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Large-Scale Screening and Molecular Characterization of EML4-ALK Fusion Variants in Archival Non–Small-Cell Lung Cancer Tumor Specimens Using Quantitative Reverse Transcription Polymerase Chain Reaction Assays

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Introduction: The objective of this study was to identify and characterize echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase fusion (EML4-ALK+) cancers by variant-specific, quantitative reverse transcription polymerase chain reaction (RT-PCR) assays in a large cohort of North American non–small-cell lung cancer (NSCLC) patients.

In summary, we here report the detection of 200 EML4-ALK fusion variants in 7344 North American NSCLC patients (2.7%) using variant-specific, quantitative RT-PCR assays. ALK expression level varied significantly among different EML4-ALK-positive variants and individual NSCLC tumors. EML4-ALK-positive tumors had a significantly lower TS RNA level compared with that of EML4-ALK-negative lung adenocarcinomas, a potential molecular basis for clinical response of ALK+ tumors to pemetrexed. Further evaluation of these variant-specific, quantitative RT-PCR assays as an adjunct to the standard FISH assay is warranted to better understand biologic variability and response patterns to ALK inhibitors. It remains to be determined how to integrate these quantitative RT-PCR assays into the cost-effective diagnostic algorithm for ALK+ tumors and whether patient tumors detected by different methods are equally sensitive to ALK inhibitors.

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Methods: We developed a panel of single and multiplex RT-PCR assays suitable for rapid and accurate detection of the eight most common EML4-ALK+ variants and ALK gene expression in archival formalin-fixed, paraffin-embedded NSCLC specimens. EGFR and KRAS genotyping and thymidylate synthase RNA level by RT-PCR assays were available in a subset of patients.

Results: Between December 2009 and September 2012, 7344 NSCLC specimens were tested. An EML4-ALK+ transcript was detected in 200 cases (2.7%), including 109 V1 (54.5%), 20 V2 (10.0%), 68 V3 (34.0%), and three V5a (1.5%) variants. Median age was 54.5 years (range, 23–89), and 104 patients (52.0%) were women. The great majority (n=188, 94.0%) of EML4-ALK+ NSCLC tumors had adenocarcinoma histology. ALK expression level varied significantly among the eight most common EML4-ALK+ variants and individual tumors. Only one case each of concurrent EGFR or KRAS mutation was detected. The median thymidylate synthase RNA level from 85 EML4-ALK+ cancers was significantly lower compared with that of EML4-ALK-negative lung adenocarcinomas (2.02 versus 3.29, respectively, p<0.001).

Conclusions: This panel of variant-specific, quantitative RT-PCR assays detects common EML4-ALK+ variants as well as ALK gene expression level in archival formalin-fixed paraffin-embedded NSCLC specimens. These RT-PCR assays may be useful as an adjunct to the standard fluorescence in situ hybridization assay to better understand biologic variability and response patterns to anaplastic lymphoma kinase inhibitors.

Key Words: Echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase fusion variants, Formalin-fixed, Paraffin-embedded, Non–small-cell lung cancer, Quantitative, Reverse transcription polymerase chain reaction.


Originally discovered in lymphomas,1,2 the overexpression of the anaplastic lymphoma kinase (ALK) gene by mutations, amplification, and translocations has since been identified as an oncogenic driver in several cancers including inflammatory myofibroblastic tumors, neuroblastomas, inflammatory breast cancer, and non–small-cell lung cancer (NSCLC).3,4 The echinoderm microtubule-associated
protein-like 4 anaplastic lymphoma kinase (EML4-ALK) fusion oncogene represents one of the newest molecular targets in NSCLC. First described by Soda et al. in 2007, the fusion oncogene results from a small inversion within chromosome 2p, in which the N-terminal half (exons 1–20) of EML4 gene is fused to the intracellular kinase domain (exons 20–29) of the ALK gene. EML4-ALK fusion (EML4-ALK+) products possess hyperactive tyrosine kinase activity and potent oncogenic activity both in vitro and in vivo. This activity can be effectively blocked by small-molecule tyrosine kinase inhibitors (TKIs) that target ALK. An increasing number of EML4-ALK+ variants have been reported in NSCLC. In addition, several other rare fusion partners for ALK gene in NSCLC have also been described, such as tropomyosin-receptor-kinase-fused gene (chromosome 3), kinesin family member 5B (chromosome 10), and kinesin light chain 1 (chromosome 14) as well as ALK–protein tyrosine phosphatase, nonreceptor Type 3 fusion oncogene.

