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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.

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 different *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.

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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*-positive tumors to pemetrexed. Further evaluation of these variant-specific, quantitative RT-PCR assays as an adjuvant 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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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).[3–7] The echinoderm microtubule-associated

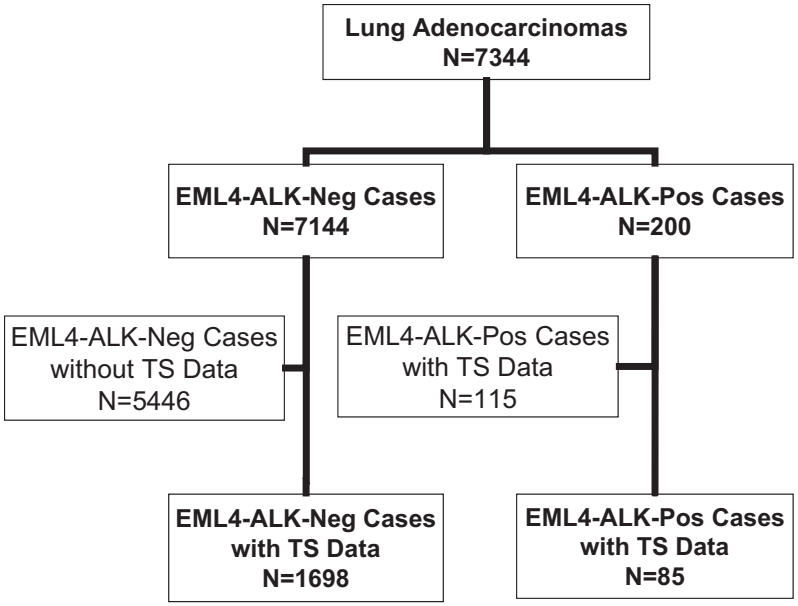


FIGURE 1. Summary of study subjects. NSCLC cases were grouped into EML4-ALK-Neg and EML4-ALK-Pos cases, then subdivided into patients who were positive or negative for TS expression. NSCLC, non–small-cell lung cancer; *EML4-ALK*, echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase; TS, thymidylate synthase.

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.[4] 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.[8] *EML4-ALK* fusion (*EML4-ALK*+) products possess hyperactive tyrosine kinase activity and potent oncogenic activity both in vitro and in vivo.[4,9] This activity can be effectively blocked by small-molecule tyrosine kinase inhibitors (TKIs) that target ALK.[9,10] An increasing number of *EML4-ALK*+ variants have been reported in NSCLC.[4,7,10–14] In addition, several other rare 5′ fusion partners for *ALK* gene in NSCLC have also been described, such as tropomyosin-receptor-kinase-fused gene (chromosome 3),[15] kinesin family member 5B (chromosome 10),[16] and kinesin light chain 1 (chromosome 14)[17] as well as ALK–protein tyrosine phosphatase, nonreceptor Type 3 fusion oncogene.[18]

Crizotinib (PF-02341066; XALKORI, Pfizer, NY) is an oral small-molecule receptor TKI against ALK, hepatocyte growth factor receptor (c-Met), and ROS1.[19,20] 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,[21–24] regardless of the number of prior treatment regimens. Survival benefit has also been suggested in retrospective analyses.[25] Although the *ALK* fluorescence in situ hybridization (FISH) test was clinically validated in early-phase crizotinib trials, alternative screening techniques,[21] most commonly immunohistochemistry (IHC) and reverse transcription polymerase chain reaction (RT-PCR), have been explored for detection of candidate patients for ALK inhibitor therapy.[25] 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.[26] 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.

