



# ALK Gene Rearrangements in Lung Adenocarcinomas: Concordance of Immunohistochemistry, Fluorescence In Situ Hybridization, RNA In Situ Hybridization, and RNA Next-Generation Sequencing Testing

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## ABSTRACT

**Introduction:** The 2018 updated molecular testing guidelines for patients with advanced lung cancer incorporated *ALK* immunohistochemistry (IHC) analysis as an equivalent to fluorescence in situ hybridization (FISH) method recommended in 2013. Nevertheless, no specific recommendation for alternative methods was proposed owing to insufficient data. The aim of this study was to compare the results of *ALK* IHC, FISH, RNA next-generation sequencing (NGS), and RNA in situ hybridization (ISH) with available clinical data.

**Methods:** A search for lung carcinomas with *ALK* testing by greater than or equal to one modality (i.e., *ALK* IHC, FISH, NGS) was performed; a subset underwent RNA ISH. When available, clinical data were recorded.

**Results:** The results were concordant among all performed testing modalities in 86 of 90 cases (95.6%). Of the four discordant cases, two were *ALK* positive by FISH but negative by IHC, RNA NGS, and RNA ISH. The remaining two cases failed RNA NGS testing, one was IHC negative, FISH positive, RNA ISH negative and the second was IHC positive, FISH positive, RNA ISH equivocal. RNA NGS identified one rare and one novel *ALK* fusion. Sufficient therapy data were available in 10 cases treated with tyrosine kinase inhibitors; three had disease progression, including one with discordant results (FISH positive, RNA NGS negative, IHC negative, RNA ISH negative) and two with concordant *ALK* positivity among all modalities.

**Conclusions:** Our results reveal high concordance among IHC, RNA NGS, and RNA ISH. In cases of discordance with

available RNA NGS, FISH result was positive whereas IHC and ISH results were negative. On the basis of our data, multi-modality testing is recommended to identify discrepant results and patients (un)likely to respond to tyrosine kinase inhibitors.

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**Keywords:** *ALK*; Fluorescent in situ hybridization (FISH); Next-generation sequencing (NGS); RNA in situ hybridization (RNA ISH); Archer; Anchored multiplex

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## Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. In the past, systemic treatment for advanced-stage disease tended to be quite circumscribed—patients with small cell carcinoma received one regimen and those with NSCLC another. After the discovery of *EGFR* driver mutations in NSCLC and results of the 2009 Iressa Pan-Asia Study trial,<sup>1</sup> which correlated a specific genotype to greatest response with a tyrosine kinase inhibitor (TKI), the management of NSCLC transformed dramatically.

From this landmark study emerged a change in paradigm. The current standard of care includes companion diagnostic biomarker testing to determine eligibility for targeted therapy. In 2007, *EML4-ALK* fusion in the short (p) arm of chromosome two was first described in NSCLC by Soda et al.,<sup>2</sup> and in 2011, crizotinib was approved after response in *ALK*-positive patients.<sup>3</sup> *ALK* rearrangements are present in approximately 3% to 8%<sup>4</sup> of lung adenocarcinomas and tend to occur more frequently in younger patients who are never or light smokers. Nevertheless, similar clinical characteristics may be found in patients with other genetic alterations, such as in *EGFR*, *ERBB2*, and *RET*. Although signet ring cytomorphology, mucinous cribriform pattern, and psammoma bodies<sup>5</sup> have been described in *ALK*-rearranged adenocarcinomas, these patterns are not uniformly present. Consequently, no treatment is based solely on (cyto)morphological pattern or clinical characteristics.

In 2013, the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology (CAP/IASLC/AMP)<sup>6</sup> set forth molecular testing guidelines to select patients eligible for TKI therapy on the basis of the presence of *EGFR* mutations or *ALK* fusions. For *ALK*, testing with fluorescence in situ hybridization (FISH) using dual-color break-apart probe was recommended, and appropriately validated immunohistochemistry (IHC) could serve as a screening modality. In 2018, the guidelines were updated, and strong granular cytoplasmic *ALK* IHC was deemed an equivalent alternative to FISH on the basis of multiple studies.<sup>7</sup> Meanwhile, there were insufficient data to support nucleic acid-based assays for detection of *ALK* fusions, though two DNA next-generation sequencing (NGS) assays (Foundation One [Foundation Medicine, Cambridge, MA] and Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets [New York, NY]) that provide comprehensive diagnostic testing were approved by the Food and Drug Administration (FDA) in 2017.

The aim of this study was to compare the *ALK* detection methodologies currently recommended by

CAP/IASLC/AMP with anchored multiplexed (AMP) RNA-based RNA NGS and RNA in situ hybridization (ISH), which have not previously been evaluated in parallel. These findings were correlated with therapy outcomes to evaluate for response in specific *ALK* variants.

## Materials and Methods

### Case Selection: *ALK* Positive and Negative

After institutional review board approval (#AAAD7936) and waiver of consent as part of a retrospective review, a search for lung carcinomas that had previously undergone testing for *ALK* by greater than or equal to one testing modality was performed. The search comprised primary and metastatic lung carcinomas diagnosed on cytology, small biopsy, and surgical resection specimens.

