Review Article

A narrative review of methods for the identification of *ALK* fusions in patients with non-small cell lung carcinoma

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Background and Objective: This narrative review is intended to provide pragmatic knowledge of current methods for the search of anaplastic lymphoma kinase (*ALK*) fusions in patients with non-small cell lung carcinoma (NSCLC). This information is very timely, because a recent survey has identified that almost 50% of patients with advanced NSCLC were not candidates for targeted therapies because of biomarker testing issues.

Methods: PubMed was searched from January 1st, 2012 to February 28th, 2023 using the following keywords: "*ALK*" and "lung", including reviews and our own work.

Key Content and Findings: Testing rates have not reached 85% among patients' candidates to ALK inhibition. The advantages and disadvantages of the different analytical options [immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), real-time polymerase chain reaction and next-generation sequencing (NGS)] are discussed. The key factor for success in *ALK* testing is a deep understanding of the concept of "molecular redundancy". This notion has been recommended and endorsed by all the major professional organizations in the field and can be summarized as follows: "laboratories should ensure that test results that are unexpected, discordant, equivocal, or otherwise of low confidence are confirmed or resolved using an alternative method or sample". In-depth knowledge of the different *ALK* testing methodologies can help clinical and molecular tumor boards implement and maintain sensible algorithms for a rapid and effective detection of predictive biomarkers in patients with NSCLC.

Conclusions: Multimodality testing has the potential to increase both the testing rate and the accuracy of *ALK* fusion identification.

Keywords: Anaplastic lymphoma kinase (ALK); fluorescence in situ hybridization (FISH); immunohistochemistry (IHC); next-generation sequencing (NGS); non-small cell lung carcinoma (NSCLC)

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Introduction

Background and rationale

The anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that plays a critical role in the development and function of the nervous system. Accordingly, there is protein expression in the nervous system during development and in some nervous tissues in adults. The *ALK* gene is located on the short arm of chromosome 2 and encodes a transmembrane protein that consists of an extracellular ligand-binding domain, a transmembrane region, and an intracellular kinase catalytic domain. ALKALs have now been reported as ligands for the ALK receptor (1,2). Upon ligand binding, activation of ALK leads to the phosphorylation of downstream signaling molecules, including the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) pathways, which regulate cell growth, survival, and differentiation. Oncogenic activating fusions were originally described in anaplastic large cell lymphomas and have subsequently been identified in a variety of malignant tumors (3,4).

In 2007, *ALK* fusions were identified in a small percentage of patients with non-small cell lung carcinoma (NSCLC) (5). The subsequent development and approval of several ALK inhibitors means that the relevance of accurately identifying *ALK* fusions in patients with NSCLC has never been greater (6,7). Thus, *ALK* fusions testing in NSCLC has increased over time, but many of the gaps on the implementation of personalized oncology can be traced back to suboptimal biomarker test results (see below) (8).

ALK gene fusions, which are found in approximately 3–5% of the overall NSCLC population, result in aberrant expression of the chimeric ALK proteins (9,10). There is a higher prevalence in never/light smokers, younger age and adenocarcinoma histology (10-13). Interestingly, a very high frequency of signet ring cells has been reported and could be a very good predictive histological feature (14-18). Accordingly, the presence of signet ring cells has been identified in 44.4% to 72% of *ALK*-positive tumors (median 56%, mean: 57.2%) (14-18). The increasing use of next generation sequencing (NGS) has identified more than 90 different fusion partners, but echinoderm microtubule-associated protein-like 4 (*EML4*) remains the most frequent partner (approximately 85%) (19,20). The most commonly reported *EML4-ALK* fusion breakpoints are v1 (30–40%), v3a/b (30–40%) and v2 (10%) (10,20-22). The individual frequencies of non-*EML4-ALK* fusions are difficult to infer but there are usually below 2%: kinesin family member 5B (*KIF5B*; range, 0.4–2.2%), striatin (*STRN*; range, 1–1.4%), huntingtin interacting protein 1 (*HIP1*; range, 1–3%) or trafficking from ER to golgi regulator (*TFG*; 1.4%) (21-28). However, some series have found higher frequencies of *STRN* (6.7%), *HIP1* (5%) or *TFG* (5%), a fact that needs to be taken into consideration when not using NGS as the primary testing modality (see below) (21,29,30).

Knowledge gap and objective

This narrative review is intended to provide pragmatic knowledge of current methods for detecting *ALK* fusions in patients with NSCLC. Although broad molecular profiling is usually the recommended testing option in most guidelines, NGS is not universally available (4). Moreover, the information presented herein is very timely, because a recent survey has identified that almost 50% of patients with advanced NSCLC were not candidates for targeted therapies because of biomarker testing issues (8). The clinical gaps can be summarized as follows (the three "Ts"): tissue (insufficient tissue or tumor cell overestimated), testing (appropriate assay was not ordered or results were inconclusive or false negative) and time (turnaround time delays) (8). The specific situation of *ALK* fusions testing in NSCLC is somewhat similar because testing rates have not surpassed 85% among patients' candidates to *ALK* inhibition and NGS is combined with single analyte assays in most regions of the world (31-35). Moreover, recent data indicate that 25% of the *ALK* positive patients begin a non-targeted treatment before receiving the results and surprisingly detection rate is influenced by the type of assay (31,36). Therefore, we hypothesized that in-depth knowledge of the advantages and disadvantages of the different *ALK* testing methodologies can help clinical and molecular tumor boards implement and maintain sensible algorithms for a rapid and effective detection of predictive biomarkers in patients with NSCLC, regardless of the histologic subtype (4,37,38). In the era of tumor-agnostic therapies, it is important to highlight

Table 1 Details of the search method for this narrative review

Items	Specification
Date of search	Apr 11th, 2023
Databases and other sources searched	PubMed, Own Work
Search terms used	"*ALK*" and "lung"
Timeframe	Jan 1st, 2012 – Feb 28th, 2023
Inclusion criteria	Original publications and reviews
Selection process	Selection by author

ALK, anaplastic lymphoma kinase.

that *ALK* fusions have also been described in many other neoplasms: 50–60% of inflammatory myofibroblastic tumors, 2.2% of papillary thyroid carcinomas, <1% of colorectal adenocarcinomas and a variety of hematolymphoid tumors, including *ALK*-positive histiocytosis (39-41). This concept is reassuring, as it indicates that we could potentially use the same testing strategies presented herein. We present this article in accordance with the Narrative Review reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-855/rc).

Methods

PubMed was searched for English-language journals from January 1st, 2012 to February 28th, 2023 using the following keywords: "*ALK*" and "lung", including reviews and our own work (*Table 1*).