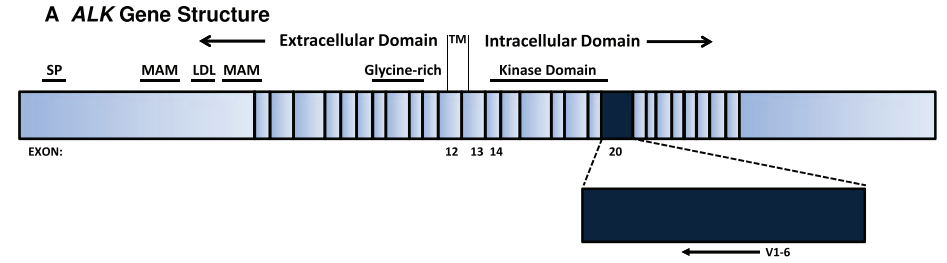
MATERIALS AND METHODS

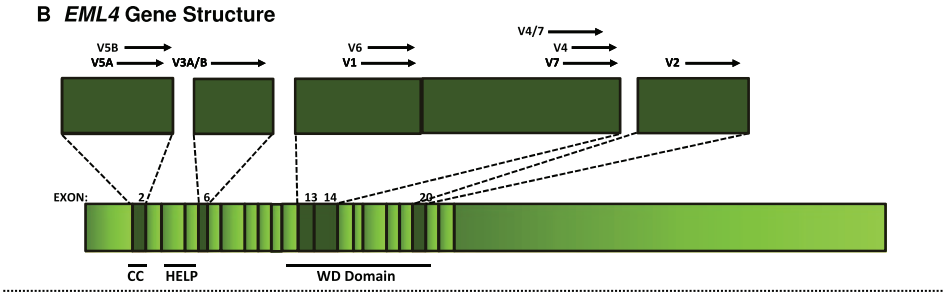
Tissue Procurement and Isolation of Nucleic Acids

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.[26,27]

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





D Sequences for *EML4-ALK* Fusion Transcripts

EML4-ALK.v1-5'	CCTGGGAAAGGACCTAAAGTG
EML4-ALK.v2-5'	GTCTAACTCGGGAGACTATGAAATATTG
EML4-ALK.v3a-5'	TAAAGATGTCATCATCAACCAAGTG
EML4-ALK.v3b-5'	GTCAACTCGCGAAAAAAACAG
EML4-ALK.v5a-5'	GGCCTCAGTGAAAAAATCAGTCT
EML4-ALK.v6-5'	CTGGGAAAGGACCTAAAGGAA
EML4-ALK.v3ab-5'	GCATAAAGATGTCATCATCAACCA
EML4ALK.v12356-3'	GAGCTTGCTCAGCTTGTACTCA
EML4-ALK.v12356-probe	6FAM-ACCAGGAGCTGCAAGCCATGC
EML4-ALK.v4-5'	GAATCCTGAAAGAGAAATAGAGATATGC
EML4-ALK.v7-5'	TGAATCCTGAAAGAGAAATAGAGCA
EML4-ALK.v47-5'	GATCATGATCTGAATCCTGAAAGAG
EML4-ALK.v47-3'	GTTGTAGTCGGTCATGATGGTC
EML4-ALK-v47-probe	6FAM-TACAAGCTGAGCAAGCTCCGCACC

E Representative PCR Products of EML4-ALK Fusion Transcripts

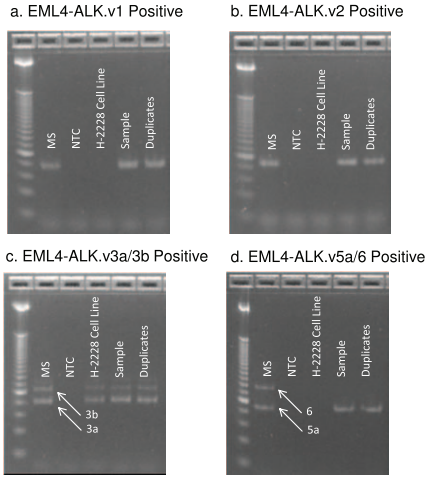


FIGURE 2. Schema of RT-PCR primer locations and sequences for the expression of *EML4-ALK*+ variants. *A*, ALK gene structure with main domains marked: SP, signal peptide; MAM, meprin, A-5 protein, receptor protein tyrosine phosphatase mu; LDL, low-density lipoprotein binding domain. The reverse RT-PCR primer for all variants is located in exon 20 as depicted. *B*, *EML4* gene structure with main domains marked: CC, coiled coil domain; HELP, hydrophobic echinoderm microtubule-associated protein-like protein; WD, WD-repeat domain. The approximate location of the forward RT-PCR primer for each variant is depicted. *C*, 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.

by recursive PCR technology.[26] Figure 2 illustrates the primer locations and sequences designed by using the synthetic fragments as templates to detect specific *EML4-ALK*+ gene fragments with a maximum amplicon of 170 bases by RT-PCR. For each variant, a specific forward (5′) primer located in the corresponding *EML4* region was used with a common reverse primer (3′), located in exon 20 of the *ALK* gene. For the RT-PCR assays, the most common variants 1 and 2 were run individually. Variants 3a and 3b, variants 4 and 7, and variants 5a and 6 were multiplexed, respectively. The multiplexed amplicons were separated by agarose gel electrophoresis and the size of the amplicons was compared with the

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, GGCTTCCATGAGGAAATCCA; 3′-ALK-T, CGTCCTGTTCAGAGCACACTTCAGGC) 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 1. Detection of *EML4-ALK*+ Transcripts in NSCLC in RGI Sample Repository

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

NSCLC, non–small-cell lung cancer; *EML4-ALK*+, echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase fusion; RGI, Response Genetics, Inc.