After the search, the cases were divided into two study groups. Group 1 (n = 66) consisted of positive and negative *ALK* samples that had successfully undergone testing with all the following methods: *ALK* FISH, *ALK* IHC, targeted RNA NGS, and DNA NGS. Group 2 (n = 7) consisted of only resection samples before the availability of RNA NGS in the laboratory but had tested positive for *ALK* rearrangement by FISH. Group 2 was selected to include additional *ALK*-rearranged cases and to evaluate success or failure of RNA ISH and RNA NGS on archived specimens. For group 2, previous DNA multiple single-gene analyses (SGAs) and IHC were recorded when available and targeted RNA NGS was subsequently performed on all samples in addition to IHC, if previously unavailable. Additional negative controls (n = 17) defined as lacking *ALK* by RNA NGS and/or having a non-*ALK* driver mutation by DNA NGS were identified.

### Case Selection: RNA ISH

RNA ISH testing was performed on a total of 51 of 90 cases. More specifically, RNA ISH testing was performed on all group 1 and group 2 cases, which had tested positive for *ALK* rearrangement by more than one testing modality, nine randomly selected group 1 negative cases, and 17 additional negative controls.

### Case Selection: Anchored Multiplexed RNA NGS

In keeping with standard practice at our institution, targeted RNA NGS was conducted only on cases that lacked an identifiable driver mutation in the DNA NGS panel in group 1 and all cases from group 2.

### Laboratory Protocols

Per laboratory protocol, all primary and metastatic lung adenocarcinomas, or when this diagnosis could not

be excluded (e.g., large cell carcinoma, NSCLC not otherwise specified, squamous cell carcinoma in a small biopsy/cytology of a never or light smoker), were reflexively submitted for testing to identify driver mutations. The tests and platforms varied with guidelines and the contemporaneous methods available in the laboratory. At the outset, *ALK* FISH was the gold standard and IHC was variably used for screening; subsequently, all specimens were subject to *ALK* IHC and FISH. Initially, multiple single-gene DNA analyses were performed; subsequently, NGS was adopted (with SGA reserved only for instances of insufficient DNA) to identify driver mutations. More recently, RNA NGS was adopted and performed (1) reflexively only on cases in which DNA NGS failed to identify a driver mutation (i.e., *KRAS*, *EGFR*, *BRAF*, *MET*, *ERBB2*) and (2) in addition to and independent of results of *ALK* IHC and *ALK* FISH.

#### **Laboratory Protocol: Detection of *ALK* Rearrangement by FISH and *ALK* Protein Expression by IHC**

FISH analysis for *ALK* rearrangement was performed using the FDA-approved Vysis *ALK* break-apart probe kit (Abbott Molecular, Des Plaines, IL) and interpreted in accordance with the manufacturer's instructions. IHC was performed using *ALK* (D5F3) rabbit monoclonal primary antibody (Ventana, Tucson, AZ) on BenchMark ULTRA system with OptiView detection kit and interpreted using manufacturer's criteria; the stain was interpreted independently before performing and availability of FISH and RNA results.

#### **Laboratory Protocol: Detection of *ALK* Fusion by RNA NGS**

After internal laboratory validation as described previously,<sup>8</sup> RNA NGS was adopted to identify specific fusions and mutations (e.g., *ALK*, *MET*, *RET*, *ROS1*, *NTRKs*) in lung carcinomas; this was performed in addition to IHC and FISH. From formalin-fixed, paraffin-embedded (FFPE) samples, the slides were cut and evaluated for tumor content, and when below approximately 20%, both macrodissection and manual microdissection (using a needle under a microscope) were used. RNA was isolated using the ALLPrep DNA/RNA kit (Qiagen) and quantified using the Qubit fluorometer. A custom NGS panel using AMP technology (Archer Dx, Boulder, CO), which targets 17 genes, was performed as previously described.<sup>9</sup> Results were interpreted after minimum quality control measures set forth by manufacturer's guidelines. A positive result "fusion" required (1) a minimum of five unique reads (2) with at least 10%

of reads greater than the wild-type transcript and (3) at least three unique start sites. Results of RNA NGS were considered gold standard on the basis of its reported high sensitivity<sup>10-12</sup> and 100% specificity compared with other methods.<sup>12</sup>

#### **Laboratory Protocol: Detection of *ALK* Rearrangement by RNA ISH**

RNA ISH testing was performed using RNAScope 2.5 LS Probe-Hs-*ALK* (Advanced Cell Diagnostics, Hayward, CA) on the Leica Bond III automated staining platform with RNA Scope DAB kit. The probes target the region of exons 19 to 29 (kinase domain and adjacent sequences). Recommended RNA scope strategies, which detect exuberant expression (rather than abnormal *ALK* protein), were used. Increase in the number of *ALK* signals, which preferentially occur in *ALK*-rearranged cells, was evaluated. A positive result by RNA ISH was defined as two signals per cell in at least 5% of cancer cells and at least one cancer cell with greater than or equal to four signals. A positive RNA control (ubiquitin) was performed on a serial section from each specimen. If the positive control slide was positive (two–three signals per cell), then a negative result on the target slide was interpreted as a true negative. If the positive control slide was negative or had minimal staining, *ALK* results were interpreted as equivocal.

#### **Laboratory Protocol: DNA SGA and NGS**

Similar to RNA NGS testing, sections were cut at 5  $\mu$ m, reviewed for tumor content, and macrodissected or microdissected when tumor content was below the required threshold. Testing was performed using Sanger sequencing for multiple SGA and TruSeq (Illumina, San Diego, CA) or a custom panel (Pillar Biosciences) for NGS, which contained mutational hotspots in several genes, including, *EGFR*, *KRAS*, *BRAF*, *ERBB2*, and *MET*, among others. A positive result required an average coverage depth of at least 500 reads and minimum 5% variant allele fraction.