Testing approaches for ALK fusions

The most frequently used tissue assays that are currently available to identify *ALK* fusions include both traditional single methodologies such as immunohistochemistry (IHC), break-apart fluorescence in situ hybridization (FISH) and real-time PCR, as well as the increasingly popular NGS (42-51). Each of these approaches has advantages and disadvantages that need to be carefully balanced to design sensible testing algorithms. *Table 2* summarizes this information (4). Except for "analytical sensitivity" and "diagnostic sensitivity", all attributes are self-explanatory. The "analytical sensitivity" (often referred to as just "sensitivity") is the limit of detection. The "diagnostic sensitivity" relates to the comprehensiveness of the assay or the percentage of all *ALK* fusions described for the gene detectable by the given assay (53).

IHC

There is currently a stand-alone IHC Food and Drug Administration (FDA) approved assay (VENTANA ALK D5F3 CDx Assay, Ventana Medical Systems, Tucson, AZ, USA) (54). Our interpretation of the workflow is presented in *Figure 1*. Staining requires three sections from the formalin-fixed paraffin embedded (FFPE) material: one hematoxylin and eosin (H&E), a second for the VENTANA anti-ALK (D5F3) CDx assay antibody, and a third for the negative reagent control. System-level controls (previously qualified appendix tissue) are included on each section. If the H&E evaluation indicates that the specimen is not acceptable, a new section should be stained. The assay should be repeated under three circumstances: (I) if the system-level controls fail to show appropriate staining, (II) if the negative reagent control demonstrates a non-specific diffuse staining, and (III) if target cells fail to show appropriate staining. The slide is interpreted using a binary scoring system: positive (strong, granular and cytoplasmic staining in any percentage of tumor cells) or negative (absence of strong granular cytoplasmic staining in tumor cells). Scoring 50 tumor cells may decrease the risk of both false negative and false positive results, but there is no agreement on the number of positive cells required for a positive IHC result (15,55,56). The main advantages of IHC are that it requires only three sections of tissue (a total of 12 µm), and that the turnaround time of its analytical phase is around 4.5 hours, which allows for "real-time" results. If samples have been properly fixed (at least 6 hours in 10% neutral buffered formalin) and both types of controls are included in every patient case (system-level and negative reagent controls), this IHC approach should have very high sensitivity and specificity (15,55). Examples of ALK IHC positive and negative samples are shown in *Figure 2A-2H*.

Table 2 Methods for detecting ALK fusions

Parameter	IHC	FISH	Real-time-PCR (RNA-based)	DNA-based NGS	RNA-based NGS
Turn-around time	Hours	1–2 days	Hours to 1–2 days	<2 weeks[a]	3 days[b] to 1 week[c]
Input material	Low	Low	Intermediate	Variable (high for hybridization capture panels)	Variable (low for some amplicon panels)
Cost	Low	Low	Low	Intermediate	Intermediate
Precise annotation of variants	No	No	Sometimes	Yes	Yes
Analytical sensitivity	High	High	High	Lower than RNA-based NGS	High
Diagnostic sensitivity	High	High	Intermediate	High	High
FDA-approved assays	Yes	Yes	No	Yes	No

[a,c], according to reference (4) and [b], reference (52). ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; NGS, next-generation sequencing; FDA, food and drug administration.

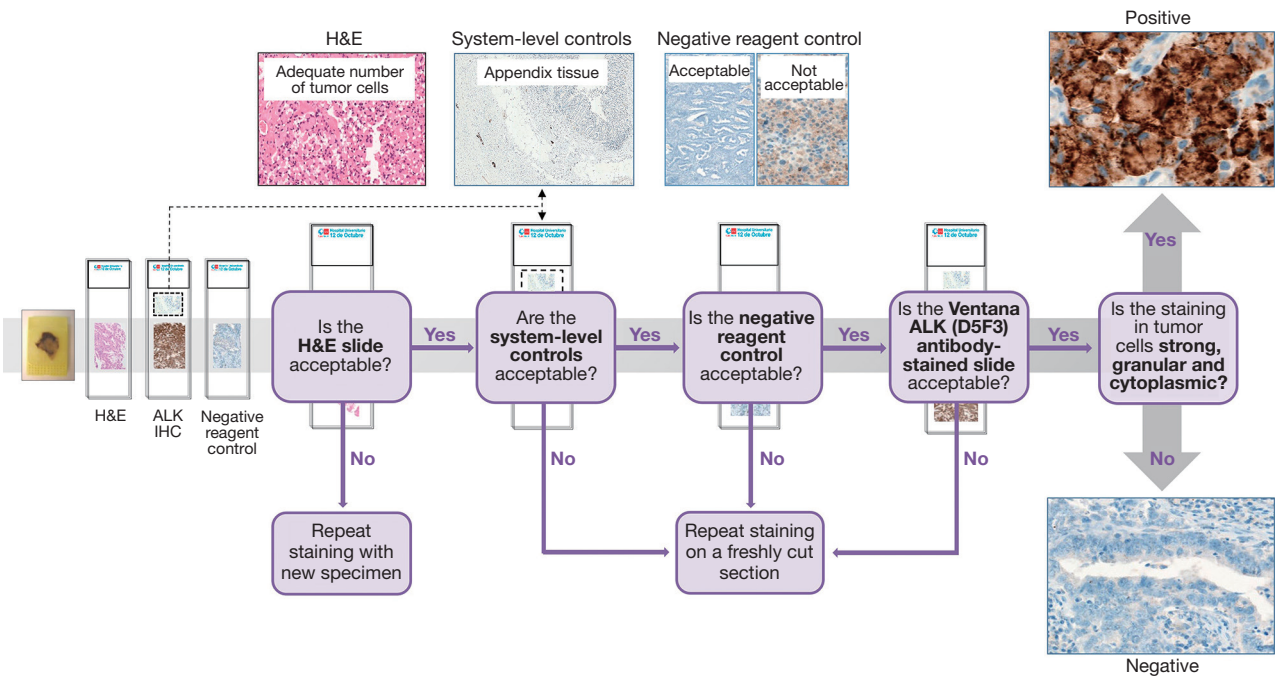

Figure 1 ALK immunohistochemistry specimen workflow using the VENTANA ALK (D5F3) CDx Assay. Magnification times: ×200. H&E, hematoxylin and eosin; ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry.

