < 0.001, Fisher exact test), for which the fusion-positive rate was 6.11%.

A total of 718 specimens were screened for EGFR mutations, with such mutations being detected in 171 cases (23.8%). Whereas most EML4-ALK–positive tumors did not harbor EGFR mutations (P = 0.002, Fisher exact test), we did detect one tumor doubly positive in this regard. EML4-ALK and EGFR mutations are largely mutually exclusive (17, 18), but, importantly, such exclusiveness may not be absolute (19). Given that the presence of EML4-ALK and EGFR mutations in our doubly positive patient was examined with cells isolated from bronchial washing fluid, which was the only available specimen for molecular analysis in this individual, we were not able to determine whether there was a genuinely double-positive tumor in the lung or there were multiple independent tumors each positive for EML4-ALK or mutated EGFR.

We also attempted to examine the mutation status of KRAS among our 32 cases positive for EML4-ALK. We were able to sequence KRAS cDNAs for 26 of these patients, none of whom showed KRAS alterations (data not shown), confirming the mutual exclusivity of EML4-ALK and KRAS mutations (17, 20, 21).

Quality assessment of samples

Complementary DNA prepared from the specimens was first subjected to RT-PCR analysis with primers (5’-
Given that we previously showed that the abundance of (RNase P) gene (GenBank accession number NM_005837).

Table 1. Characteristics of subjects positive for EML4-ALK by the RT-PCR diagnostic system.

<table>
<thead>
<tr>
<th>Identification number</th>
<th>Sex/age</th>
<th>Pathologic classification</th>
<th>EML4-ALK variant</th>
<th>Smoking history (pack-years)</th>
<th>TNM classification</th>
<th>Clinical stage</th>
<th>EGFR mutation</th>
<th>KRAS mutation</th>
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<td>E13;A20</td>
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<td>+</td>
<td>-</td>
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<td>4</td>
<td>+</td>
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<td>E1a/bcA20</td>
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<td>F/62</td>
<td>Adenocarcinoma</td>
<td>E13;A20</td>
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<td>cT4N2M0</td>
<td>3B</td>
<td>ND</td>
<td>-</td>
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<td>E13;A20</td>
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<td>1A</td>
<td>ND</td>
<td>-</td>
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<td>cT1N3M1</td>
<td>3B</td>
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<td>ND</td>
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<tr>
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<td>cT2N2M1</td>
<td>4</td>
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<td>-</td>
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</table>

Abbreviations: F, female; M, male; NA, not available; ND, not determined.

CTGTTGGAGGCTGAACCTGGATC-3' and 5'-TCATCAACAG-GCTCCACGGTG-3' specific for the human ribonuclease P (RNase P) gene (GenBank accession number NM_005837). Given that we previously showed that the abundance of RNase P mRNA is similar to that of EML4-ALK mRNA in NSCLCs (data not shown), we used the successful amplification of RT-PCR products for RNase P as a threshold for selection of specimens for further analysis. Exclusion of small cell lung cancer specimens and filtering on the basis of RNase P mRNA abundance resulted in the isolation of 808 specimens of primary NSCLCs obtained from 754 individuals.

As shown in Supplementary Fig. S1, bronchial washing fluid, including bronchoalveolar lavage fluid and washing fluid for the brush, needle, forceps, and other implements used in bronchoscopy, constituted 66.3% of the 808 eligible samples, with the remaining specimens including pleural effusion (12.8%); surgically resected tumor (7.05%); sputum (4.33%); tumor biopsy tissue including that obtained by transbronchial lung biopsy and transbronchial needle aspiration (3.71%); peripheral blood (3.71%); cardiac effusion, spinal fluid, or ascites (1.36%); and metastatic lesions of NSCLCs (0.74%).