#### **Clinical Follow-Up**

When available, clinical data, including response to therapy and progression-free survival (PFS), defined as a new metastatic lesion or unequivocal growth of existing lesion(s), were recorded.

#### **Statistical Analysis and Concordance and Discordance**

The sensitivity and specificity of each testing modality were analyzed using RNA NGS as the gold standard. Two cases of RNA NGS testing failure were not included

Table 1. Clinical Characteristics

	Group 1	Group 2	Negative Controls	Overall
Total cases	66	7	17	90
Male, n (%)	33 (50.0)	3 (42.9)	8 (47.1)	44 (48.9)
Female, n (%)	33 (50.0)	4 (57.1)	9 (52.9)	46 (51.1)
Average age, y	72	60	73	69
Median age (age range)	69.5 (29-90)	58 (45-71)	72 (62-87)	69 (29-90)

in calculations. RNA ISH sensitivity and specificity calculation were based on 32 cases (discluded 17 negative control cases lacking corresponding RNA NGS testing). IHC and FISH sensitivity and specificity calculation were based on 71 cases (all cases with available results for IHC, FISH, and RNA NGS). Concordance and discordance across the various testing modalities were detailed.

## Results

The study consisted of 90 lung adenocarcinomas from 46 women and 44 men (median age = 69 y; range: 29–90 y) who underwent testing for an *ALK* gene rearrangement with greater than one testing modality. Specimen types evaluated included resections (n = 60, 66.7%), small biopsies (n = 23, 25.6%), and cytology (pleural effusion [n = 1, 1.1%]; fine-needle aspiration [n = 6, 6.7%]). The patients were subdivided into group 1 (n = 66), group 2 (n = 7), and RNA ISH-negative controls (n = 17) on the basis of previously detailed criteria (Table 1). Overall, 25 patient cases were positive by at least one modality and 65 patient cases were negative by all modalities (Fig. 1A–E). Results of all studies are summarized in Figure 2.

### Concordance and Discordance

Using RNA NGS as the gold standard, the sensitivity and specificity of *ALK* IHC, FISH, and RNA ISH are as follows: 100% and 100%, 100% and 96%, and 100% and 100%, respectively.

There was complete concordance in all but four cases. Two discordant cases had all testing modalities completed for comparison. In both cases, FISH results were positive for *ALK* rearrangement whereas IHC, RNA NGS, and RNA ISH were negative (Fig. 3A–D). The remaining two cases, designated as case A and case B and both from group 2, failed RNA NGS testing. The completed testing modalities in these cases had the following: case A: IHC negative, FISH positive, RNA ISH negative and case B: IHC positive, FISH positive, RNA ISH equivocal. Cases A and B were both resection specimens from 8 and 13 years before, respectively. Evaluation of corresponding hematoxylin and eosin slides revealed a relatively low tumor content (~20% of slide content)

and a marked lymphocyte infiltrate in both cases and background fibrosis in case B. Among the remaining four cases in group 2, testing was successfully performed on FFPE samples retrieved from up to 10 years before, though with comparatively higher tumor content (~90% of slide content) and no considerable lymphoid component.

### RNA NGS: Novel and Rare Fusion Partners

With targeted RNA NGS, one rare and one novel *ALK* fusion partner, *TFG* (exon 6) and *SLMAP* (exon 12 and exon 13 isoforms), respectively, were identified. The remaining 19 positive cases had *ALK* exon 20 with the most often described fusion, *EML4* (variable) (Fig. 4). The two most frequent variants detected by RNA NGS were variant 1 (11 of 20; 55%) and variant 3a/b (5 of 20; 25%).

### Immunohistochemistry

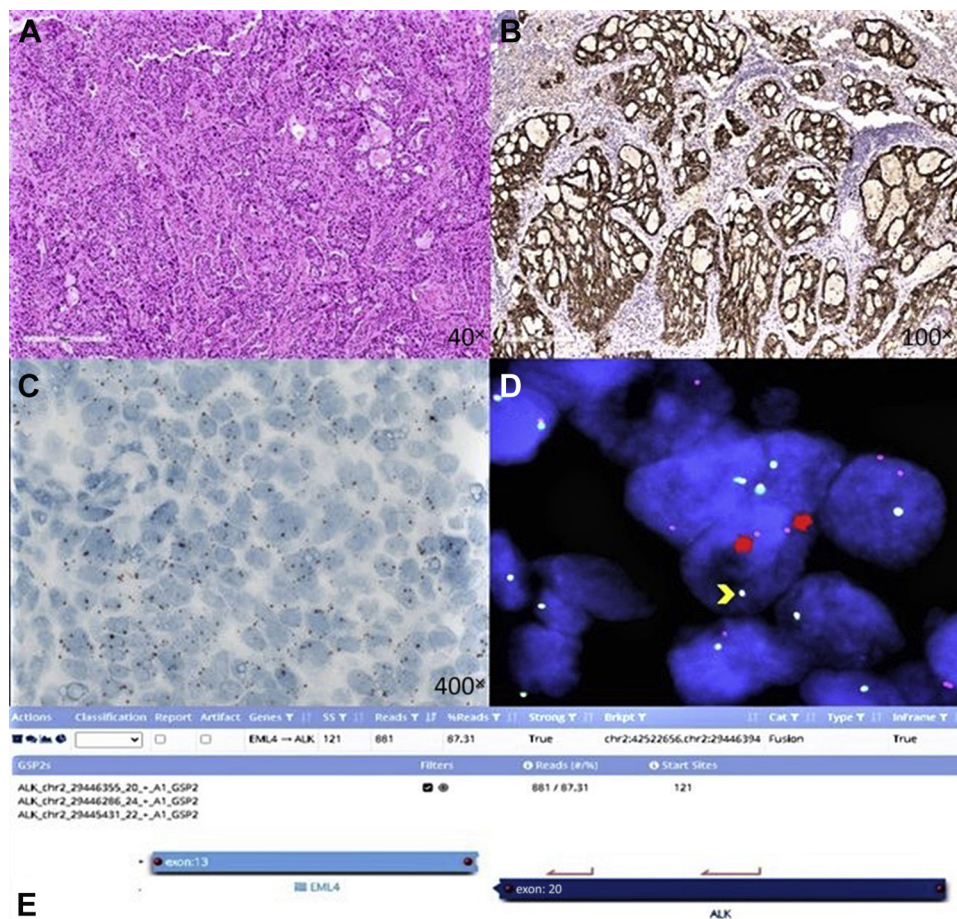
IHC studies were reviewed for all positive cases and a subset of negative cases. All positive cases had some degree of moderate to strong staining intensity; all negative cases reviewed revealed no staining.