FISH

FISH using break-apart probes used to be the reference method for the identification of fusions, and as such it is still widely available and recommended by international guidelines (6,7). There is currently an FDA-approved assay (Vysis *ALK* Break Apart FISH Probe Kit, Abbott Molecular, IL, USA) (57). Our interpretation of the workflow is presented in *Figure 3*. The assay requires two sections from the FFPE block: one H&E and a second for the FISH assay. If the H&E evaluation shows that the specimen is not acceptable, a new section should be stained. A pathologist should select the best tumor area for hybridization and scoring. If all previous steps are adequate, the stained

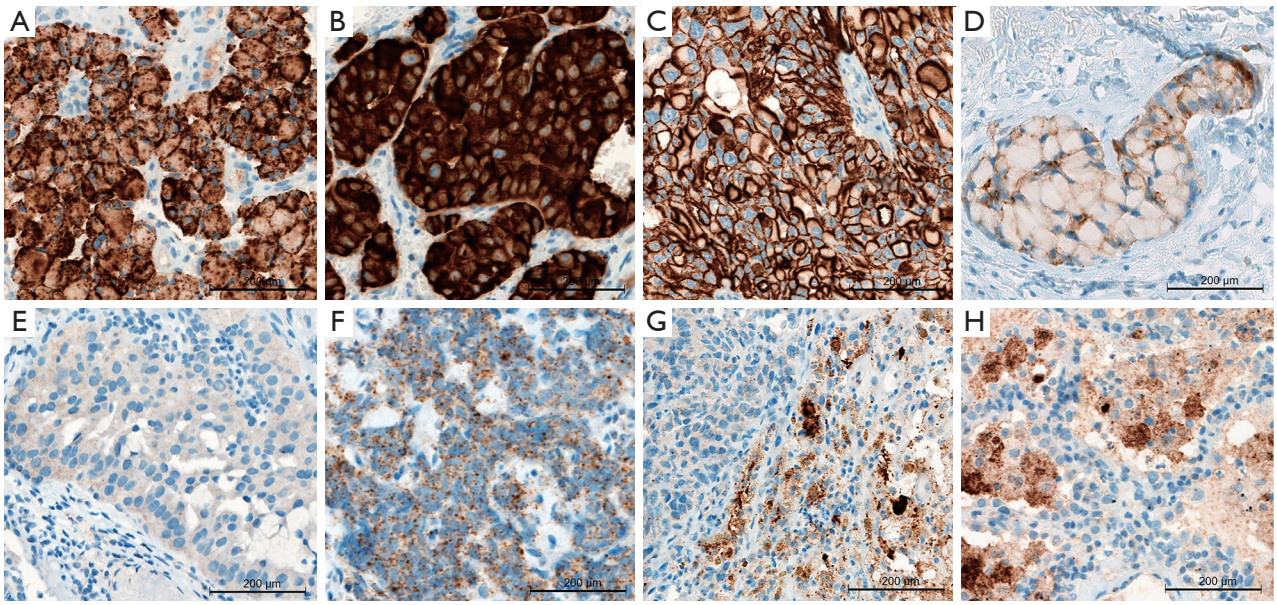

Figure 2 Examples of ALK immunohistochemistry positive (A-D) and negative (E-H) cases using the VENTANA ALK (D5F3) CDx Assay. (A) An ALK-positive tumor showing the typical staining pattern (strong, granular and cytoplasmic staining) (original magnification: ×200); (B,C) positive cases exhibiting atypical staining patterns (B, homogeneous strong but diffuse cytoplasmic staining and C, linear membranous accentuation) (original magnification: ×200); (D) an ALK positive adenocarcinoma with signet ring cells showing a weaker staining (the cytoplasm is replaced by mucin) (original magnification: ×200); (E) an ALK-negative lung adenocarcinoma showing a weak cytoplasmic staining (original magnification: ×200); (F) a small cell lung carcinoma exhibiting an aberrant ALK expression (i.e., fusion negative) (original magnification: ×200); (G) brain metastasis from a lung adenocarcinoma showing positivity in neural tissue but no staining in tumor cells (original magnification: ×200); (H) positivity in alveolar macrophages (original magnification: ×200). ALK, anaplastic lymphoma kinase.

specimen is scored as depicted in *Figure 3*. There are two patterns of positivity: (I) break-apart or typical pattern, with one fused signal [orange (i.e., 3') and green (i.e., 5') are either overlapping, adjacent or are less than 2 signal diameters apart] and separated orange and green signals by 2 or more signal diameters; and (II) isolated orange signal or atypical pattern, with one fused signal and one orange signal without the corresponding green signal. The main advantages of FISH are that only two sections of tissue are required, and interpretation is straightforward with very high sensitivity and specificity, particularly if the *ALK* FDA-approved image analysis algorithm is used (Duet System, BioView, Rehovot, Israel) (15). The use of automated digital scoring workflows has highlighted the prevalence of the atypical pattern, either alone (45%) or in combination with the typical pattern (34%) (15). Finally, we must emphasize two important facts: (I) a precise annotation of the fusion variant is not possible when using IHC or FISH; and (II) detailed IHC and FISH data for most of the rare or uncommon *ALK* variants is currently lacking (19). Examples of *ALK* FISH positive and negative samples are shown in *Figure 4A-4H*.

Real-time PCR

RNA-based real-time PCR assays are very popular in regions with a very high prevalence of epidermal growth factor receptor (*EGFR*) mutations or when tumor content, turnaround time and/or cost preclude the use of NGS (21,43,45,58,59). Taking into consideration the molecular epidemiology of *ALK* fusions (see above), the design (i.e., breadth) of these kits is probably not as relevant for this discussion as it is for the detection of other actionable fusions (60). Nonetheless, users of these assays should be constantly aware that "*pseudo* false negatives" (i.e., due to rare or uncommon fusion partners not included in the design of an assay) are unavoidable because the most widely used real-time PCR assays are designed to only identify some of the non-*EML4-ALK* fusion partners (21,43,45,58,59). Finally, it must be overemphasized