**Multiplex RT-PCR analysis of EML4-ALK and KIF5B-ALK**

Each specimen (with the exception of resected tumors) was mixed with an equal volume of RLT buffer at the Institute at which it was harvested. The resulting mixture was sent to Jichi Medical University, where DNA and RNA were extracted with the use of an automated BioRobot EZ1 workstation (Qiagen). The isolated RNA was subjected to RT with a ReverTra Ace qPCR RT kit (Toyobo), and the resulting cDNA was subjected to PCR for 50 cycles of incubation at 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute with AmpliTaq Gold DNA polymerase (Applied Biosystems) and with 2 μmol/L of each of the following primers:
primers: F-1, 5′-GCCTTCGGGAAATGAAGGGAAGG-3′; F-2, 5′-TCTACAGGTTCGCTGTTAAGGACAAAGG-3′; F-3, 5′-GTGCTGATGACGTCTTGCTGGG-3′; F-4, 5′-ACCTACTACACACCACCACCTGGG-3′; F-5, 5′-TCAAGCACATCCTCAAGGACGCAATG-3′; F-6, 5′-TCCTGGCGGAACACTATTCAGTGG-3′; F-7, 5′-GACAGTTGGAGGAATCTGTCGATG-3′; F-8, 5′-CTTGATGAGGGAAGTCGGACGAGGG-3′; F-9, 5′-GCTTTCCCCGCAAGATGGACGG-3′; F-10, 5′-ATCCTGCGGAACACTATTCAGTGG-3′; F-11, 5′-TACCAGTGCTGTCTCAATTGCAGG-3′; F-12, 5′-ATCCTGCGGAACACTATTCAGTGG-3′; F-13, 5′-GCTTTCCCCGCAAGATGGACGG-3′; F-14, 5′-ATCCTGCGGAACACTATTCAGTGG-3′.

Results

Multiplex RT-PCR system

In addition to the original EML4-ALK fusion cDNA in which exon 13 of EML4 is fused to exon 20 of ALK in an in-frame manner (designated the E13;A20 variant by analogy with karyotype nomenclature; see http://atlasgeneticsoncology.org/Tumors/inv2p21p23NSCCLungID5667.html), 14 different variants of EML4-ALK have been described (1, 14, 21–27). Seven exons of EML4 are theoretically capable of in-frame fusion with exon 20 of ALK (Fig. 1A), and all but the E1;A20 variant would be expected to produce an oncogenic EML4-ALK protein, given that the coiled-coil domain encoded by exon 2 is required for constitutive dimerization of EML4-ALK. In addition, 6 different exons of KIF5B are theoretically capable of in-frame fusion with exon 20 of ALK (Fig. 1A).

To detect any such EML4-ALK or KIF5B-ALK fusion mRNAs, we developed a multiplex RT-PCR system. We had previously screened our archive of frozen tumors by RT-PCR analysis with 2 forward primers targeted to EML4 and 1 reverse primer targeted to ALK (24), but such PCR conditions resulted in the amplification of products as large as ~1,300 bp for some variants. In this prospective study, we were faced with the analysis of a large number of samples with different levels of RNA quality. If the size of PCR products varied substantially among different EML4-ALK or KIF5B-ALK variants, some variants with large PCR products might not be amplified efficiently from specimens with low RNA quality. To be able to diagnose all possible fusions even with such samples, we therefore designed 4 forward primers for each of EML4 and KIF5B so that the size variation among all possible RT-PCR products is minimal (Fig. 1A). This new multiplex system faithfully detected all known fusion variants from EML4-ALK–positive specimens in our previous archive of NSCLCs (data not shown).

To examine the sensitivity of our RT-PCR system, we mixed EML4-ALK–expressing BA/F3 cells (0 to 1 × 10⁶) with EML4-ALK–negative cells (1 × 10⁶) and then subjected them to RT-PCR analysis. A fusion cDNA was readily identified even with 10 positive cells (0.001%) among 1 × 10⁶ negative cells (Fig. 1B), showing the high sensitivity of the RT-PCR system.

To confirm the potential of our RT-PCR–based system, we compared it with a sensitive immunohistochemical approach and with FISH for the diagnosis of our archive of surgically resected and freshly frozen tumors with high RNA quality. Fifteen NSCLC specimens that previously stained positive by our sensitive immunohistochemical approach, which is based on an intercalated antibody-enhanced polymer (iAEP) method (14), were analyzed by RT-PCR and FISH together with 96 iAEP-negative specimens in a blinded manner. RT-PCR analysis of all these specimens (n = 111) yielded a diagnosis identical to that obtained with the iAEP method (P = 7.3 × 10⁻⁹; Fisher exact test; data not shown). Analysis of the same sample set by a split FISH assay with Vysis probes (Abbott Laboratories) revealed that all of the iAEP-positive cases showed a rearranged ALK locus, whereas one iAEP-negative sample gave a discordant result (negative by iAEP and RT-PCR but positive by FISH; Supplementary Fig. S2). The reason for this discrepant result remains unclear, but the multiple signals obtained with the 3′-ALK probe in the FISH analysis are indicative of amplification of the ALK gene or its adjacent region. Despite this discrepancy, the RT-PCR and iAEP data were highly concordant with the FISH results (P = 1.2 × 10⁻¹⁷; Fisher exact test). Compared with the iAEP method, therefore, both the sensitivity and specificity of our RT-PCR system were 100%. In comparison with the Vysis FISH, the sensitivity and specificity of RT-PCR was 93.8% and 100%, respectively.