### DNA SGA and NGS

Because driver mutations are mutually exclusive, targeted DNA SGA and NGS served as controls. In all concordant *ALK*-positive cases and the four discordant cases, no driver mutation was detected by targeted DNA NGS. In the one discordant case, *STK11*: c.1027G>A, p.D343N, a variant of uncertain significance (VUS) was detected. This mutation was reported 8 times in ClinVar as a VUS and is therefore not likely a driver of the tumor. Among the concordant negative cases, no mutation was detected by DNA NGS in 32 of 48 cases (66.7%), *KRAS* mutation was present in six cases (12.5%), *MET* mutation in three cases (6.3%), *EGFR* mutation in three cases (6.3%), *STK11* mutation in three cases (6.3%), and *BRAF* mutation in one case (2.1%).

### Clinical Follow-Up

Clinical data were sought for all cases with an *ALK* rearrangement detected by greater than one testing modality. A total of 10 cases with sufficient clinical data



**Figure 1.** Images of testing modalities in one *ALK*-positive concordant case. Image (A) reveals cribriform pattern frequently encountered in, though not diagnostic of, *ALK*-rearranged NSCLC on H&E-stained sample. Image (B) reveals strong homogeneous staining with *ALK* IHC. Image (C) reveals positive RNA ISH study; positivity is defined by two signals per cell in at least 5% of cancer cells and at least one cancer cell with four or more signals. Image (D) reveals corresponding FISH study: fused (yellow arrowhead) signal represents unrearranged gene and red arrows represent rearranged *ALK* with loss of 5' *ALK* (green signal). Image (E) reveals the RNA NGS (Archer) results: schematic of the *EML4* (exon 13)-*ALK* (exon 20) fusion. The chromosomal breakpoint of the fusion is chr2:42522656, chr2:29446394. FISH, fluorescence in situ hybridization; H&E, hematoxylin and eosin; IHC, immunohistochemistry; ISH, in situ hybridization; NGS, next-generation sequencing.

after initiation of TKI therapy were identified. Of cases with available clinical data, three had progression on first-line therapy. Specifically, among patients with concordant positive findings among all *ALK* studies, one case with variant 3a/b had progression on alectinib at 4 months and one case with variant 2 had progression on crizotinib at 5 months followed by response on ceritinib at 21-month follow-up. The third case of documented progression was a previously described discordant case (IHC negative, FISH positive, RNA ISH negative, RNA NGS negative), in which disease progression was noted at 3-month follow-up (Table 2).

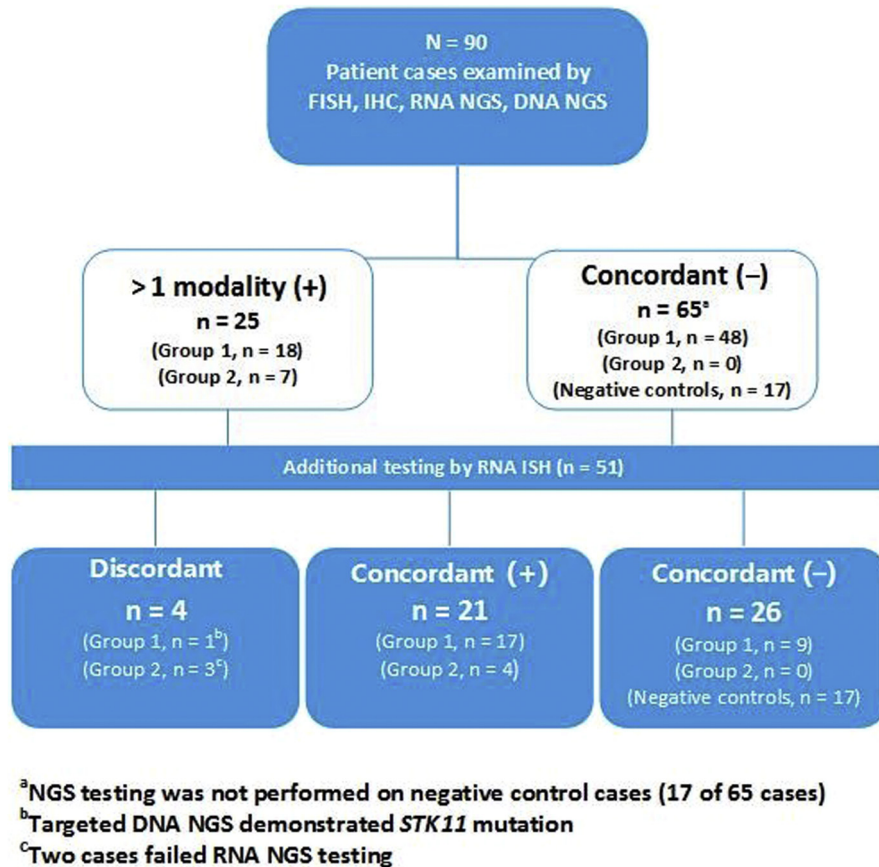
## Discussion

To date, the FDA has approved rearrangement by FISH or reactivity with IHC as primary testing methods to identify *ALK* fusions, and the updated CAP/IASLC/

