Clinical Validation of a Novel Commercial Reverse Transcription–Quantitative Polymerase Chain Reaction Screening Assay for Detection of *ALK* Translocations and Amplifications in Non–Small Cell Lung Carcinomas

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• ***Context.***—**_EGFR_ mutations and anaplastic lymphoma kinase (_ALK_) translocations have significant biologic and therapeutic implications in lung cancers, particularly lung adenocarcinomas. _ALK_ translocations are less frequent compared with _EGFR_ mutations; interestingly, these two abnormalities are most commonly mutually exclusive. The 2013 College of American Pathologists/Association for Molecular Pathology/International Association for the Study of Lung Cancer molecular testing guideline for lung cancers recommend a testing algorithm in which detection of _ALK_ translocations using fluorescence in situ hybridization (FISH) is to be performed following testing for _EGFR_ mutations. Such an algorithm is cost-effective but potentially slows down turnaround time; and as a secondary test, _ALK_ FISH assay may not be completed because it requires the use of additional tissue, and the small biopsies or cytology specimens may have been exhausted in the extraction of nucleic acid for _EGFR_ mutation screening.**

Objective.—**To provide efficient testing of both _EGFR_ and _ALK_ genetic alterations in small biopsies and cytology specimens.**

Design.—**We validated a highly sensitive _ALK_ reverse transcription–quantitative polymerase chain reaction (RT-qPCR) assay as a screening tool for _ALK_ translocations and amplifications.**

Results.—**We performed a retrospective review of cases previously tested by FISH and found that all FISH _ALK_ translocation–positive specimens were RT-qPCR positive, and all FISH _ALK_ translocation–negative cases were RT-qPCR negative (the sensitivity and specificity of the _ALK_ RT-qPCR assay were 100%).**

Conclusion.—**This assay allows rapid identification of _ALK_ alterations, can be performed in conjunction with _EGFR_ testing, and does not require use of valuable additional tumor tissue.**

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Epidermal growth factor receptor (*EGFR*) gene mutations and Anaplastic lymphoma kinase (*ALK*) gene translocations are found to have significant biologic and therapeutic implications in lung cancers, particularly lung adenocarcinomas, and may respond to treatment with tyrosine kinase inhibitors that have been approved by the Food and Drug Administration (FDA). *ALK* gene translocations (also referred to as gene fusions) occur in 3% to 5% of non–small cell lung carcinomas.[1,2] Most *ALK*-associated oncogenic changes are caused by *ALK* translocations, and to date at least 22 *ALK* partners have been identified, most commonly involving the echinoderm microtubule-associated protein-like 4 (*EML4*) gene as a fusion partner. These translocations involve the *ALK* kinase domain, and the resultant fusion protein shows oncogenic *ALK* tyrosine kinase (ALK-TK) activity. All *ALK* fusion proteins involve the kinase domain, regardless of the fusion partner. Crizotinib (trade name Xalkori, Pfizer, New York, New York) was the first drug approved by FDA that antagonizes the ALK-TK activity. Recently, next-generation ALK-TK inhibitors, such as ceritinib (Zykadia, Novartis Pharmaceuticals Corp, Cambridge, Massachusetts), were approved by FDA[3] to combat crizotinib-resistant tumors. The detection of *ALK* genetic alterations is therefore crucial in selecting patients for ALK-TK inhibitor therapy. Currently, the College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology (CAP/IASLC/AMP) guideline recommends using fluorescence in situ hybridization (FISH) as the gold standard for detecting *ALK* translocations; reverse transcription–polymerase chain reaction (RT-PCR) is not recommended because of concerns regarding a higher failure rate of an RNA-based assay in routine formalin-fixed, paraffin-embedded (FFPE) pathology material, as well as the risk of false negatives due to variability in the *EML4-ALK* fusion structure