Crizotinib (PF-02341066; XALKORI, Pfizer, NY) is an oral small-molecule receptor TKI against ALK, hepatocyte growth factor receptor (c-Met), and ROS1. Early clinical experience demonstrated that treatment with crizotinib yields a response rate of 51% to 61% and a median progression-free survival of 8 to 10 months in patients with ALK+ advanced NSCLC, regardless of the number of prior treatment regimens. Survival benefit has also been suggested in retrospective analyses. Although the ALK fluorescence in situ hybridization (FISH) test was clinically validated in early-phase crizotinib trials, alternative screening techniques, most commonly immunohistochemistry (IHC) and reverse transcription polymerase chain reaction (RT-PCR), have been explored for detection of candidate patients for ALK inhibitor therapy. We were among the first group of laboratories that developed quantitative RT-PCR assays that were optimized for use of clinical samples, before the U.S. Food and Drug Administration (FDA) approved the Vysis ALK Break-Apart FISH Probe Kit (Abbott Molecular Inc., Des Plaines, IL) for detection of ALK gene rearrangements. We report here the detection of EML4-ALK+ and ALK gene expression in archival North American NSCLC tumor specimens in the Response Genetics, Inc. (RGI) sample repository by a panel of variant-specific, quantitative RT-PCR assays and implications for clinical application.

MATERIALS AND METHODS

Tissue Procurement and Isolation of Nucleic Acids

Formalin-fixed, paraffin-embedded (FFPE) archival tumor specimens from patients with advanced NSCLC sent to the Clinical Laboratory Improvement Amendments (CLIA)–certified RGI for genotyping and the expression of molecular biomarkers predictive of drug sensitivity were included in this study. Figure 1 summarizes the study subjects included in this report. A hematoxylin and eosin–stained section of all FFPE specimens from each NSCLC patient was evaluated by a board-certified pathologist (GZ) for diagnosis confirmation and tumor content. Tumor specimens were obtained from core or fine-needle aspiration, surgical resection, or cell blocks from body fluids on a case-by-case basis. Adjacent sections of the tumor were sectioned and stained with nuclear fast red (NFR) for visualization for gross microdissection. Tumor cells were microdissected by laser capture from 10-µm nuclear fast red–stained slides if present in less than 50% of a specimen. After isolation and lysis of the tumor cells, RNA and DNA were isolated separately from the specimen by RGI-patented methods, and the RNA was then reverse transcribed to cDNA for subsequent RT-PCR assays as previously described.

RT-PCR Assays for EML4-ALK Fusion Genes and ALK Expression

Synthetic fragments representing the eight EML4-ALK+ variants 1, 2, 3a, 3b, 4, 5a, 6, and 7 were generated

![FIGURE 1. Summary of study subjects. NSCLC cases were grouped into EML4-ALK-Neg and EML4-ALK-Pos cases, then subdivided into patients who were positive or negative for TS expression. NSCLC, non–small-cell lung cancer; EML4-ALK, echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase; TS, thymidylate synthase.](image-url)
by recursive PCR technology. Figure 2 illustrates the primer locations and sequences designed by using the synthetic fragments as templates to detect specific EML4-ALK+ variants. The reverse RT-PCR primer for each variant is located in exon 20 as depicted. The resulting EML4-ALK fusion gene structure is shown. D, Sequences for EML4-ALK fusion transcript RT-PCR primers used. E, Representative PCR products of EML4-ALK fusion transcripts. The RT-PCR amplicons were separated by agarose gel electrophoresis and the size of specific amplicons was compared with the known molecular weight and synthetic positive controls. EML4-ALK variants 1, 2, 3a and 3b, and 5a and 6 are represented in gels a, b, c, and d, respectively. First lane: MS = marker standard (positive control); second lane: NTC = no template control; third lane: H-2228, an EML4-ALK variant 3a/3b-positive lung adenocarcinoma cell line; fourth and fifth lanes = patient samples run in duplicate. RT-PCR, reverse transcription polymerase chain reaction; EML4-ALK, echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase; TS, thymidylate synthase.
known molecular weight and synthetic positive controls. The amplicons of any unexpected size and selected cases were confirmed by direct sequencing by using ABI 3730 DNA Analyzer (Applied Biosystems Inc, Foster City, CA). ALK RNA level was detected by an RT-PCR assay by using a set of primers (3′-ALK-F, CCCTGCAAGTGGCTGTGA; 3′-ALK-R, GGCTTCCATGAGGAATCCA; 3′-ALK-T, CGTCCCTGGTCAGGACACCTTCAGGC) amplifying a 3′ nonfusion fragment of the ALK gene in the tyrosine kinase domain at the junction of exons 21 and 22. Beta-actin was used as the reference gene for quality and quantity of cDNA.