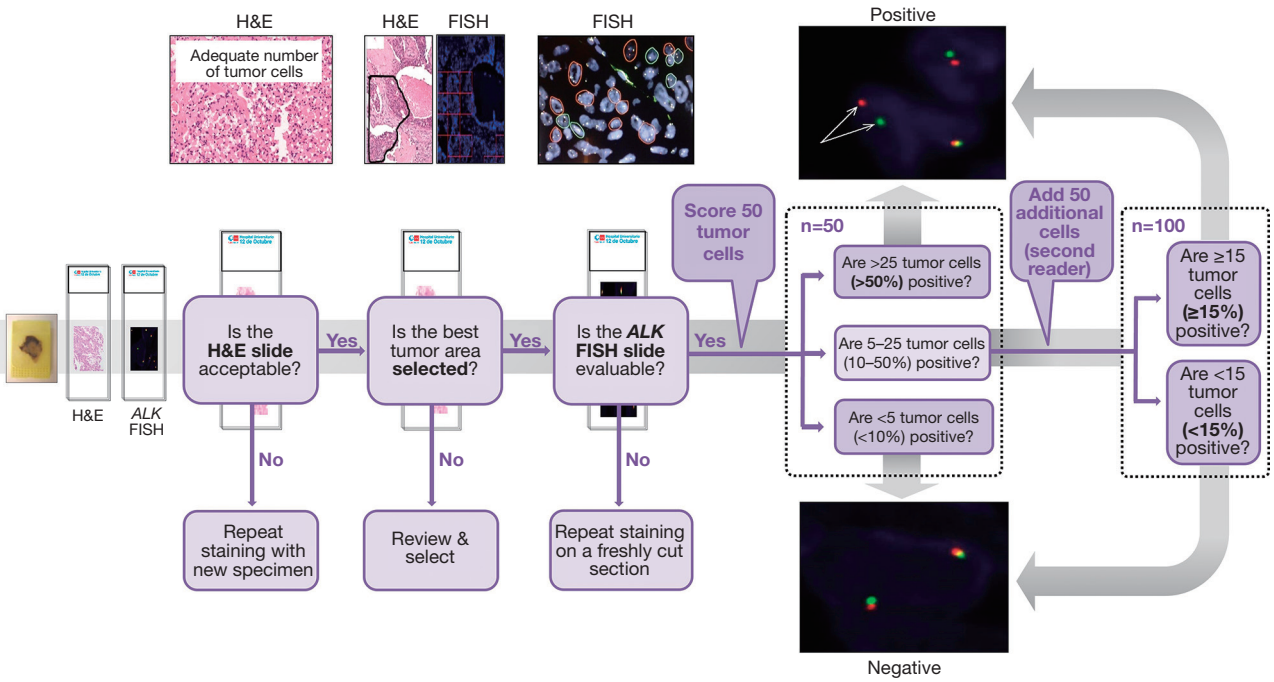

Figure 3 *ALK* fluorescence in situ hybridization specimen workflow using the Vysis *ALK* Break Apart FISH Probe Kit. H&E, hematoxylin and eosin; FISH, fluorescence in situ hybridization; ALK, anaplastic lymphoma kinase.

that sometimes the fusions are detected by imbalance assessment, a strategy that is a potential source of confusion because its lower specificity (see below).

NGS

NGS should be performed using a clinically validated NGS panel, which ideally should include all guideline recommended biomarkers for patients with advanced NSCLC (6,7). Although there are no recent head-to-head comparisons of all the main available platforms using a large cohort of real-world *ALK* positive patients, most NGS panels should theoretically have a high performance for the detection of *ALK* fusions (21,22,29). From a pragmatic stand-point some comments are useful when assessing NGS options or discussing their results. DNA-only hybrid capture methods are fusion-partner agnostic but their sensitivity for the detection of actionable fusions has been challenged (61,62). This situation has prompted many institutions to combine DNA-only NGS with RNA sequencing at least in driver-negative patients. Regarding RNA-based NGS, it is worth considering three facts: (I) is the sequencing method partner-agnostic? (II) is the panel affected by a low quantity and/or quality of the RNA? and (III) what is the turnaround time, including hands-on time and interpretation time? (63). In general, hybrid-capture NGS and anchored multiplex PCR are fusion-partner agnostic but usually require higher quantity and/or quality of RNA, with longer turnaround times (63). In contradistinction, the recent arrival of ultrafast amplicon-based NGS panels is bridging the gap between real-time PCR and targeted NGS (21,52). Finally, a precise annotation of the *ALK* variant and the molecular context (i.e., the presence of a *TP53* mutation) is becoming increasingly important for treatment selection. For example, tumors with *EML4-ALK* variant 3 are more prone to the development of resistance mutations (24). Accordingly, patients with variant 3 have a worse outcome (64), and frequently *TP53* co-mutations are identified within this subgroup (65,66). Nonetheless, the topic is still controversial because other studies have found the same degree of benefit across variants (67), and the predictive value of rare *EML4-ALK* and non-*EML4-ALK* fusions is slowly emerging (20,25,68).

Liquid biopsies

Although liquid biopsies encompass a wide range of components, circulating tumor DNA (ctDNA) is the

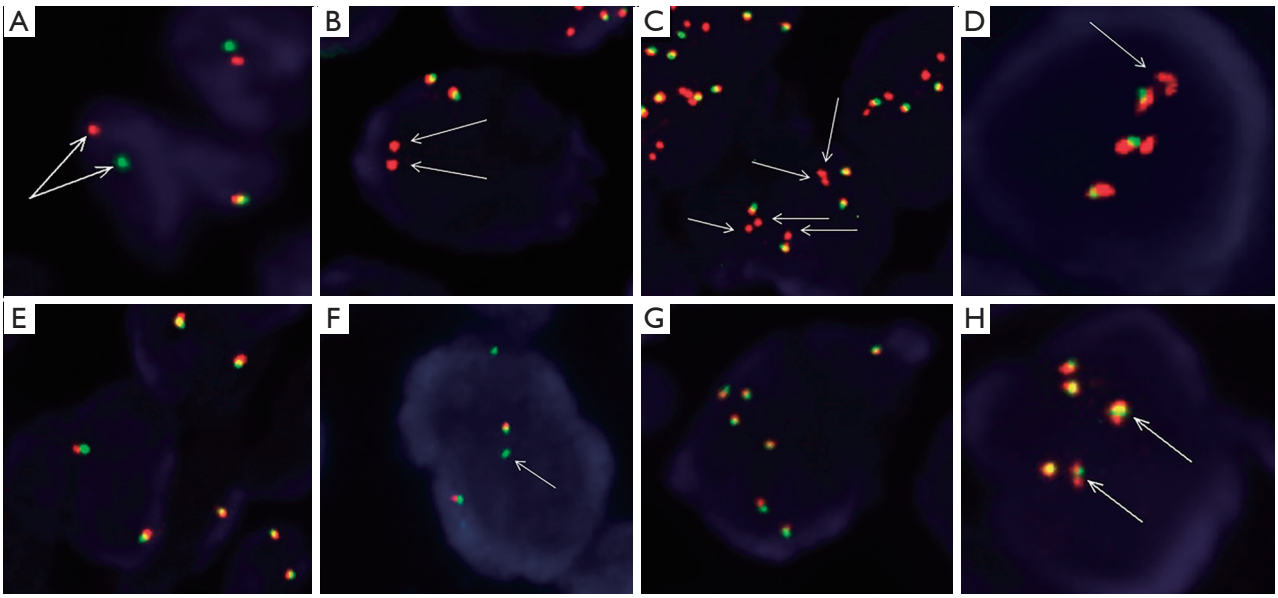

Figure 4 Examples of *ALK* fluorescence in situ hybridization positive (A-D) and negative (E-H) cases using the Vysis *ALK* Break Apart FISH Probe Kit. (A) Typical positive or break apart 3'-5' pattern (orange and green signals are separated by 2 or more signal diameters). In some positive cases, tumor nuclei can show more than 1 set of break apart signals; (B) atypical positive or isolated 3' pattern (single orange signals); (C) atypical positive or isolated 3' pattern with *ALK* copy number gain; (D) atypical positive or isolated 3' pattern. The case also shows a duplication of the *ALK* 3'end (3'/5'/3') and copy number gain. (E) negative or fused pattern; (F) negative or isolated 5'pattern (single green signal); (G) negative or fused pattern with *ALK* amplification (≥6 *ALK* fusion signals per cell in ≥10% of analyzed cells); (H) negative pattern with a duplication of the *ALK* 3'end (3'/5'/3'). The case also shows a copy number gain (3–5 *ALK* fusion signals in ≥10% of analyzed cells); *ALK* FISH assays were scored with an image analysis algorithm (Duet System, BioView, Rehovot, Israel). ALK, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization.