AMP guidelines consider the two equivalents. Additional methodologies to detect *ALK* fusions have been developed, investigated, or variably adopted, and first, we evaluated two of these—RNA NGS and RNA ISH—against the currently available FISH and IHC standards. Second, we correlated results of the various methods, including specific variants identified by RNA NGS, with clinical outcomes.

### Concordant and Discordant Cases

Our study reveals discordance among *ALK* IHC, FISH, RNA ISH, and RNA NGS in a subset of cases. Among the “positives,” all four methods were concordant in 21 of 23 cases (91.3%). If RNA NGS is considered to be the gold standard, greatest correlation was equally found with IHC and RNA ISH methods, whereas there were discrepancies in a small subset of cases with FISH—the



**Figure 2.** Summary of the results. A total of 90 cases (group 1, n = 66; group 2, n = 7; negative controls, n = 17) underwent testing for *ALK* gene rearrangement. RNA ISH was performed on 51 cases, including all cases positive by greater than one testing modality, 17 negative control cases, and nine randomly selected concordant negative cases from group 1. FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; ISH, in situ hybridization; NGS, next-generation sequencing.

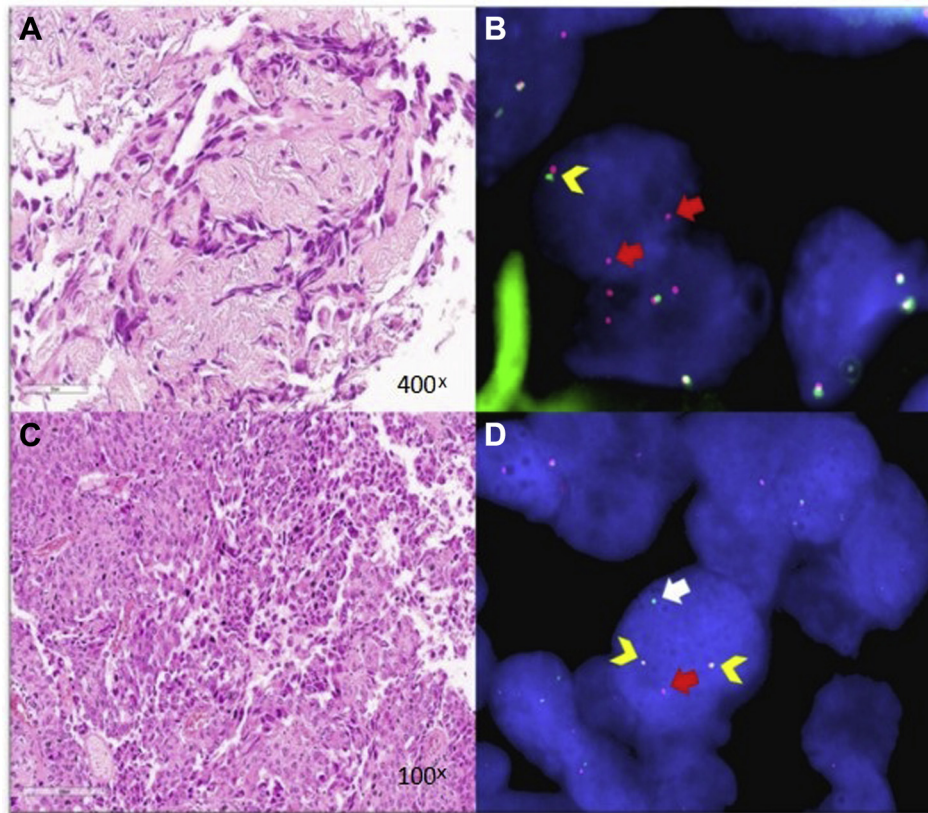
currently accepted gold standard. Meanwhile, the “negatives” were concordant among IHC, FISH, and RNA NGS results in 48 of 48 cases (100%); nine such cases with available RNA ISH were all negative.

Among the four cases in which there were discordances, two had all testing modalities successfully completed for comparison and were found to be FISH positive and otherwise *ALK* negative with IHC, RNA ISH, and RNA NGS. DNA NGS and SGA results evaluated in these two cases had *STK11* mutation in one case (Table 2; case 9) and no *KRAS* or *EGFR* mutations in the second case. Clinical follow-up was available for case 9 who had disease progression on TKI therapy at 3 months.