**RT-PCR Assays for Genotyping and Thymidylate Synthase Expression Level**

The EGFR RGQ PCR Kit (QIAGEN, Valencia, CA) was used to detect 29 specific somatic mutations, insertions, and deletions in the epidermal growth factor receptor (EGFR) gene using real-time polymerase chain reaction (PCR) on the Rotor-Gene Q 5plex HRM instrument (QIAGEN).27 The EGFR RGQ PCR kit enables the detection of EGFR E19del, L858R, L861Q, T790M, G719S/A/C, S768I, and E20ins mutations against a background of wild-type genomic DNA. Kirsten rat sarcoma (KRAS) mutation analysis was performed with an RGI in-house mutation RT-PCR assay using specifically designed primers and probes to detect each of the following mutations: Gly12Ala (GGT>GCT) 522; Gly12Asp (GGT>GAT) 521; Gly12Arg (GGT>CGT) 518; Gly12Cys (GGT>TGT) 516; Gly12Ser (GGT>AGT) 517; Gly12Val (GGT>GTT) 520; Gly13Asp (GGC>GAC) 532. The expression level of thymidylate synthase (TS) gene expression was determined by an established RT-PCR assay by using specific primers and analyzed through an Excel template using the 2(-Delta Delta threshold cycle (Ct) method as previously described.26,27

**Statistical Analysis**

All statistical tests were performed using the SAS statistical program, version 9.3. Descriptive statistics were used to characterize patient’s clinical-pathological features. Gene expression levels of the ALK and TS genes were log-transformed to render them normally distributed. Analysis of variance was used to test the difference in ALK RNA level across different EML4-ALK+ variants. The difference in TS RNA levels by EML4-ALK+ status was compared using the Wilcoxon rank sum test as the data were not normally distributed. All tests were two-sided, with a significance level of 0.05.

**RESULTS**

**Large-Scale Screening for EML4-ALK+ Variants**

This current report combines the results of EML4-ALK RT-PCR analysis using both residual tumor nucleic acids from the RGI sample repository,28 and subsequent tumor specimens analyzed prospectively.28,29 Together, between December 2009 and September 2012, 7344 NSCLC specimens in the RGI sample repository were tested for the presence of EML4-ALK+ variants (Fig. 1).29 We found that 200 NSCLC cases (2.7%) harbored one of the EML4-ALK+ variants (Table 1).

<table>
<thead>
<tr>
<th>Period of RGI database</th>
<th>December 2009–September 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of NSCLC cases</td>
<td>7344</td>
</tr>
<tr>
<td>No. of EML4-ALK+ cases</td>
<td>200</td>
</tr>
<tr>
<td>Prevalence of EML4-ALK+ cases</td>
<td>2.7%</td>
</tr>
<tr>
<td>Sex: female (%)</td>
<td>109 (52%)</td>
</tr>
<tr>
<td>Median age, yrs (range)</td>
<td>54.5 (23–89)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>188 (94.0%)</td>
</tr>
<tr>
<td>Large-cell</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>4 (2.0%)</td>
</tr>
<tr>
<td>Adenosquamous cell carcinomas</td>
<td>3 (1.5%)</td>
</tr>
<tr>
<td>Not specified</td>
<td>4 (2.0%)</td>
</tr>
</tbody>
</table>

**TABLE 1. Detection of EML4-ALK+ Transcripts in NSCLC in RGI Sample Repository**

The median age of patients with EML4-ALK+ variants was 54.5 (range, 23–89) years old. One hundred and four (52.0%) were women. The majority (94.0%) of EML4-ALK+ tumors were adenocarcinomas. However, EML4-ALK+ variants were also rarely detected in squamous cell carcinomas (n=4), adenosquamous cell carcinomas (n=3), and large-cell carcinomas (n=1) (Table 1).