most widely used for detection of actionable findings in patients with NSCLC. Several methodologies for ctDNA analysis have been implemented in clinical practice and research purposes, including PCR-based assays and NGS-based methodologies (69-71). PCR-based approaches have more limited multiplexing capabilities but may deliver faster results with a higher sensitivity for the target genes. NGS-based approaches can provide comprehensive tumor genotyping at a higher turnaround time, cost and complexity (72). Three strategies have been proposed in patients with advanced NSCLC: plasma-first, sequential tissue-first and complementary tissue-first (69). Regardless of the testing strategy, three facts need to be considered to optimize the use of liquid biopsies: (I) ctDNA detection is influenced by the stage of disease (i.e., intrathoracic and intracranial disease have been associated with a higher risk of false-negative results) (73), (II) the sensitivity of ctDNA assays for the detection of *ALK* fusions ranges from 65% to 78% (74,75), and (III) liquid biopsies are a very effective method for assessing *ALK* mutations after ALK inhibition (76,77).

Discussion

Tables 3,4 provide real-world considerations to avoid false negative and false positive results when using IHC, FISH, real-time PCR or NGS (4,21-23,55,56,59,62,63,78-81). Although the main potential solutions are shown in the tables and briefly discussed below, the key factor for success in *ALK* testing is a deep understanding of the concept of "molecular redundancy". This notion has been recommended and endorsed by all the major professional organizations in the field and can be summarized as follows: "laboratories should ensure that test results that are unexpected, discordant, equivocal, or otherwise of low confidence are confirmed or resolved using an alternative

Table 3 Practical considerations for ALK testing: avoiding false negative results

Methodology	Potential reasons for failure or false negative results	Potential solutions
IHC	Suboptimal sample fixation	Do not stain samples with pre-analytical issues
	Suboptimal analytical phase	Always check the system level controls
	Use of pre-cut slides	Use slides that have been sectioned recently
	IHC slide must contain at least 50 evaluable tumor cells	Retest another block (same specimen), retest another specimen or propose a rebiopsy
FISH	Scoring a non-neoplastic area	Always score *ALK* FISH after looking at the H&E slide
	FISH slide must contain at least 50 evaluable tumor cells	Retest another block (same specimen), retest another specimen or propose a rebiopsy
	Presence of uncommon FISH patterns	Seek a second opinion
		Use molecular redundancy (including image analysis algorithms)
	Suboptimal hybridization quality (nuclei morphology, background, probe signal intensity, etc.)	Retest another block (same specimen), retest another specimen or propose a rebiopsy
Real-time PCR	Overestimation of tumor content	Always check the tumor content on a new H&E after RNA extraction
	Low quantity and/or quality of input RNA	Retest another block (same specimen), retest another specimen or propose a rebiopsy
	Design of the kit (breadth)	Use molecular redundancy in driver negative patients
DNA-based NGS	Overestimation of tumor content	Always check the tumor content on a new H&E after DNA extraction
	Low quantity and/or quality of input DNA	Retest another block (same specimen), retest another specimen or propose a rebiopsy
	Higher input requirement for some panels	Retest another block (same specimen), retest another specimen or propose a rebiopsy
	Lower sensitivity for the detection of fusions	Use molecular redundancy in driver-negative patients
RNA-based NGS	Overestimation of tumor content	Always check the tumor content on a new H&E after RNA extraction
	Higher RNA quantity and/or quality requirement for some panels	Retest another block (same specimen), retest another specimen or propose a rebiopsy
	RNA workflow fails and only DNA results are reported	Use molecular redundancy in driver-negative patients
		Retest another block (same specimen), retest another specimen or propose a rebiopsy
	Design of the panel (breadth)	Use molecular redundancy in driver-negative patients

ALK, anaplastic lymphoma kinase; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; H&E, hematoxylin and eosin; NGS, next-generation sequencing; PCR, polymerase chain reaction; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

method or sample" (82).

Avoiding false negative results (Table 3)

The use of pre-cut slides is a well-known source of differences in the intensity of IHC staining that can even cause unequivocally false negative results (81). Pathologists should not score ALK IHC slides with less than 50 tumor cells (56). Scoring uncommon FISH patterns can be very challenging, so the use of "molecular redundancy" is advised when encountering such cases (79,80). When using PCR-based assays (real-time PCR or NGS), a precise knowledge of the breadth of the kit/panel and its real-world performance can help rule-out a false negative result (63,78,79). From a practical standpoint, the most important question we need to consider in driver-negative NSCLC patients is: do I need to persevere in the search of actionable findings? In such circumstances, reflex testing

Table 4 Practical considerations for ALK testing: avoiding false positive results

Methodology	Potential reasons for false positive results	Potential solutions
IHC	Scoring non-specific background in tumor cells	Compare the intensity of the staining with the one observed in the negative reagent control
		Use molecular redundancy
	Scoring normal tissue elements (alveolar macrophages, benign glandular epithelium, etc.)	Always score ALK IHC after looking at the H&E slide
	Aberrant ALK IHC expression in lung neuroendocrine carcinomas and neural tissue	Always score ALK IHC after looking at the H&E slide
FISH	Using a lower cut-off for positivity when scoring the initial 50 tumor cells (15% instead of 50%)	Follow the manufacturer´s instructions
	Exclusive presence of the atypical FISH positive pattern (isolated orange signal)	Seek a second opinion
		Use molecular redundancy (including image analysis algorithms)
	Suboptimal hybridization quality (nuclei morphology, background, probe signal intensity, etc.)	Retest another block (same specimen), retest another specimen or propose a rebiopsy
Real-time PCR	Lower specificity for imbalance assessment	Use molecular redundancy
DNA-based NGS	Fusion detected with a low number of reads	Follow the manufacturer's instructions
		Use molecular redundancy if tumor content is low
RNA-based NGS	Fusion detected with a low number of reads	Follow the manufacturer's instructions
		Use molecular redundancy if tumor content is low
	Lower specificity for imbalance assessment	Use molecular redundancy

IHC, immunohistochemistry; ALK, anaplastic lymphoma kinase; H&E, hematoxylin and eosin; FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction; NGS, next-generation sequencing; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

with IHC, FISH or a different/larger NGS panel is strongly recommended (4).