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,

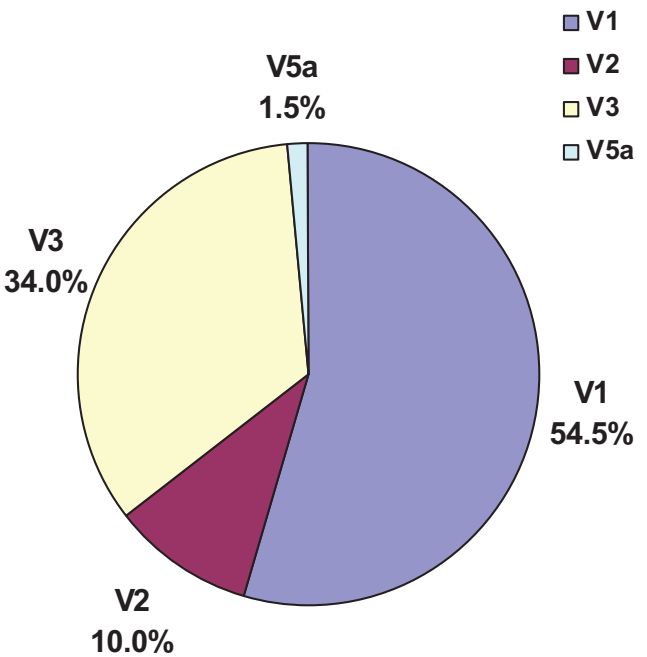


FIGURE 3. *EML4-ALK*+ variants in NSCLC tumors (N=200). Distribution of various EML4-ALK fusion variant. *EML4-ALK*, echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; SCC, squamous cell carcinoma; AD, adenocarcinoma; LC, large cell carcinoma; NOS, not otherwise specified.

TABLE 2. Demographics and Genotypes of *EML4-ALK*+ NSCLC Tumors in this Study

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

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

68 cases (34.0%) were V3 variant, and three cases (1.5%) were V5a variant (Figure 3, Table 2). In addition, by using V3 primers we identified two cases harboring an 18-base-pair insertion between exon 6 of *EML*4 and exon 20 of *ALK* genes.[14] We further determined the expression of ALK RNA level by a separate quantitative RT-PCR, which spans the junction of exon 21 and 22 of the *ALK* tyrosine kinase domain (Fig. 2). *ALK* expression levels varied significantly between the common *EML4-ALK*+ variants (Fig. 4*A*). About 2.5% of *EML4-ALK*-negative tumors had high ALK RNA expression level (Fig. 4*B*), which might because of the presence of other fusion partners or because of the high expression of full-length ALK transcript. Although no direct cross comparison with the ALK FISH assay is possible in this case series, in the recent New York State validation test, this panel of EML4-ALK RT-PCR assays had 93.3% concordance with ALK FISH assay done at an external CLIA-certified institution, with the sensitivity of 83.3% (*n*=6) and specificity of 100% (*n*=23).

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. 3*A*). All *EML4-ALK*+ NSCLC cases tested had wild-type *EGFR* and *KRAS* genes, except one 79-year-old man had lung adenocarcinoma harboring an *EGFR* exon 19 deletion concurrently with an *EML4-ALK*+ V1 variant and one 60-year-old man with lung adenocarcinoma harboring an *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.[30] ALK-negative lung adenocarcinomas served as an appropriate control group, based on our previous report.[31] 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).[32]

DISCUSSION

Two unmet needs in the clinical application of ALK TKIy 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,[26] 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,[4,10,13,21] 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.[33–36] 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,[37] 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,[38,39] and represent drug