Of the 200 EML4-ALK+ NSCLC tumors, 109 cases (54.5%) were V1 variant, 20 cases (10.0%) were V2 variant, 54.5% of patients harbored EML4-ALK+...
Table 2. Demographics and Genotypes of EML4-ALK+ NSCLC Tumors in this Study

<table>
<thead>
<tr>
<th>EML4-ALK Fusion Variants: (N=200)</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>V5</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases (%):</td>
<td>109 (54.5%)</td>
<td>20 (10.0%)</td>
<td>68 (34.0%)</td>
<td>3 (1.5%)</td>
</tr>
<tr>
<td>Age, yr: median (range)</td>
<td>52.5 (23–79)</td>
<td>57.8 (36–77)</td>
<td>57.4 (33–89)</td>
<td>56.5 (50–73)</td>
</tr>
<tr>
<td>Gender: female (%)</td>
<td>60 (55.0%)</td>
<td>10 (50.0%)</td>
<td>32 (47.0%)</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>Non-adenocarcinoma histology</td>
<td>2 SCC, 1 AS, 1 LC, 2 NOS</td>
<td>1 SCC</td>
<td>1 SCC, 2 AS, 1 NOS</td>
<td>1 NOS</td>
</tr>
<tr>
<td>EGFR mutation (N=189):</td>
<td>1 (E19del)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KRAS mutation (N=85):</td>
<td>0</td>
<td>0</td>
<td>1 (Gly12Asp)</td>
<td>0</td>
</tr>
</tbody>
</table>

SCC, squamous cell carcinoma; AS, adenosquamous; LC, large cell; NOS, not otherwise specified.

RT-PCR Assays for Genotyping and TS RNA Level in EML4-ALK+ NSCLC Tumors

Of the 200 EML4-ALK+ NSCLC cases, EGFR and KRAS genotype data were available from 189 (94.5%) and 90 (45.0%) cases, respectively (Fig. 3A). All EML4-ALK+ NSCLC cases tested had wild-type EGFR and KRAS genes, except one 79-year-old man had lung adenocarcinoma harboring an EML4-ALK+ exon 19 deletion concurrently with an EML4-ALK+ V1 variant and one 60-year-old man with lung adenocarcinoma harboring a KRAS Gly12Asp mutation concurrently with an EML4-ALK+ V3 variant (Fig. 3). TS mRNA levels were available from 85 EML4-ALK+ lung adenocarcinoma cases (Fig. 5), with low TS RNA expression defined as a level of less than 2.33, as previously described. ALK-negative lung adenocarcinomas served as an appropriate control group, based on our previous report. The median TS RNA level was significantly lower in EML4-ALK+ lung adenocarcinomas compared with that of EML4-ALK- lung adenocarcinomas (2.02 versus 3.29, respectively, p<0.001; Fig. 5).12

Discussion

Two unmet needs in the clinical application of ALK TKI agents are: (1) to establish a rapid and cost-effective diagnostic algorithm to identify this uncommon molecular subset of NSCLC; and (2) to understand molecular heterogeneity in responsiveness of ALK+ NSCLC tumors to optimize treatment strategies in the era of personalized cancer therapy. Several diagnostic techniques, including FISH, immunohistochemistry (IHC), and RT-PCR, have been explored for identifying candidate patients for ALK inhibitor therapy. Here we report development of a panel of quantitative monoplex and multiplex RT-PCR assays for the rapid detection of EML4-ALK+ variants and high ALK gene expression in a large (N=7344) cohort of archival FFPE NSCLC specimens in a commercial laboratory sample repository. It is worth emphasizing that our group was among the first to optimize RT-PCR assays suitable for clinical testing on archival tumor specimens in 2010, before the Vysis ALK Break-Apart FISH Probe Kit (Abbott Molecular Inc., Des Plaines, IL) for detecting ALK gene rearrangements was approved by the U.S. FDA as a companion diagnostic of ALK inhibitor crizotinib. To our knowledge, this is the largest cohort of EML4-ALK+ cases in unselected NSCLC tumors reported to date.