Detection of EML4-ALK

Screening of the 808 eligible specimens with our multiplex RT-PCR system identified positive products in 36 samples (4.46%) obtained from 32 different individuals.
Nucleotide sequencing of each PCR product identified 19 cases positive for the E13;A20 variant, 10 cases for E6a/b;A20, a single case each for E18;A20, E20;A20, and a novel variant. 

EML4-ALK was detected in a wide range of specimens including bronchial washing fluid (n = 11), tumor biopsy (n = 8), resected tumor (n = 7), pleural effusion (n = 5), sputum (n = 4), and metastatic lymph node (n = 1). We did not detect any KIF5B-ALK cDNAs, confirming the rarity of this fusion gene.

Importantly, an E13;A20 product was consistently identified in both of the sputa obtained at different time points from patient J-#1. Likewise, an E13;A20 product was detected in both the tumor biopsy and sputum from patient J-#53 as well as in the pleural effusion and 2 resected tumor specimens from patient J-#330, supporting the reliability of our RT-PCR approach.

Sequence determination for the RT-PCR product from patient J-#189 revealed that exon 14 of EML4 is thus spliced to a TT sequence that is located within EML4 intron 14 and which is directly ligated to intron 19 of ALK. This splicing event results in an in-frame fusion between the mRNA sequences derived from EML4 and ALK. Furthermore, a full-length cDNA for this variant, here designated E14::ins2;ins56A20, was isolated by RT-PCR analysis (Supplementary Fig. S3), and the potent transforming ability of the encoded protein was confirmed with an in vitro focus formation assay (Supplementary Fig. S4).

Comparison between multiplex RT-PCR and sensitive IHC

Finally, we applied the iAEP method to the EML4-ALK–positive cases for which FFPE specimens were also available (n = 15). All but 2 cases (J-#393 and J-#927) manifested clear immunoreactivity with antibodies to ALK (HE), subjected to immunohistochemical analysis by the iAEP method, and examined by split or fusion FISH. The color of fluorescence for the probes in each hybridization is indicated below the FISH images. Nuclei are stained blue with 4',6-diamidino-2-phenylindole (DAPI).
were not able to determine whether the PCR result was a false-positive. For J-#927, however, PCR analysis of genomic DNA with a forward primer targeted to EML4 exon 6 and a reverse primer to ALK exon 20 resulted in the amplification of an approximately 8.8-kbp genomic fragment, nucleotide sequencing of which revealed a fusion event between intron 6 of EML4 and intron 19 of ALK (Supplementary Fig. S5). Isolation of the genomic fusion point thus indicates that J-#927 indeed harbors an EML4-ALK–positive tumor.

Discussion
We have conducted a large-scale, prospective screening for EML4-ALK with an RT-PCR–based approach. Whereas RNA extraction and cDNA synthesis add extra labor to the diagnostic procedure, certain introns of EML4 are too large (intron 6 spans >16 kbp, for instance) for reliable amplification by genomic PCR. We therefore adopted RT-PCR as the method for our prospective screening. Specific PCR products were successfully isolated from different types of specimen, even from sputum (J-#1, J-#53, J-#215) and washing fluid of a tumor biopsy needle (J-#530). Multiple positive results obtained with different specimens of the same individuals further reinforce the reliability of our multiplex RT-PCR system as a diagnostic tool for EML4-ALK–positive tumors. Importantly, a subset of EML4-ALK–positive individuals diagnosed in the present study entered a clinical trial for crizotinib, and the response rate of the evaluable patients (n = 9) was 100% with this drug, again verifying the accuracy of our RT-PCR–based diagnosis.

The frequency of EML4-ALK in our cohort was 4.24% for all NSCLC cases and 6.11% for lung adenocarcinoma, values similar to those obtained in previous studies (20, 21). However, our prevalence data might be overestimates because the knowledge of mutual exclusiveness for EGFR and ALK mutations may have affected patient selection for our specimen collection. Indeed, EGFR mutation frequency among our cohort (23.8%) is slightly lower than that (30.9%) determined in a previous large-scale screening in Japan (28).

The clinicopathologic features of patients with EML4-ALK–positive tumors determined in the present study are also in agreement with those previously described, with a bias toward a young age, adenocarcinoma histology, and never or light smoking. Whereas a previous large-scale screening for EML4-ALK based on FISH did not detect a sex preference for the fusion gene (7), our cohort revealed a significant female preference. Such a sex difference was evident even among individuals below 40 years of age (P = 0.03, Fisher’s exact test) and among those with an adenocarcinoma histology (P = 0.005, Fisher’s exact test). Further large-scale studies are warranted to determine whether this uneven sex distribution of EML4-ALK is related to particular clinicopathologic features or ethnic groups.

