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Evaluation of NGS and RT-PCR Methods for *ALK* Rearrangement in European NSCLC Patients: Resultsfrom the European Thoracic Oncology Platform Lungscape Project

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ABSTRACT

Introduction: The reported prevalence of ALK receptor tyrosine kinase gene (*ALK*) rearrangement in NSCLC rangesfrom 2% to 7%. The primary standard diagnostic methodis fluorescence in situ hybridization (FISH). Recently, immu-nohistochemistry (IHC) has also proved to be a reproducibleand sensitive technique. Reverse-transcriptase polymerase chain reaction (RT-PCR) has also been advocated, and mostrecently, the advent of targeted next-generation sequencing(NGS) for *ALK* and other fusions has become possible. This study compares anaplastic lymphoma kinase (ALK) evalua-tion with all four techniques in resected NSCLC from the large European Thoracic Oncology Platform Lungscape cohort.

Methods: A total of 96 cases from the European Thoracic Oncology Platform Lungscape iBiobank, with any ALK immunoreactivity were examined by FISH, central RT-PCR, and NGS. An H-score higher than 120 defines IHC positiv- ity. RNA was extracted from the same formalin-fixed, paraffin-embedded tissues. For RT-PCR, primers covered the most frequent *ALK* translocations. For NGS, the Onco- mine Solid Tumour Fusion Transcript Kit (Thermo Fisher Scientific, Waltham, MA) was used. The concordance was assessed using the Cohen k coefficient (two-sided a $\leq$ 5%).

Results: NGS provided results for 77 of the 95 cases tested (81.1%), whereas RT-PCR provided results for 77 of 96(80.2%). Concordance occurred in 55 cases of the 60 cases tested with all four methods (43 ALK negative and 12 ALK positive). Using ALK copositivity for IHC and FISH as the criterion standard, we derived a sensitivity for RT-PCR/NGSof 70.0%/85.0%, with a specificity of 87.1%/79.0%. When either RT-PCR or NGS was combined with IHC, the sensitivity remained the same, whereas the specificity increased to 88.7% and 83.9% respectively.

Conclusion: NGS evaluation with the Oncomine Solid TumourFusion transcript kit and RT-PCR proved to have high sensi-tivity and specificity, advocating their use in routine practice. For maximal sensitivity and specificity, *ALK* status should beassessed by using two techniques and a third one in discordantcases. We therefore propose a customizable testing algorithm. These findings significantly influence existing testing paradigms and have clear clinical and economic impact.

Introduction

Progress in understanding of the molecular mecha- nisms driving NSCLC has led to the development of new targeted drugs, mainly tyrosine kinase inhibitors (TKIs), and opened the field to new treatment strategies in molecularly selected cases.[1] Biomarkers have been identified to select patients eligible for these clinically validated therapies, including frontline application of gefitinib,[2] erlotinib, and afatinib for *EGFR*-mutated NSCLC, crizotinib therapy[3–6] for ALK receptor tyrosine kinase gene (*ALK*)- or *ROS1*-rearranged NSCLC, and (recently) alectinib for *ALK*-rearranged NSCLC.[7]

Around 2% to 7% of NSCLCs are characterized by the presence of an inversion or translocation of chromosome 2p involving the *ALK* gene, resulting in a transforming fusion gene echinoderm microtubule associated protein like 4 gene (*EML4*)-*ALK*,[8] which can be targeted with ALK TKIs. We previously reported the prevalence and clinical outcomes for resected stage I to III adenocarcinoma with ALK positivity assessed by immunohistochemistry (IHC) in the Lungscape patient cohort.[9] Reported prevalence of such alterations varies across studies as a conse- quence of both patient selection criteria and the spe- cific detection methods used.[3,10–12]

Depending on the type of molecular alteration (mutation, translocation or amplification), a variety of techniques can be used to evaluate predictive bio- markers. Mutational status is usually assessed by po-lymerase chain reaction (PCR) and/or sequencing with a diversity of accepted techniques limited by the spectrum of PCR and sequencing methods available. In contrast, rearrangements including, but not limited to, *ALK* rearrangements can be identified with a wider range of techniques including fluorescence in situ hybridization (FISH), reverse-transcriptase PCR (RT- PCR), next-generation sequencing (NGS), or IHC. For each of these methods, both commercial kits and validated laboratory-developed tests may be used, subject to local regulations and practice.