Consistent with previous reports, we found that EML4-ALK+ variants are present in a small subset (2.7%) of North American NSCLC patients whose tumors were tested for molecular biomarkers (Table 1). Although the incidence of EML4-ALK+ variants in our series might be slightly lower than that reported using ALK FISH overall, or series enriched by adenocarcinoma histology only, and/or never smokers and former light smokers, it is within the expected range. Of 200 EML4-ALK+ NSCLC tumors, V1 variant was the most common variant (54.5%) detected, followed by V3 (34.0%), V2 (10.0%), and V5a (1.5%; Fig. 3). Although the majority (n=188, 94.0%) of the EML4-ALK+ NSCLC tumors in our study had lung adenocarcinoma histology, EML4-ALK+ transcripts were detected in other histologic types of NSCLC, including squamous cell carcinomas (n=4), adenosquamous cell carcinomas (n=3), and large-cell carcinoma (n=1).

Of interest, we detected one case each of concordant ALK+ and either EGFR or KRAS mutation, respectively. A similar phenomenon, that is, the simultaneous detection of both an ALK gene rearrangement and an EGFR or KRAS mutation, has been previously reported by several investigators. A recent report demonstrated simultaneous detection of an EML4-ALK+ variant and an EGFR exon 19 deletion in a lung cancer case of combined small-cell and lung adenocarcinoma components, suggesting that neither tumor type nor histology alone are absolute determinants for guiding cancer genetic testing. These additional mutations may serve as mechanisms of primary or acquired resistance of ALK+ NSCLC tumors to ALK TKI therapy, and represent drug
targets for subsequent therapy. Importantly, EGFR mutant ALK+ tumors may respond to an EGFR TKI, similarly to those EGFR mutant ALK-negative tumors.36,38 Our results support the increasing use of multiplex genotyping and genomic profiling tests in individual NSCLC patients for selecting molecularly targeted therapy,40 an approach that needs to be proven cost effective because of the low prevalence of druggable genetic anomalies and high test cost. Currently, both the National Comprehensive Cancer Network and American Society of Clinical Oncology guidelines recommend EGFR mutation and ALK+ testing on all NSCLCs that contain an adenocarcinoma component, regardless of histologic grade or dominant histologic subtype.41,42

Of particular interest, we found that ALK expression level varied significantly among different EML4-ALK variants (Fig. 4A) and individual tumors (Fig. 4B). A recent study suggests that different EML4-ALK variants as well as other ALK fusion genes exhibit differential sensitivity to ALK inhibition, likely because of variability in overall fusion protein stability.43 The biological and clinical significance of variable expression of different EML4-ALK variants and the threshold of ALK expression level for predicting response to an ALK

FIGURE 4. ALK RNA levels in EML4-ALK+ variants (N=113). A, ALK RNA levels in different EML4-ALK fusion variants. RNA expression level for EML4-ALK variants divided by expression of β-actin control is shown for variant 1 (V1), variant 2 (V2), and variant 3 (V3). B, Variable intertumor ALK RNA level in NSCLC. High ALK expression was detected in 2.5% of EML4-ALK negative cases, shown in blue, when compared with EML4-ALK-positive expression levels. EML4-ALK, echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase.

A TS RNA Levels

<table>
<thead>
<tr>
<th>TS RNA Level</th>
<th>N</th>
<th>Median</th>
<th>95% CI</th>
<th>Range</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EML4-ALK-Pos Cases</td>
<td>85</td>
<td>2.02</td>
<td>1.64-2.13</td>
<td>0.55-19.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EML4-ALK-Neg Cases</td>
<td>1698</td>
<td>3.29</td>
<td>3.11-3.42</td>
<td>0.36-53.51</td>
<td></td>
</tr>
</tbody>
</table>

*Wilcoxon rank sum test.