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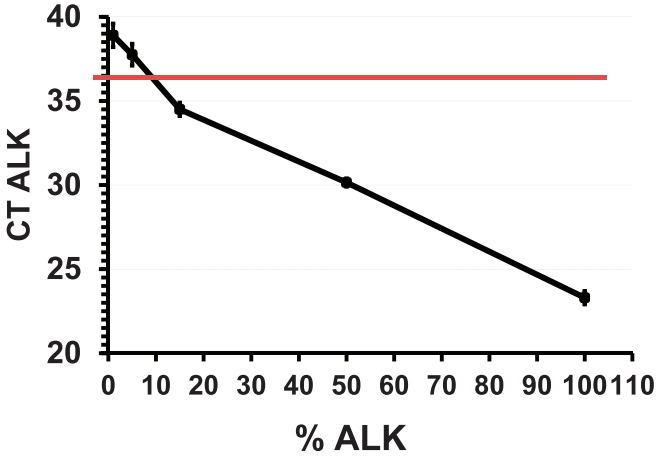

Figure 1. *Analytical sensitivity of* ALK *reverse transcription–quantitative polymerase chain reaction.*

and the existence of other *ALK* fusion partners.[4] To overcome these problems, we validated a novel, highly sensitive commercial RT–quantitative PCR (RT-qPCR) kit that amplifies the *ALK* kinase domain that detects overexpression of *ALK* caused by translocation.

MATERIALS AND METHODS

Patients and Samples

A total of 43 samples were tested for *ALK* translocation and amplification utilizing the ALK RGQ RT-PCR Kit (Qiagen, Valencia, California). A total of 20 samples were positive for *ALK* translocations: 17 patient samples, 2 cell lines (H2228 with *EML4-ALK* t(6;20) and U118 with *EML4-ALK* t(13;20)) obtained from ATCC (Manassas, Virginia), and 1 plasmid-positive control provided with an ALK RGQ RT-PCR Kit (Qiagen). A total of 23 samples were negative for *ALK* translocations: 21 patient samples, 1 commercial RNA-negative control (normal lung, Ambion/Life Technologies, Grand Island, New York), and 1 plasmid-negative control provided with an ALK RGQ RT-PCR Kit (Qiagen).

The histologic diagnoses of all 38 patient samples (17 *ALK*-positive and 21 control samples) were confirmed by pathologist review of hematoxylin-eosin and/or immunohistochemistry performed on FFPE tissue. *ALK* translocation–positive patient samples included 5 cases collected from Brigham and Women's Hospital (Boston, Massachusetts), 4 collected from Florida Hospital (Orlando, Florida), 2 collected from Highland Park NorthShore University Hospital (Highland Park, Illinois), 1 collected from Northwestern Memorial Hospital (Chicago, Illinois), and 5 collected from Houston Methodist Hospital (Houston, Texas). The outside cases' accession years ranged from 2005 to 2015, whereas the accession years of the Houston Methodist cases ranged from 2014 to 2015. Of the 38 patient samples, 7 had insufficient recovery of high-quality RNA based on lack of detection of internal control RNA for *ABL1*, and therefore could not be used for analysis (data not shown). A total of 31 patient samples were included in the final validation.

The *ALK* translocations of the patient samples were previously confirmed by FISH performed either in house (Brigham and Women's, Northwestern, and NorthShore) or at a commercial laboratory, including Genzyme (Florida Hospital cases; Cambridge, Massachusetts) or Clarient Diagnostic Services Inc (Houston Methodist Hospital cases; Aliso Viejo, California).

Nucleic Acid Extraction

A single hematoxylin-eosin slide for each case was examined by a pathologist (B.P.P. or P.T.C.); the section with the highest tumor content was circled and a tumor percentage was assigned. Tumor tissues were microdissected from 4 to 10 unstained sections of 10-μm slices of FFPE tissue using an Agencourt FormaPure FFPE kit (Beckman Coulter Inc, Brea, California). The quantity of RNA was measured using a Qubit 2.0 Fluorometer (Invitrogen/Life Technologies, Carlsbad, California) following the manufacturer instructions.

ALK RT-qPCR

The RT-qPCR was performed using the ALK RGQ RT-PCR Kit. The ALK RGQ RT-PCR Kit uses Scorpions technology and contains 2 assays in a single multiplex reaction that enables the detection of RNA transcripts encoding the *ALK*-TK domain and the control region of the Abelson murine leukemia viral oncogene homolog 1 (*ABL1*) RNA transcript (ALK RGQ RT-PCR Kit Handbook, Qiagen). It comprises a 1-step procedure during which RNA is reverse transcribed and tested using real-time PCR. The *ALK* assay detection is via a carboxyfluorescein-labeled Scorpion probe, and the *ABL1* internal control assay detection is via a HEX-labeled Scorpion probe. The internal control *ABL1* is an endogenous control that monitors the quantity of RNA and also the quality of RNA, by checking for the presence of inhibitors that may lead to false-negative results.