### IHC and FISH

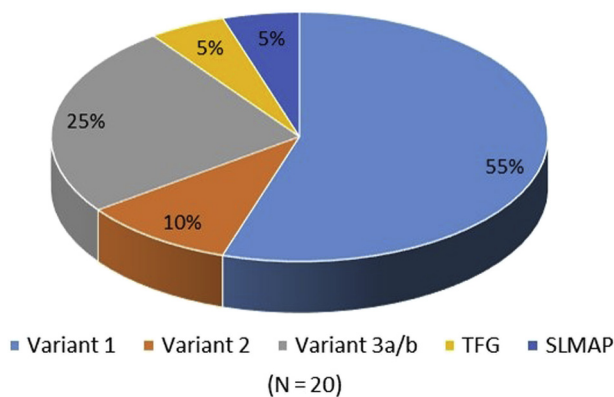
Previous studies have compared FISH and IHC with and without other *ALK* detection methods—DNA NGS and several non-Archer, RNA-based techniques—and illustrated concordance in most but not all cases.<sup>13–15</sup> Discordances may be due to interpretation of borderline FISH cases (i.e., positivity on the recommended  $\geq 15\%$

cutoff) or heterogeneity in *ALK* FISH patterns (i.e., [1] fusion and split green signals, [2] fusion and single orange, and [3] single orange or green signals). The discrepancies between various methods in our case can also be due to complex biological events, including transcriptional and post-translocation events, rather than technical issues that could modulate gene expression and potentially affect response to therapy. Such discrepant cases have been reported previously.<sup>15,16</sup> Furthermore, among FISH-negative, IHC-positive and FISH-positive, IHC-negative<sup>15,17–19</sup> cases, studies have revealed response<sup>13,17,20</sup> and worse outcome in both scenarios.<sup>21</sup> Marchetti et al.<sup>17</sup> in a review of the literature found that among FISH-positive, IHC-negative discordant cases, there was only a 46% response rate to therapy. Although this response is not insignificant, a shift away from FISH as the gold standard<sup>15,18,22,23</sup> and incorporation of NGS as standard clinical practice has been proposed.<sup>24</sup> Our study data support this proposal and have the added strength of additional testing modalities for comparison. Nevertheless, discordance and disease



**Figure 3.** H&E and FISH studies of discrepant cases. A high-powered H&E-stained image of a tumor present in one discordant case (Table 2; case 9) is illustrated in image (A). The corresponding FISH is illustrated in image (B): fused (yellow arrowhead) signal represents unrearranged gene and red arrows represent rearranged *ALK* with loss of 5' *ALK* (green signal). FISH positivity was found in 88% of the diploid and tetraploid cells. A high-powered H&E image of a tumor present in the second discordant case is illustrated in image (C). The corresponding FISH is illustrated in image (D): fused (yellow arrowhead) signals represent unrearranged gene, and red and white arrows represent rearranged *ALK*. FISH positivity was found in 68% of the cells. FISH, fluorescence in situ hybridization; H&E, hematoxylin and eosin.

progression require further investigation and optimization of current protocols to segregate eligible patients from those unlikely to respond to TKIs.



**Figure 4.** *ALK* gene fusion variants observed. Variant 1 = *EML4* (ex 13); variant 2 = *EML4* (ex 20); variant 3a/b = *EML4* (ex 6); *TFG* (ex 6); *SLMAP* (ex 12/13). All indicated variants represent fusions with *ALK* ex 20. For one positive case evaluated, ex data were unavailable. ex, exon.

### Targeted RNA NGS

In our cohort, RNA NGS was particularly advantageous in three scenarios. These encompass, first, identification of a novel gene fusion, second, confirmation (or lack thereof) of canonical and noncanonical *ALK* partners, and third, identification of specific rearrangements with potential impact on susceptibility to TKIs.

In the first scenario, RNA NGS uncovered *SLMAP-ALK*, a novel partner, and the results were concordant with IHC, FISH, RNA ISH, and DNA NGS (no hotspot mutations detected). In the second scenario, a FISH-positive result (IHC negative) prompted treatment with alectinib but without response leading to discontinuation. This case had a VUS in the *STK11* gene that is unlikely a driver mutation. In general, pathogenic *STK11* mutations are associated with poor prognosis in NSCLC and have been reported rarely with an *ALK* rearrangement<sup>25</sup>; as a VUS, it may not be consistently reported.<sup>26,27</sup> More recent studies have used RNA NGS AMP.<sup>11,28</sup> Similar to our analysis, others have uncovered (1) rare and (2) novel

Table 2. Therapy Outcomes

Case	IHC	FISH	RNA NGS	Variant	DNA NGS	RNA ISH	First-Line Therapy	Documented Progression	Second-Line Therapy	Follow-Up (mo)
1	P	P	<i>EML4</i> (ex 13)- <i>ALK</i> (ex 20)	1	N	P	Alectinib	N	—	4
2	P	P	<i>EML4</i> (ex 13)- <i>ALK</i> (ex 20)	1	N	P	Alectinib	N	—	9
3	P	P	<i>EML4</i> (ex 6)- <i>ALK</i> (ex 20)	3 a/b	N	P	Alectinib	N	—	10
4	P	P	<i>EML4</i> (ex13)- <i>ALK</i> (ex20)	1	N	P	Alectinib	N	—	10
5	P	P	<i>EML4</i> (ex 13)- <i>ALK</i> (ex 20)	1	N	P	Alectinib	N	—	6
6	P	P	<i>EML4</i> (ex 6)- <i>ALK</i> (ex 20)	3 a/b	F1174L	P	Alectinib	@ 4 mo, 12 d	Lorlatinib	6
7	P	P	<i>EML4</i> (ex 13)- <i>ALK</i> (ex 20)	1	N	P	Alectinib	N	—	13
8	P	P	<i>EML4</i> (ex 20)- <i>ALK</i> (ex 20)	2	N <sup>a</sup>	P	Crizotinib	@ 5 mo	Ceritinib	21
9	N	P	N	—	<i>STK11</i>	N	Alectinib	@ 3 mo, 5 d	Pembrolizumab Carboplatin- pemetrexed	18
10	P	P	<i>SLMAP</i> (ex 12/13)- <i>ALK</i> (ex 20)	—	N	P	Crizotinib <sup>b</sup>	N	—	24

<sup>a</sup>SGA KRAS/EGFR.