B EML4-ALK-Pos Lung Adenocarcinomas

C EML4-ALK-Neg Lung Adenocarcinomas

FIGURE 5. TS RNA levels in EML4-ALK+ and EML4-ALK-negative lung adenocarcinomas. A, TS RNA levels. Of 200 EML4-ALK+ cases, TS expression data were available for 85 cases with a median expression of 2.02. In the EML4-ALK-negative cases, TS expression data were available for 1698 of 7144 cases, with a median expression of 3.29. B, EML4-ALK-pos lung adenocarcinomas. Overall TS expression in the 85 EML4-ALK-pos lung adenocarcinomas. C, EML4-ALK-neg lung adenocarcinomas. Overall TS expression in the 1698 EML4-ALK-neg lung adenocarcinomas. EML4-ALK, echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase; TS, thymidylate synthase.
inhibitor is unknown, but warrants further evaluation in pre-clinical models and prospective clinical trials, respectively.

Although crizotinib offers a new standard of care for patients with ALK+ NSCLC tumors, only about 60% of patients exhibit Response Evaluation Criteria in Solid Tumors response and eventually all patients progress, with a median progression-free survival of about 8 to 10 months. As cytotoxic chemotherapy remains the mainstay of treatment for patients with advanced NSCLC, it is important to discern potential differential sensitivity patterns of ALK+ tumors to chemotherapy. Several reports have suggested that pemetrexed is particularly active in patients with ALK+ tumors,46,46 but this is controversial.47 To investigate this premise, we analyzed TS gene expression levels, a purported biomarker of pemetrexed sensitivity, in our series. We found that TS expression was significantly lower in ALK+ tumors compared with ALK− lung adenocarcinoma controls, although the interpatient range of expression was wide (Fig. 5). This provides a potential molecular mechanism for increased clinical benefit seen in ALK+ NSCLC patients treated with pemetrexed. These data remain hypothesis-generating and require prospective validation.

The use of RT-PCR assays on nucleic acids from archival FFPE tumor specimens has been standardized for genotyping and molecular profiling studies in CLIA-certified laboratory. RT-PCR assays allow maximal use of scant nucleic acids from limited archival tumor specimens or blood and other body fluids for molecular diagnostic tests.48 Lessons learned from molecular testing in chronic myeloid leukemia reveal that quantitative RT-PCR is a more sensitive test than FISH for monitoring residual disease during TKI treatment, detecting 1-2-log reduction and ~5 log reduction, respectively.49 In addition to high sensitivity and specificity, advantages of the multiplex RT-PCR assay include rapid turnaround time, less technical operating challenges, easy interpretation, low cost, and the RNA/cDNA from each tumor could be used for simultaneous assessment of other molecular biomarkers,50,51 and most importantly, for DNA- or RNA-based genomic tests by next-generation sequencing technologies.

Nevertheless, our study has several limitations. First, patient samples were from an archival commercial sample repository, and the project was started before specific ALK FISH test received U.S. FDA approval as a companion diagnostic for crizotinib in 2011. No additional NSCLC specimens were available for the validation study of ALK FISH assay or IHC stain. Moreover, IHC test did not receive approval from the U.S. FDA. Nevertheless, our panel of RT-PCR assays passed the New York State validation test. Over the past 18 months, we performed both RT-PCR and FISH assays in approximately 900 consecutive NSCLC tumors in our CLIA-certified laboratory. We found about 30 ALK+ cases, and the concordance between the two tests was 98% (RGI database on file). Furthermore, our quantitative RT-PCR assay targeting a conserved region of the ALK fusion partner allows assessment of the expression level of all ALK gene rearrangements regardless of their 5′ fusion partners. The clinical utility of this quantitative RT-PCR assay could be evaluated in reference to ALK expression by FISH and IHC in prospective clinical studies. Second, only limited deidentified demographics (i.e., age and sex), were available. Smoking history and ethnicity information were not collected. Third, no clinical outcome data are available. Thus, a threshold of ALK expression level that predicts response to ALK inhibitors needs to be defined in future studies.

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