Although RT-PCR was the first published method[8] fordetermining *ALK* status, FISH, a widely available tech-nique for formalin-fixed, paraffin-embedded (FFPE) specimens known to preserve tissue architecture, has supplanted it. *ALK* FISH was approved as a companion diagnostic by the U.S. Food and Drug Administration. In the context of multiple retrospective comparative biomarker studies, IHC has proved to be a very sensitive technique for anaplastic lymphoma kinase (ALK) proteinexpression, which is usually absent from normal lung tissues. It has recently been adopted[6,13–17] as a primary screening method for detection of patients with *ALK* rearrangement and treatment selection.

This study compares ALK evaluation with multiple technologies, FISH, RT-PCR, and targeted NGS, in the Lungscape cohort by using any degree of ALK IHC pos- itivity as a selection criterion.

We also reviewed literature and discussed the limi- tations and potential advantages inherent to each tech- nique to refine and optimize diagnostic strategies.

Material and Methods

The research was conducted according to the Lung- scape master and ALK subproject protocols with adher-ence to country-specific ethics, regulatory requirements, and Reporting Recommendations for Tumor Marker Prognostic Studies.[18]

Case Selection

This is a cohort study of surgically resected stage I toIII NSCLC cases from the European Thoracic Oncology Platform Lungscape.[19] ALK IHC analysis was implementedin a large subset of the Lungscape cohort with FISH performed on IHC-positive cases and matched negativecontrols. RT-PCR and NGS were performed on all IHC-positive cases, irrespective of extent or intensity of staining. For *ALK* FISH and IHC, all sites participated in an external quality assessment for the laboratory analyses, and all samples were analyzed at their site of origin, following the same methods.

IHC and FISH

ALK IHC using clone 5A4 antibody (Novocastra, LeicaBiosystems, Buffalo Grove, IL) and the Novolink detec-tion system (Leica Biosystems) and FISH using Vysis ALK break-apart FISH probe (Abbott Molecular, Des Plaines, IL) were performed and evaluated according to previ- ously published methodology.[9]

RNA Extraction for RT-PCR and NGS

After macrodissection for tumor enrichment, RNA was extracted from unstained slides using the Qiagen RNeasy FFPE Kit (Qiagen, Hombrechtikon. Switzerland) according to producer instructions. RNA was quantified by Qubit Fluorimeter 2.0 (Invitrogen, Carlsbad, CA) Quant-iT RNA assay Kit (Invitrogen). RNA from the same extraction was used for both RT-PCR and NGS testing. RNA extraction details are provided in the Supplementary

Methods.

RT-PCR

The obtained RNA was eluted by using 20 to 30 mL ofwater and RNasin Plus (final concentration w1%). Beta-actin forward and reverse primers were used to amplify a region of 196 bp of the actin beta gene (*ACTB*) as an internal positive control. RT-PCR was performed startingfrom 50 ng of total RNA by using two PCR mixes (mix 1 and mix 2) including Superscript One-Step RT-PCR and Platinum Taq (Invitrogen) in a Primus 25 thermocycler (MWG-BIOTECH Europe, Ebersberg, Germany) with the following programs: initial RT reaction at 50°C for 30 minutes; 95°C for 2 minutes followed by PCR reaction (40 cycles) consisting of 95°C for 30 seconds, 57°C for 45 seconds, and 72°C for 1 minute and 15 seconds; and final elongation at 72°C for 10 minutes. *EML4-ALK* primers (see Supplementary Table 1, which provides RT-PCR primer information) were used at a concentration of 10 rmol/mL and those for beta-actin were used at aconcentration of 2 rmol/mL. Analysis of the obtained product was done by using capillary electrophoresis on QIAxcel with a QIAxcel DNA Screening Kit. All tests wereperformed in duplicate (Fig. 1; NGS data visualization in Supplementary Fig 1).