Avoiding false positive results (Table 4)

Aberrant ALK expression (i.e., rearrangement negative) has been described in lung neuroendocrine carcinomas (including small cell lung carcinomas) and neural tissue (22,55). Because ALK IHC is a standalone test, reviewing the H&E before scoring the ALK IHC slide is mandatory. FISH slides that only show the atypical pattern of positivity (isolated orange signals) should ideally be scored with an image analysis algorithm to rule out a suboptimal or a very low signal intensity of the green probe, an overlooked reason for *ALK* FISH false positive results (23,80). Regarding PCR-based methods (real-time PCR or NGS), when an *ALK* fusion is identified by expression imbalance or with a low number of reads, orthogonal testing is recommended to improve specificity (21,59,63,78,79).

Strengths and limitations of this review

The main strength of this review is that we provide pragmatic and technology-agnostic solutions to avoid false negative and false positive results when detecting *ALK* fusions (or other actionable fusions for that matter) in patients with NSCLC.

The main limitation is that the available real-world evidence on the performance of the different methods is scarce, due to the constantly changing world of lung cancer biomarker testing.

Conclusions

Through this paper we have described the most frequently used *ALK* testing modalities: IHC, FISH, real-time PCR

and NGS. Although most guidelines recommended broad molecular profiling for patients with advanced NSCLC, single-gene assays are still widely used across the globe. Therefore, the key factor for success (i.e., increase testing rates and avoid false positive and false negative results) is to develop sensible testing algorithms, that level the advantages and disadvantages of the different methodologies. This approach can be reconciled with the fact that RNA sequencing is now becoming the gold standard for fusion identification, because of its superior sensitivity.

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Footnote

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References

1. Fadeev A, Mendoza-Garcia P, Irion U, et al. ALKALs are in vivo ligands for ALK family receptor tyrosine kinases in the neural crest and derived cells. Proc Natl Acad Sci U S A 2018;115:E630-8.
2. Li T, Stayrook SE, Tsutsui Y, et al. Structural basis for ligand reception by anaplastic lymphoma kinase. Nature 2021;600:148-52.
3. Schneider JL, Lin JJ, Shaw AT. ALK-positive lung cancer: a moving target. Nat Cancer 2023;4:330-43.
4. IASLC Atlas of Molecular Testing for Targeted Therapy in Lung Cancer | IASLC. [Accessed February 28, 2023] Available online: https://www.iaslc.org/iaslc-atlas-molecular-testing-targeted-therapy-lung-cancer
5. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007;448:561-6.

6. NCCN Guidelines NSCLC Version 2.2023. [Accessed February 26, 2023] Available online: https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1450
7. Hendriks LE, Kerr KM, Menis J, et al. Non-oncogene addicted metastatic non-small-cell lung cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. Ann Oncol 2023; 34:358-376.
8. Sadik H, Pritchard D, Keeling DM, et al. Impact of Clinical Practice Gaps on the Implementation of Personalized Medicine in Advanced Non-Small-Cell Lung Cancer. JCO Precis Oncol 2022;6:e2200246.
9. Thai AA, Solomon BJ, Sequist LV, et al. Lung cancer. Lancet 2021;398:535-54.
10. Remon J, Pignataro D, Novello S, et al. Current treatment and future challenges in ROS1- and ALK-rearranged advanced non-small cell lung cancer. Cancer Treat Rev 2021;95:102178.
11. Park HS, Lee JK, Kim DW, et al. Immunohistochemical screening for anaplastic lymphoma kinase (ALK) rearrangement in advanced non-small cell lung cancer patients. Lung Cancer 2012;77:288-92.
12. Mack PC, Klein MI, Ayers KL, et al. Targeted Next-Generation Sequencing Reveals Exceptionally High Rates of Molecular Driver Mutations in Never-Smokers With Lung Adenocarcinoma. Oncologist 2022;27:476-86.
13. Fan L, Feng Y, Wan H, et al. Clinicopathological and demographical characteristics of non-small cell lung cancer patients with ALK rearrangements: a systematic review and meta-analysis. PLoS One 2014;9:e100866.
14. Conde E, Angulo B, Izquierdo E, et al. The ALK translocation in advanced non-small-cell lung carcinomas: preapproval testing experience at a single cancer centre. Histopathology 2013;62:609-16.
15. Conde E, Suárez-Gauthier A, Benito A, et al. Accurate identification of ALK positive lung carcinoma patients: novel FDA-cleared automated fluorescence in situ hybridization scanning system and ultrasensitive immunohistochemistry. PLoS One 2014;9:e107200.
16. Rodig SJ, Mino-Kenudson M, Dacic S, et al. Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. Clin Cancer Res 2009;15:5216-23.
17. Yoshida A, Tsuta K, Nakamura H, et al. Comprehensive histologic analysis of ALK-rearranged lung carcinomas. Am J Surg Pathol 2011;35:1226-34.
18. Nishino M, Klepeis VE, Yeap BY, et al. Histologic and cytomorphologic features of ALK-rearranged lung adenocarcinomas. Mod Pathol 2012;25:1462-72.
19. Ou SI, Zhu VW, Nagasaka M. Catalog of 5' Fusion Partners in ALK-positive NSCLC Circa 2020. JTO Clin Res Rep 2020;1:100015.
20. Zhang SS, Nagasaka M, Zhu VW, et al. Going beneath the tip of the iceberg. Identifying and understanding EML4-ALK variants and TP53 mutations to optimize treatment of ALK fusion positive (ALK+) NSCLC. Lung Cancer 2021;158:126-36.
21. Hofman V, Heeke S, Bontoux C, et al. Ultrafast Gene Fusion Assessment for Nonsquamous NSCLC. JTO Clin Res Rep 2022;4:100457.
22. Zhao R, Guo L, Zhang B, et al. Identification and therapeutic evaluation of ALK rearrangements in non-small-cell lung cancer. J Pathol Clin Res 2022;8:538-49.
23. Tan DS, Thomas M, Kim DW, et al. Genetic landscape of patients with ALK-rearranged non-small-cell lung cancer (NSCLC) and response to ceritinib in ASCEND-1 study. Lung Cancer 2022;163:7-13.
24. Lin JJ, Zhu VW, Yoda S, et al. Impact of EML4-ALK Variant on Resistance Mechanisms and Clinical Outcomes in ALK-Positive Lung Cancer. J Clin Oncol 2018;36:1199-206.
25. Kang J, Zhang XC, Chen HJ, et al. Complex ALK Fusions Are Associated With Better Prognosis in Advanced Non-Small Cell Lung Cancer. Front Oncol 2020;10:596937.
26. Li M, Hou X, Chen J, et al. ALK fusion variant 3a/b, concomitant mutations, and high PD-L1 expression were associated with unfavorable clinical response to second-generation ALK TKIs in patients with advanced ALK-rearranged non-small cell lung cancer (GASTO 1061). Lung Cancer 2022. [Epub ahead of print]. doi: 10.1016/j.lungcan.2022.01.006.
27. Noh KW, Lee MS, Lee SE, et al. Molecular breakdown: a comprehensive view of anaplastic lymphoma kinase (ALK)-rearranged non-small cell lung cancer. J Pathol 2017;243:307-19.
28. Wang T, Wei L, Lu Q, et al. Landscape of potentially targetable receptor tyrosine kinase fusions in diverse cancers by DNA-based profiling. NPJ Precis Oncol 2022;6:84.
29. Canterbury CR, Fernandes H, Crapanzano JP, et al. ALK Gene Rearrangements in Lung Adenocarcinomas: Concordance of Immunohistochemistry, Fluorescence In Situ Hybridization, RNA In Situ Hybridization, and RNA Next-Generation Sequencing Testing. JTO Clin Res Rep 2021;2:100223.
30. Tabbò F, Muscarella LA, Gobbini E, et al. Detection of ALK fusion variants by RNA-based NGS and clinical