<sup>b</sup>Patient initially received cisplatin and pemetrexed for four cycles, after RNA NGS testing *ALK* fusion was identified and patient was switched to crizotinib. @, at the rate; ex, exon; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; N, negative; NGS, next-generation sequencing; P, positive; RNA ISH, RNA in situ hybridization; SGA, single-gene analysis.

fusions<sup>28</sup> and noted discordance between the current methods and RNA NGS.<sup>11</sup>

In the third scenario, variable response to TKIs was noted among nine patients with positive concordance among *ALK* IHC, FISH, RNA ISH, and RNA NGS. Currently, there are only rare studies suggesting differences in response to *ALK* fusion variants.<sup>25-27</sup> Emerging data reveal that specific *ALK* variants may be inherently aggressive<sup>25-28</sup> or responsive. An analysis of 54 tumors revealed that compared with variant 3a/b or 5a, variants 1/2/others have higher PFS.<sup>29</sup> Most cases in our cohort had PFS as of most recent clinical evaluation. One exception is case 8 with variant 2, which revealed progression at 5 months; the reported 2-year PFS is 76% in crizotinib-treated patients.<sup>27</sup> The second exception was in cases 3 and 6 both with variant 3a/b—one (case 3) responded to alectinib and one (case 6) with a known *ALK* resistance mutation (F1174L) that progressed.<sup>30</sup> These suggest that *ALK* positivity across all testing modalities may still portend suboptimal TKI response. We postulate, similar to previous reports, that there may be either variable response to specific variants or an underlying resistance mutation, akin to *EGFR* exon 20 mutations that have been found to convey de novo resistance to approved TKIs. Nevertheless, additional studies using RNA NGS are warranted to confirm the extent of correlation between *ALK* variant and clinical response after TKIs.

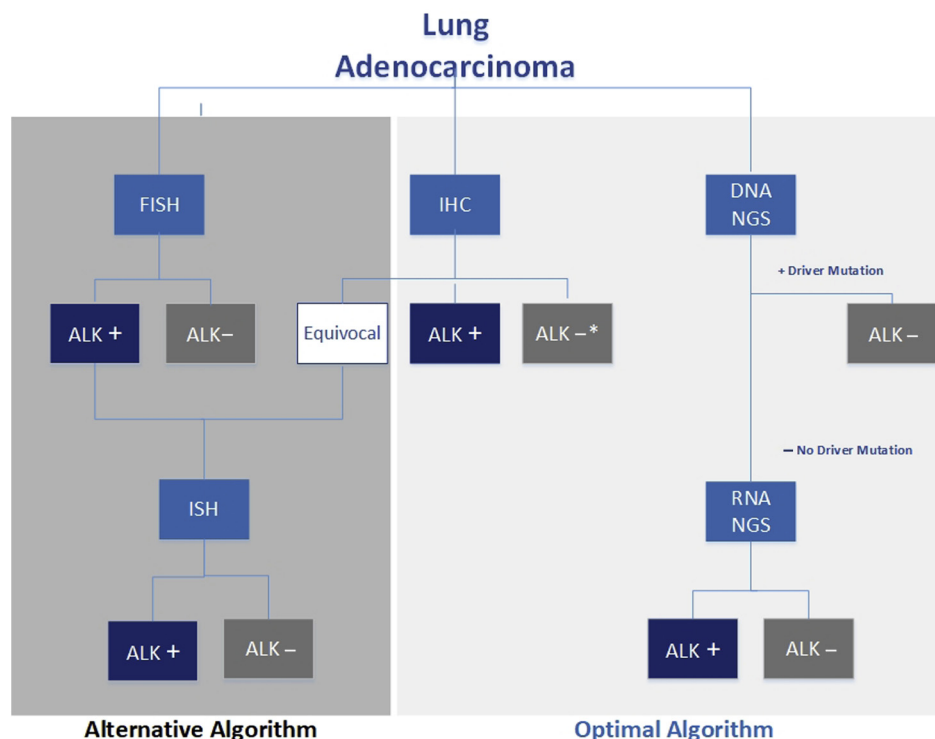
Several RNA-based methods are available, and we used AMP Archer FusionPlex.<sup>12</sup> First, a major advantage

to this method is the ability to identify specific fusion partners, including novel ones. In contrast to DNA NGS and other RNA methods, RNA NGS (AMP) uses gene-specific primers known to occur adjacent to fusion breakpoints and permits detection of rearrangements, including those in noncoding regions and novel ones, with a single primer. This is relevant as *ALK* rearrangements are heterogeneous and some may predict (greater) responsiveness than others as described previously. Second, RNA NGS has the ability to multiplex and identify other targetable fusions/alterations (e.g., *MET* exon 14 skipping, *RET*, *ROS1*, and *NTRK* fusions) without additional cost or tumor tissue for each additional test. Third, our ability to successfully perform Archer in most archived FFPE samples for the past 5 years makes RNA degradation a less likely hurdle when a sufficient contemporaneous sample is available. Nevertheless, RNA NGS (AMP) is neither widely available nor widely implemented. Although DNA NGS and reverse transcriptase-polymerase chain reaction are alternatives, they can only identify known variants.