Targeted NGS

Library preparation was performed according to the Oncomine Solid Tumor Fusion transcript kit protocol (Thermo Fisher Scientific, Waltham, MA), starting with 10 ng of DNase-treated RNA (see Supplementary Table 2, which provides Oncomine Solid Tumor Fusion Transcript information). Samples were barcoded with 16 distinct adapter sequences to enable multiplexing. Final RNA li- braries were quantified by quantitative PCR by using theIon Library Quantitation Kit and then diluted and pooledin equimolar amounts; 25 mL of a 16-pM pool of RNA li- braries was mixed with ion sphere particles and clonallyamplified in an emulsion PCR performed in accordance with the Ion PGM Template OT2 200 Kit protocol by using the Ion OneTouch 2 instrument (Thermo Fisher Scienti- fic). Template-positive ion sphere particles were then enriched using the Ion OneTouch ES instrument. Enriched samples were loaded onto Ion 318 chips and sequenced by using the Ion PGM System, following the Ion PGM Sequencing 200 Kit v2 protocol.

Torrent Suite software, version 4.4.2, was used toremove low-quality reads, trim adapter dimers, and align to a reference genome (hg19). RNA fusion datawere then uploaded to Ion Reporter 4.6 and analyzedby using the AmpliSeq RNA Lung Fusion v4.6 workflow.

Statistics

The concordance between the alternative methods was assessed through multiway tables. The statistical significance of paired associations was based on the Cohen k coefficient (two-sided $\alpha \leq 5\%$). A waterfall plot was used to graphically compare the four methods. The sensitivity and specificity of each method were calculated (on cases with available results for IHC [H-score], FISH, and either RT-PCR or NGS) by using the combined result of IHC H-score and FISH as the criterion standard.True positive cases were those with a positive result by both methods (H-score >120 and FISH positivity). Median RNA concentrations between groups of cases were compared on the basis of the nonparametric Wil- coxon test. Statistical analyses were performed with SAS software (version 9.3, SAS Inc., Cary NC) and R language for statistical computing (version 3.2.2, R Foundation forStatistical Computing, Vienna, Austria).[20]

Results

Study Cohort

From the 2709 cases registered in the Lungscape iBiobank, 1772 were screened for ALK by IHC. All patients selected for this study were considered ALK IHC–positive on the basis of IHC intensity scoring of 1+ and higher (N = 96 [5.4%]; H-score was not available for four cases). Among them, 76 patients (79.2%) had adenocarcinoma, 13 (13.5%) had squa- mous cell carcinoma, four (4.2%) had large cell carcinoma, and three (3.1%) had combined (mixed) carcinoma.

IHC

All cases were at least focally positive for ALK by IHC, with variable maximum intensity: 1+ in 58 cases (60.4%), 2 in 16 (16.7%), and 3 in 22 (22.9%). As previously reported,[9] an H-score cutoff of 120 correlated with FISH status and was used to classify IHC as positive in the rest of the study. H-Score was retrieved from the Lungscape iBiobank database and was available for 92 cases of the 96 cases positive for ALK by IHC (four cases from a single center had missing H-scores). Twenty- three cases (25%) had an H-score higher than 120 and 69 (75%) had an H-score of 120 or lower.

FISH

FISH results were available for 88 cases: 24 FISH- positive (27.3%) and 64 FISH-negative (72.7%) cases.

Tumor Cellularity and RNA Yield

The median tumor cellularity and RNA yield were 70% (range 10%–90%) and 47 ng/mL (range 9–300), respectively.

The median RNA concentration was significantly lower in cases in which RT-PCR failed than in cases in which RT-PCR was successfully implemented (34 versus 55 ng/mL [p 0.0056]), whereas no significant difference was found between groups of cases by NGS failure or success (p 0.17) (see Supplementary Table 3, which shows RNA concentration by RT-PCR and NGS result).

RT-PCR

The assay was successful in 77 of the 96 cases analyzed (80.2%). In 19 cases in which no beta-actin could be co-amplified, RNA quality was considered as insufficient for result validation. *ALK* rearrangement was identified in 16 cases (20.8%). Translocations were characterized in 13 rearranged cases: V1 in five cases (38.5%), V2 in one (7.7%), and V3a/b in seven (53.8%).