outcome correlation in NSCLC patients treated with ALK-TKI sequences. Eur J Cancer 2022;174:200-11.
31. Lin HM, Wu Y, Yin Y, et al. Real-world ALK Testing Trends in Patients With Advanced Non-Small-Cell Lung Cancer in the United States. Clin Lung Cancer 2023;24:e39-49.
32. Griesinger F, Eberhardt W, Nusch A, et al. Biomarker testing in non-small cell lung cancer in routine care: Analysis of the first 3,717 patients in the German prospective, observational, nation-wide CRISP Registry (AIO-TRK-0315). Lung Cancer 2021;152:174-84.
33. Kerr KM, Bibeau F, Thunnissen E, et al. The evolving landscape of biomarker testing for non-small cell lung cancer in Europe. Lung Cancer 2021;154:161-75.
34. Adizie JB, Tweedie J, Khakwani A, et al. Biomarker Testing for People With Advanced Lung Cancer in England. JTO Clin Res Rep 2021;2:100176.
35. Lee DH, Tsao MS, Kambartel KO, et al. Molecular testing and treatment patterns for patients with advanced non-small cell lung cancer: PIvOTAL observational study. PLoS One 2018;13:e0202865.
36. Lin C, Shi X, Yang S, et al. Comparison of ALK detection by FISH, IHC and NGS to predict benefit from crizotinib in advanced non-small-cell lung cancer. Lung Cancer 2019;131:62-8.
37. Kazdal D, Hofman V, Christopoulos P, et al. Fusion-positive non-small cell lung carcinoma: Biological principles, clinical practice, and diagnostic implications. Genes Chromosomes Cancer 2022;61:244-60.
38. Chakravarty D, Johnson A, Sklar J, et al. Somatic Genomic Testing in Patients With Metastatic or Advanced Cancer: ASCO Provisional Clinical Opinion. J Clin Oncol 2022;40:1231-58.
39. Lasota J, Chłopek M, Wasąg B, et al. Colorectal Adenocarcinomas Harboring ALK Fusion Genes: A Clinicopathologic and Molecular Genetic Study of 12 Cases and Review of the Literature. Am J Surg Pathol 2020;44:1224-34.
40. Kemps PG, Picarsic J, Durham BH, et al. ALK-positive histiocytosis: a new clinicopathologic spectrum highlighting neurologic involvement and responses to ALK inhibition. Blood 2022;139:256-80.
41. Cao Z, Gao Q, Fu M, et al. Anaplastic lymphoma kinase fusions: Roles in cancer and therapeutic perspectives. Oncol Lett 2019;17:2020-30.
42. Conde E, Taniere P, Lopez-Rios F. The anaplastic lymphoma kinase testing conundrum. Expert Rev Mol Diagn 2015;15:161-3.
43. Chu YH, Barbee J, Yang SR, et al. Clinical Utility and Performance of an Ultrarapid Multiplex RNA-Based Assay for Detection of ALK, ROS1, RET, and NTRK1/2/3 Rearrangements and MET Exon 14 Skipping Alterations. J Mol Diagn 2022;24:642-54.
44. Tachon G, Cortes U, Richard S, et al. Targeted RNA-sequencing assays: a step forward compared to FISH and IHC techniques? Cancer Med 2019;8:7556-66.
45. Kuang Y, Xu P, Wang J, et al. Detecting ALK Rearrangement with RT-PCR: A Reliable Approach Compared with Next-Generation Sequencing in Patients with NSCLC. Mol Diagn Ther 2021;25:487-94.
46. Sepúlveda-Hermosilla G, Freire M, Blanco A, et al. Concordance Analysis of ALK Gene Fusion Detection Methods in Patients with Non-Small-Cell Lung Cancer from Chile, Brazil, and Peru. J Mol Diagn 2021;23:1127-37.
47. Clavé S, Rodon N, Pijuan L, et al. Next-generation Sequencing for ALK and ROS1 Rearrangement Detection in Patients With Non-small-cell Lung Cancer: Implications of FISH-positive Patterns. Clin Lung Cancer 2019;20:e421-9.
48. Vendrell JA, Taviaux S, Béganton B, et al. Detection of known and novel ALK fusion transcripts in lung cancer patients using next-generation sequencing approaches. Sci Rep 2017;7:12510.
49. Kuang S, Fung AS, Perdrizet KA, et al. Upfront Next Generation Sequencing in Non-Small Cell Lung Cancer. Curr Oncol 2022;29:4428-37.
50. Frankel D, Nanni I, Ouafik L, et al. Comparison between Immunocytochemistry, FISH and NGS for ALK and ROS1 Rearrangement Detection in Cytological Samples. Int J Mol Sci 2022;23:10556.
51. Dalurzo ML, Avilés-Salas A, Soares FA, et al. Testing for EGFR Mutations and ALK Rearrangements in Advanced Non-Small-Cell Lung Cancer: Considerations for Countries in Emerging Markets. Onco Targets Ther 2021;14:4671-92.
52. Hernandez S, Conde E, Alonso M, et al. The Utopia study: ultrafast next-generation sequencing testing in patients with non-small cell lung carcinomas. Virchows Arch 2023 (in press).
53. Pennell NA, Arcila ME, Gandara DR, et al. Biomarker Testing for Patients With Advanced Non-Small Cell Lung Cancer: Real-World Issues and Tough Choices. Am Soc Clin Oncol Educ Book 2019;39:531-42.
54. VENTANA ALK (D5F3) CDx Assay. [Accessed February 28, 2023] Available online: https://diagnostics.roche.com/us/en/products/tests/ventana-alk-_d5f3-cdx-assay0.