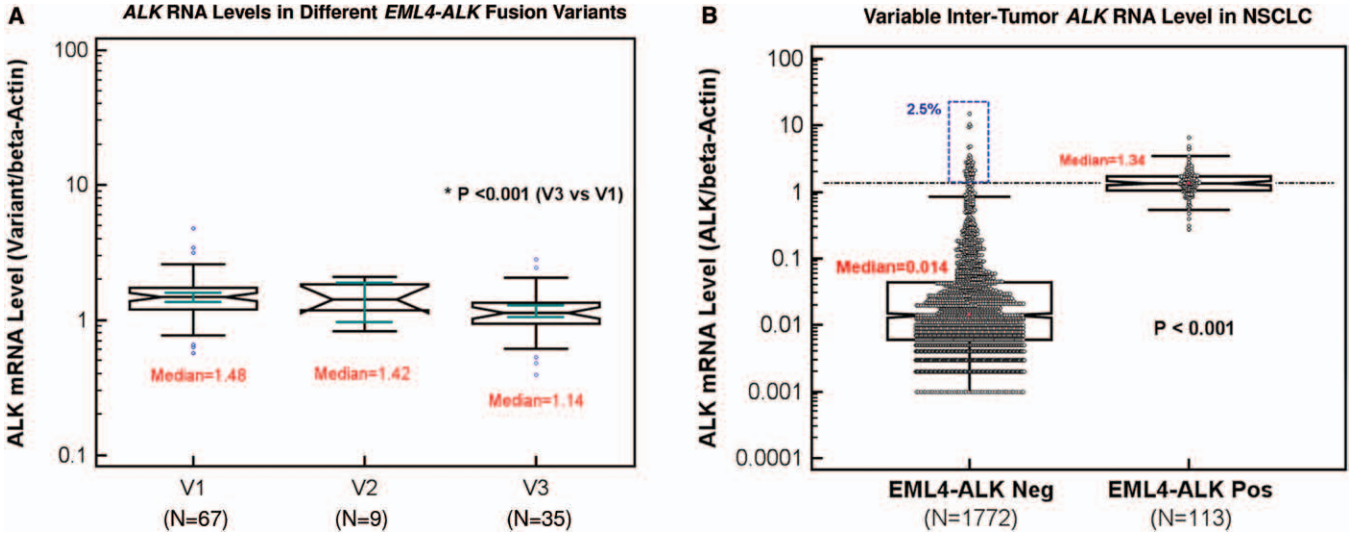


FIGURE 4. *ALK* RNA levels in *EML4-ALK*+ variants (N=113). *A*, ALK RNA levels in different *EML4-ALK* fusion variants. RNA expression level for *EML4-ALK* variants divided by expression of β-actin control is shown for variant 1 (V1), variant 2 (V2), and variant 3 (V3). *B*, Variable intertumor ALK 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.

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. 4*A*) and individual tumors (Fig. 4*B*). 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

A *TS* RNA Levels

TS RNA Level	N	Median	95% CI	Range	*p Value
***EML4-ALK*-Pos Cases**	85	2.02	1.64-2.13	0.55-19.44	<0.001
***EML4-ALK*-Neg Cases**	1698	3.29	3.11-3.42	0.36-53.51	

*Wilcoxon rank sum test.

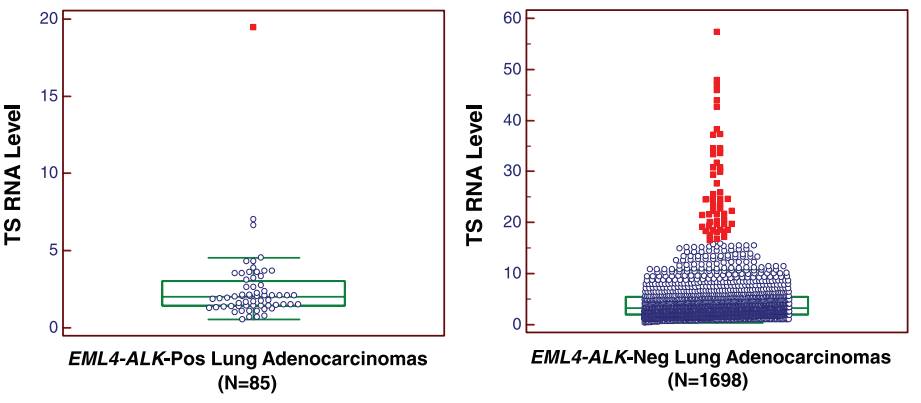


FIGURE 5. *TS* RNA levels in *EML4-ALK*+ and *EML4-ALK*-negative lung adenocarcinomas. A, TS RNA levels. Of 200 *EML4-ALK*+ cases, TS expression data were available for 85 cases with a median expression of 2.02. In the *EML4-ALK*-negative cases, TS expression data were available for 1698 of 7144 cases, 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 preclinical 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,[44–46] 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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