The RT-qPCR was performed on the 7500 Real-Time PCR System (Applied Biosystems/Life Technologies). The PCR cycling conditions were: RT, 50°C, 30 minutes; denaturation and *Taq* activation, 95°C, 15 minutes; and cycling (40 cycles), denaturation at 95°C for 30 seconds and annealing at 60°C for 1 minute.

RESULTS

Analytic Sensitivity

The RNA from cell line H2228 was titrated into RNA from normal lung at 100%, 50%, 15%, 5%, 1%, and 0% dilution ratios, respectively. *ALK* transcript was detected at 100%, 50%, and 15% dilution (Figure 1). The cutoff of *ALK* assay was set at Ct $\leq$36.9, and the cutoff of *ABL1* assay was Ct $\leq$36.4.

Accuracy

All 15 *ALK* translocation–positive samples, including 12 patient specimens, 2 cell line specimens, and 1 positive RNA control, showed positive *ALK* transcript in the presence of amplification control *ABL1* (Figure 2). The clinical sensitivity of the assay was 100% (15/15; 95% confidence interval, 78%–100%).

All 21 *ALK* translocation–negative samples, including 19 patient specimens (Figure 2), 1 commercial normal lung RNA sample, and 1 negative RNA control, showed no amplification of *ALK* in the presence of amplification control *ABL1*. The clinical specificity was 100% (21/21; 95% confidence interval, 83.8%–100%).

All 36 samples showed correlation with the prior FISH result. The accuracy of the assay was 100% (36/36).

Precision

Two samples were duplicated 5 times on the same run, and all duplicates showed the same result. The intrarun reproducibility was 100% (10/10).

A total of 29 samples were repeated in multiple runs performed by the same technologist and all results correlated. The interrun reproducibility was 100% (29/29).

A total of 8 samples were repeated in a different run by a different technologist, and all results correlated. The intertechnologist reproducibility was 100% (8/8).

COMMENT

The development of an RT-qPCR–based detection method for *ALK* detection based on measuring expression of the