html#productSpecs
55. Conde E, Hernandez S, Prieto M, et al. Profile of Ventana ALK (D5F3) companion diagnostic assay for non-small-cell lung carcinomas. Expert Rev Mol Diagn 2016;16:707-13.
56. Sholl LM, Weremowicz S, Gray SW, et al. Combined use of ALK immunohistochemistry and FISH for optimal detection of ALK-rearranged lung adenocarcinomas. J Thorac Oncol 2013;8:322-8.
57. Vysis ALK Break Apart FISH Probe Kit. [Accessed February 28, 2023] Available online: https://www.molecular.abbott/int/en/products/oncology/vysis-alk-break-apart-fish-probe-kit
58. Mitsudomi T, Tan D, Yang JC, et al. Expert Consensus Recommendations on Biomarker Testing in Metastatic and Nonmetastatic NSCLC in Asia. J Thorac Oncol 2023;18:436-46.
59. Leone A, Muscarella LA, Graziano P, et al. Robust Performance of the Novel Research-Use-Only Idylla GeneFusion Assay Using a Diverse Set of Pathological Samples with a Proposed 1-Day Workflow for Advanced NSCLC Evaluation. Cancers (Basel) 2022;15:292.
60. Hernandez S, Conde E, Molero A, et al. Efficient Identification of Patients With NTRK Fusions Using a Supervised Tumor-Agnostic Approach. Arch Pathol Lab Med 2023. [Epub ahead of print]. doi: 10.5858/arpa.2022-0443-OA.
61. Davies KD, Aisner DL. Wake Up and Smell the Fusions: Single-Modality Molecular Testing Misses Drivers. Clin Cancer Res 2019;25:4586-8.
62. Benayed R, Offin M, Mullaney K, et al. High Yield of RNA Sequencing for Targetable Kinase Fusions in Lung Adenocarcinomas with No Mitogenic Driver Alteration Detected by DNA Sequencing and Low Tumor Mutation Burden. Clin Cancer Res 2019;25:4712-22.
63. Heydt C, Wölwer CB, Velazquez Camacho O, et al. Detection of gene fusions using targeted next-generation sequencing: a comparative evaluation. BMC Med Genomics 2021;14:62.
64. Christopoulos P, Endris V, Bozorgmehr F, et al. EML4-ALK fusion variant V3 is a high-risk feature conferring accelerated metastatic spread, early treatment failure and worse overall survival in ALK(+) non-small cell lung cancer. Int J Cancer 2018;142:2589-98.
65. Camidge DR, Kim HR, Ahn MJ, et al. Brigatinib Versus Crizotinib in ALK Inhibitor-Naive Advanced ALK-Positive NSCLC: Final Results of Phase 3 ALTA-1L Trial. J Thorac Oncol 2021;16:2091-108.
66. Felip E, Martini J-F, Mazieres J, et al. 1008P Resistance mechanisms to lorlatinib or crizotinib in treatment-naive patients (pts) with ALK+ advanced non-small cell lung cancer (NSCLC). Ann Oncol 2022;33:S1014.
67. Camidge DR, Dziadziuszko R, Peters S, et al. Updated Efficacy and Safety Data and Impact of the EML4-ALK Fusion Variant on the Efficacy of Alectinib in Untreated ALK-Positive Advanced Non-Small Cell Lung Cancer in the Global Phase III ALEX Study. J Thorac Oncol 2019;14:1233-43.
68. Xiang Y, Zhang S, Fang X, et al. Therapeutic Advances of Rare ALK Fusions in Non-Small Cell Lung Cancer. Curr Oncol 2022;29:7816-31.
69. Rolfo C, Mack P, Scagliotti GV, et al. Liquid Biopsy for Advanced NSCLC: A Consensus Statement From the International Association for the Study of Lung Cancer. J Thorac Oncol 2021;16:1647-62.
70. Pisapia P, Costa JL, Pepe F, et al. Next generation sequencing for liquid biopsy based testing in non-small cell lung cancer in 2021. Crit Rev Oncol Hematol 2021;161:103311.
71. Krebs MG, Malapelle U, André F, et al. Practical Considerations for the Use of Circulating Tumor DNA in the Treatment of Patients With Cancer: A Narrative Review. JAMA Oncol 2022;8:1830-9.
72. Malapelle U, Pisapia P, Addeo A, et al. Liquid biopsy from research to clinical practice: focus on non-small cell lung cancer. Expert Rev Mol Diagn 2021;21:1165-78.
73. Pascual J, Attard G, Bidard FC, et al. ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO Precision Medicine Working Group. Ann Oncol 2022;33:750-68.
74. Mezquita L, Swalduz A, Jovelet C, et al. Clinical Relevance of an Amplicon-Based Liquid Biopsy for Detecting ALK and ROS1 Fusion and Resistance Mutations in Patients With Non-Small-Cell Lung Cancer. JCO Precis Oncol 2020;4:PO.19.00281.
75. Dagogo-Jack I, Ritterhouse LL. The role of plasma genotyping in ALK- and ROS1-rearranged lung cancer. Transl Lung Cancer Res 2020;9:2557-70.
76. McCoach CE, Le AT, Gowan K, et al. Resistance mechanisms to targeted therapies in ROS1þ and ALKþ non–small cell lung cancer. Clin Cancer Res 2018;24:3334-47.
77. Dagogo-Jack I, Brannon AR, Ferris LA, et al. Tracking the Evolution of Resistance to ALK Tyrosine Kinase Inhibitors through Longitudinal Analysis of Circulating

Tumor DNA. JCO Precis Oncol 2018;2018:PO.17.00160.
78. Letovanec I, Finn S, Zygoura P, et al. Evaluation of NGS and RT-PCR Methods for ALK Rearrangement in European NSCLC Patients: Results from the European Thoracic Oncology Platform Lungscape Project. J Thorac Oncol 2018;13:413-25.
79. McLeer-Florin A, Duruisseaux M, Pinsolle J, et al. ALK fusion variants detection by targeted RNA-next generation sequencing and clinical responses to crizotinib in ALK-positive non-small cell lung cancer. Lung Cancer 2018;116:15-24.
80. Kerr KM, López-Ríos F. Precision medicine in NSCLC and pathology: how does ALK fit in the pathway? Ann Oncol 2016;27 Suppl 3:iii16-24.
81. von Laffert M, Warth A, Penzel R, et al. Multicenter immunohistochemical ALK-testing of non-small-cell lung cancer shows high concordance after harmonization of techniques and interpretation criteria. J Thorac Oncol 2014;9:1685-92.
82. Kalemkerian GP, Narula N, Kennedy EB, et al. Molecular testing guideline for the selection of patients with lung cancer for treatment with targeted tyrosine kinase inhibitors: American society of clinical oncology endorsement of the college of American pathologists/ international association for the. J Clin Oncol 2018; 36:911-9.

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