Given that EML4-ALK and EGFR mutations are almost mutually exclusive and that the fusion gene is enriched in lung adenocarcinoma with an early onset, it should prove to be clinically beneficial to pay special attention to such subsets of patients. Indeed, EML4-ALK was detected in 27.7% of EGFR mutation–negative adenocarcinomas in individuals of younger than 50 years and in 50.0% of those in individuals of younger than 40 years in our cohort. Given the marked efficacy of ALK inhibitors in patients with EML4-ALK–positive NSCLCs (7), however, physicians should not dismiss the diagnosis in other subsets of patients. For example, EML4-ALK was even detected in an 80-year-old woman and in another woman with an intense smoking history (82 pack-years; Table 1).

Multiplex RT-PCR has both advantages and disadvantages compared with other techniques. Importantly, the accuracy of RT-PCR–based diagnosis depends markedly on the RNA quality of specimens. In our cohort, for instance, 71 (7.75%) of the initial 916 specimens were excluded from EML4-ALK screening because of a failure to obtain PCR products for RNase P (the other 37 samples were excluded because they were not NSCLCs). Low RNA quality thus clearly hampers reliable RT-PCR–based diagnosis. Also, as expected, there was a large variation in the PCR cycle number required for successful amplification among specimens. In our cohort, 50 cycles of PCR allowed detection of PCR products for all positive cases, but such extensive amplification may also generate nonspecific products (as shown in Fig. 2A). Further optimization of primer sequences or combinations may minimize the generation of such byproducts. Furthermore, whereas our system should be able to capture all in-frame fusions of ALK to EML4 or KIF5B, it is not capable in its present form of detecting ALK fusions to other partners, such as KLC1-ALK, which was recently shown to be present infrequently in NSCLCs (29).

On the other hand, RT-PCR can be readily applied to specimens such as sputum, bronchial washing fluid, or pleural effusion that may not be suitable for preparation of FFPE samples. Whereas the latter 2 specimen types can be used for the preparation of cell blocks suitable for analysis by FISH or IHC, this procedure may not be as widely adopted in the clinic as is FISH or IHC. More importantly, it is difficult to generate cell blocks or FFPE samples from sputum. Our current prospective screening identified 4 EML4-ALK–positive sputa of 35 samples (Table 1, Supplementary Fig. S1), showing that sputum is a suitable specimen for RT-PCR analysis. Indeed, sputum was the only available specimen from patient J-#215 both for the diagnosis of NSCLCs and for the detection of EML4-ALK. If RT-PCR had not been applied to this patient’s sputum, we would not have been able to identify her tumor as positive for EML4-ALK, and she would not have had the chance to receive treatment with an ALK inhibitor in Japan.

Furthermore, PCR-based detection of EML4-ALK should have a higher analytic sensitivity compared with IHC or FISH (Fig. 1B). Even with sputum obtained from a patient with chronic bronchitis, RT-PCR was able to readily detect EML4-ALK at a concentration of 10 positive cells/mL (1). Thus, provided that RNA is not substantially degraded, RT-PCR–based diagnosis is expected to have a strong advantage.
with regard to the detection of low numbers of EML4-ALK-positive cells.

Ideally, every NSCLC case should be examined for the presence of EML4-ALK, with a sensitive and accurate diagnostic strategy for the oncogenic fusion being essential for the adoption of ALK inhibitors in the clinic. Given the reliable detection of EML4-ALK mRNA by multiplex RT-PCR shown in the present study, we propose the following scheme for the comprehensive diagnosis of EML4-ALK-positive NSCLCs. For sputum, bronchial lavage fluid, pleural effusion, or other specimens that may not be suitable for the preparation of FFPE tissue, multiplex RT-PCR should be applied to detect ALK fusion mRNAs. In contrast, given that FFPE specimens usually have fragmented RNA, they should be subjected to FISH and to sensitive immunohistochemical analysis such as that described previously (14, 15). Furthermore, FISH or IHC can be applied to cell blocks prepared from some non-FFPE specimens. No single technique is therefore able to detect EML4-ALK in all types of specimen, and appropriate tests should be chosen on the basis of the specimens available for a given patient.

Disclosure of Potential Conflicts of Interest
H. Mano is the CEO of CareGene Co., Ltd., has commercial research grant from Illumina, Inc., and Astellas Pharma Inc.; has ownership interest (including patents); and is on the consultant/advisory board of Chugai Pharmaceutical, Astellas Pharma Inc., and Daichi Sankyo Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

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