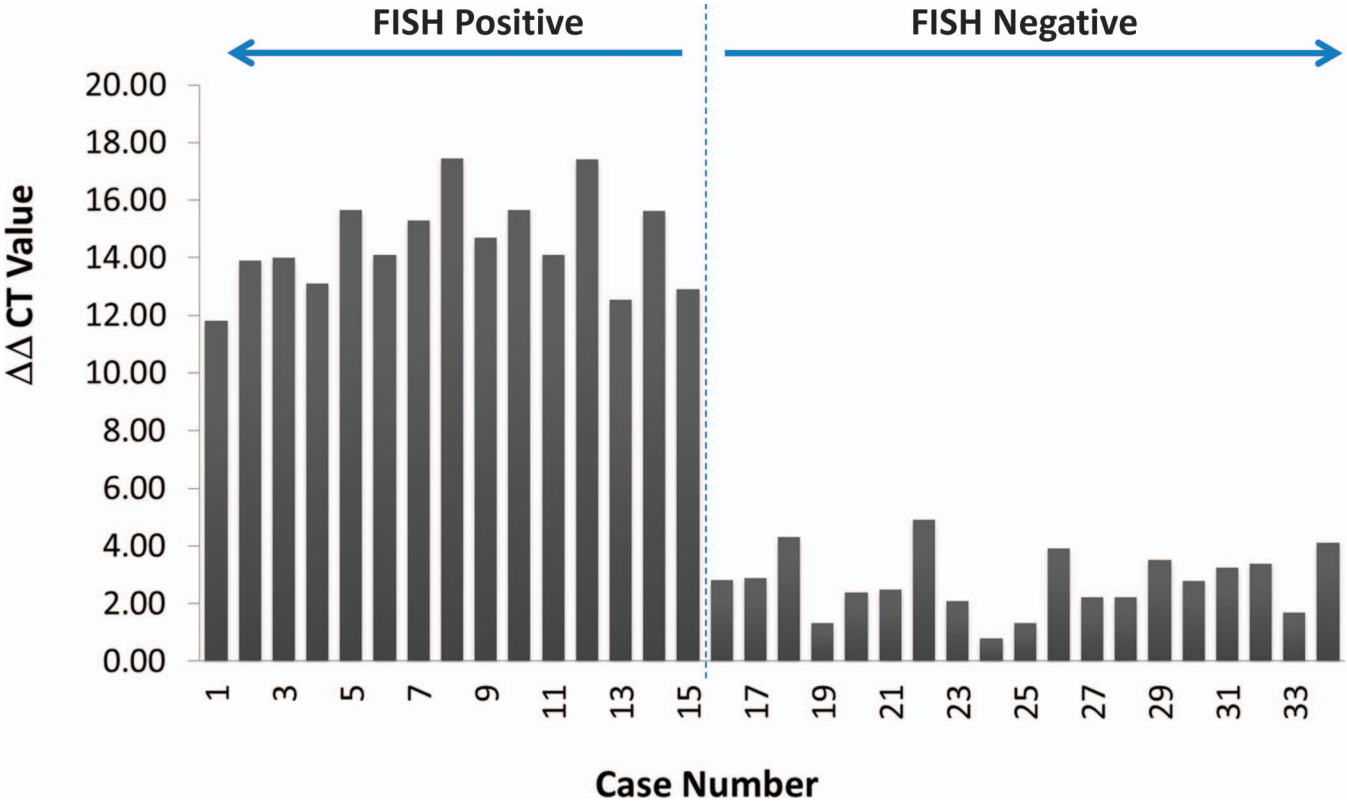

Figure 2. *Correlation of* ALK *reverse transcription–quantitative polymerase chain reaction with ALK fluorescence in situ hybridization (FISH).*

translocation-conserved critical kinase domain resulted in rapid detection of *ALK* translocation/amplification. Recently, the FDA approved the use of immunohistochemistry as a screening method for *ALK* overexpression.[5] Overexpression of *ALK* is now considered a surrogate for detecting *ALK* translocation. A comparison of immunohistochemistry versus RT-qPCR as a screening tool for *ALK* translocation remains to be performed. Nonetheless, a consistent goal of clinical molecular pathology is to minimize the material required for testing, to provide comprehensive testing for well-characterized biomarkers, such as *EGFR* and *ALK*, from small biopsies or cytology specimens, and to shorten the overall turnaround time to allow decisions about targeted therapy as quickly as possible.

The current CAP/IASLC/AMP guideline recommends prioritizing testing of *EGFR* mutations followed by *ALK* FISH assay. Although this strategy is prudent in that more non–small cell lung carcinoma cases will possess an *EGFR* mutation rather than an *ALK* translocation/amplification, it has been shown that these two events are not completely mutually exclusive.[6,7] This algorithm holds the risk of (1) potentially missing a rare case harboring both an *EGFR* mutation and *ALK* translocation; (2) exhausting the small amount of testing material from small biopsies and cytology specimens, rendering the possibility of the *ALK* FISH assay not being performed; and (3) increasing the overall turnaround time for molecular result reporting.

There are multiple RT-qPCR assays that are reported to be capable of detecting the *ALK* translocations in lung cancers.[8–11] However, so far there is no report on the validation of an easily obtainable commercial kit as an *ALK* mutation screening tool for practical purposes. We clinically validated the kit using total nucleic acid extracted from FFPE tissues. The same DNA/RNA extracts can be used for both *EGFR* mutation testing and detection of *ALK* translocation/amplification. In fact, after the validation, we have been routinely using the total nucleic acids from FFPE tissues derived from small biopsies and cytology cell blocks to perform both an *EGFR* mutation test (a custom single-nucleotide polymorphism genotyping test) and *ALK* RT-qPCR. The failure rate is very low (~2%). This strategy enables better use of small quantities of clinical specimens, and it increases the likelihood that both tests are completed. *ALK* gene translocation–negative cases can be reported immediately, bypassing the cost and time required to perform FISH analysis. In our laboratory, we were able to report the negative *ALK* RT-PCT result within 48 hours of sample collection, and we reduced the number of FISH send-out tests by 80%, from approximately 20 cases per month to 4 cases per month. With the RT-qPCR ALK assay we have lowered our testing cost and improved overall turnaround time. We therefore highly recommend using this novel, sensitive *ALK* RT-qPCR assay as a screening tool for detection of *ALK* translocations and amplifications in non–small cell lung carcinomas.

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