### RNA ISH

In the two cases that failed RNA NGS, owing possibly to low quantity/quality of RNA or inadequate tumor cellularity, RNA ISH was negative in one case and equivocal in the other. In the remaining cohort, RNA ISH correlated 100% with the results of RNA NGS testing. To the best of our knowledge, only a single study has





**Figure 5.** Proposed algorithm for lung NSCLC (i.e., adenocarcinoma, large cell carcinoma, NSCLC, NOS): *ALK* testing. Optimal algorithm (light gray background, right): Perform *ALK* IHC and targeted DNA NGS testing for lung NSCLC in parallel. If a positive result is obtained by IHC (in the absence of other driver mutations—*EGFR*, *KRAS*, *BRAF*, *ERBB2*, and *MET*), follow with RNA NGS, when sufficient tumor material and resources are available. Alternative algorithm (dark gray background, left): RNA ISH may serve as an alternative secondary testing method for the confirmation of IHC results in cases without sufficient material for NGS or for clarity in cases of equivocal staining by IHC. If FISH is the predominant testing modality, additional confirmatory testing, which includes parallel IHC and/or NGS testing followed by RNA ISH in cases of equivocal IHC or insufficient material for NGS, can exclude a potential false-positive result. Note: The proposed algorithm is for first-line *ALK* testing; its application for identifying acquired *ALK* rearrangements that may develop after treatment with TKI was not evaluated.<sup>33</sup> \*RNA NGS is performed on *ALK* IHC-negative cases without driver mutations on DNA NGS to identify other potential targetable rearrangements (e.g., *MET*, *RET*, *ROS1*, *NTRKs*). FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; ISH, in situ hybridization; NGS, next-generation sequencing; NOS, not otherwise specified; TKI, tyrosine kinase inhibitor.

evaluated *ALK* RNA ISH. In it, Nakajima et al.<sup>31</sup> describe 16 cases with complete correlation between RNA ISH and either IHC or FISH positivity; NGS methods (DNA, RNA) were not performed.

Relative to RNA NGS, RNA ISH is more accessible and has a shorter turnaround time. In addition, RNA ISH analysis can provide quantitative scoring of gene expression by enumerating the number of punctuate dots present within each cell. This process provides an objective means of evaluation of positivity (in contrast to IHC) and can also be automated using RNAScope software.<sup>32</sup> Similar to RNA NGS, the ability to successfully perform ISH on older paraffin-embedded samples, at least 5 years before, makes RNA degradation a less likely possibility, when sufficient sample is available. A noteworthy challenge in evaluation of RNA ISH is localizing the brown signal at low magnification when present focally and distinguishing it from anthracotic pigment or entrapped fine air bubbles. An amplified and

red chromogen that provides sharper contrast would address both concerns.

### Proposed Optimized Algorithm

To capture all (in)eligible patients, we propose an algorithm that incorporates greater than one *ALK* testing modality (Fig. 5<sup>33</sup>). In this algorithm, we suggest *ALK* IHC followed by RNA NGS when no driver mutation is identified by DNA NGS, and sufficient tumor and resources are available to identify novel *ALK* fusion partners and other potential targetable fusion/rearrangements (e.g., *MET*, *RET*, *ROS1*, *NTRKs*). This combination provides a reportable result in a short turnaround time (initial IHC), followed by confirmation with a second testing method (RNA NGS) that has the advantage of identifying the specific *ALK* fusion partner. It is acknowledged that the overall turnaround time and cost of both testing modalities (IHC and RNA NGS) may be slightly increased in comparison to performing FISH alone or FISH and IHC;

however, the improvement in specificity and avoidance of treating false positives or potential specific variants with ineffective therapy greatly outweigh this shortcoming. In addition, RNA ISH may serve as an alternative secondary testing method in the following three scenarios: (1) confirmation of IHC results in cases with insufficient material to conduct RNA NGS testing; (2) clarity in cases of equivocal staining by IHC; and (3) inaccessibility to RNA NGS (complementary techniques [DNA NGS and RNA ISH] serve as alternatives). Although IHC (FDA-approved Ventana clone D5F3) is recommended in the optimal algorithm, FISH is a standard and FDA-approved alternative. A negative result by RNA NGS or RNA ISH in an archived specimen may require further investigation. Finally, in scenarios with negative *ALK* IHC (and/or FISH) and insufficient tissue for DNA NGS and RNA NGS, the 2018 CAP/IASLC/AMP minimum testing guidelines for lung adenocarcinomas include *EGFR* and *ROS1* (in addition to *ALK*).

### Strengths and Limitations

Strengths of this study are evaluation and comparison of four *ALK* testing modalities and the greatest numbers of positive and negative cases compared with previous studies evaluating AMP RNA NGS and RNA ISH. Though the results were compared with clinical outcomes, a limitation is that not all patients received targeted therapy or had available follow-up to evaluate response to various rearrangements.

### Conclusions

This study highlights that a small subset of results of IHC, FISH, RNA ISH, and RNA NGS are discordant and gold standard designation for FISH necessitates reassessment. An alternative to the current recommendations is IHC followed by RNA NGS, but it requires further investigation.

### CRedit Authorship Contribution Statement

**Carleigh R. Canterbury:** Data curation, Writing—original draft, Writing—review and editing.

**Helen Fernandes:** Investigation, Resources, Writing—review and editing.

**John P. Crapanzano, Vundavalli V. Murty, Mahesh M. Mansukhani:** Investigation, Writing—review and editing.

**Catherine A. Shu:** Data curation, Writing—review and editing.

**Matthias Szabolcs:** Methodology, Resources, Writing—review and editing.

**Anjali Saqi:** Conceptualization, Investigation, Writing—original draft, Writing—review and editing.

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