NGS

e assay passed quality control and was initially successful in 90 of the 95 cases (94.7%) analyzed. A specific *ALK* rearrangement fusion partner transcript was initially identified in 15 cases (16.7%) and a 3'/5' imbalance value was assessed in cases in which no specific *ALK* rearrangement was detected (see Supplementary Fig. 2, which shows imbalance scores against criterion standard assessment of positivity). The imbalance assay is a method to screen for *ALK* gene (or other) rearrangements by assessing the ratio of 3' to 5' amplicon reads standardized against all control gene reads. Essentially, the imbalance assay gives an indication of the likelihood that a fusion is present. When a rearrangement is present, there is preferential loss of the 5' amplicon, leading to 3'/5' imbalance. The imbalance is expressed in this equation: (3' reads – 5' reads)/(sum of all control reads), with values close to zero (0.001) showing no evidence of rearrangement, values between 0.001 and 0.010 showing uncertain evidence, and values of 0.010 or higher implying strong evidence of a fusion. Strong evidence of imbalance (score 0.010) was observed in six cases, uncertain evidence (score in 0.001–0.010) in 11 cases, and no evidence (score 0.001) in 56. Cases with strong evidence of imbalance were considered highly likely to be *ALK* NGS–positive but suggesting additional validation by using another technology. Cases with uncertain evidence of imbalance were considered failed assays because the software could not determine positivity or negativity. Thus, we finally proceeded to further analyses with 77 available results (an 81.1% success rate), with 21 NGS positive (27.3%) and 56 NGS negative (72.7%).

Of the 15 cases with characterized *ALK* translocations, V1 was found in four cases (26.7%), V2 in one (6.7%), and V3a/b in 10 (66.7%). Seven cases had more than one isoform: three cases had two isoforms and four cases had three. In six cases with strong evidence of imbalance no specific translocations could be identified.

Concordance of Methodologies

Figure 2 illustrates and summarizes all individual technique results for each case (information on the number of available cases by each combination of methods is pro-vided in Supplementary Table 4 and Supplementary Fig. 3).

IHC and FISH. Concordance of IHC (H-score cutoff) with FISH was evaluated in 87 cases: 84 cases (97%) were concordant (22 positive and 62 negative) and three were discordant (one FISH-negative with an H-score >120, two FISH-positive with H-score ≤120) with a k coefficient of 0.91 ($p < 0.001$ [data not shown]).

RT-PCR. Paired concordance of RT-PCR with IHC, H- score, and FISH was evaluated in 77, 73, and 73 cases, respectively (Table 1). RT-PCR was highly concordant with an H-score and FISH with a k coefficient of 0.88 and 0.85, respectively ($p < 0.001$ for all). Within the RT-PCR– positive group, 93.8% are strongly IHC positive (2+ or 3+) with an H-score higher than 120 and FISH positivity. Joint concordance of RT-PCR with IHC (H-score) and FISH was evaluated in 72 cases (Table 2), of which 67 (93.1%) had complete agreement among all three methods.

NGS. Paired concordance of NGS with IHC, H-score, FISH, and RT-PCR was evaluated in 77, 73, 71, and 65 cases (see Table 1). NGS was highly concordant with H-score, FISH, and RT-PCR, with k coefficients of 0.90, 0.86, and
0.91, respectively ($p < 0.001$ for all). In the NGS-positive group, 85.7% are strongly IHC positive (2+ or 3+) with an H-score higher than 120 and FISH positivity and 87.5% are RT-PCR–positive. Joint concordance of NGS with both IHC (H-score) and FISH results was evaluated in 70 cases (see Table 2). Agreement of the three methods was achieved in 67 cases (95.8%).

Joint Comparison of All Methods. Joint concordance of all four methods was evaluated in 60 cases (Table 3). Agreement of all methods (H-score, FISH, RT-PCR, NGS) was achieved in 55 cases (91.7%), of which 12 were positive (75.0% of all 16 NGS-positive) and 43 were negative (97.7% of all 44 NGS-negative).

The sensitivity and specificity of each stand-alone method and of the combination of IHC with RT-PCR or NGS is based on 82 cases with available results for IHC, FISH, and either RT-PCR or NGS. The criterion standard was determined by the combined result of IHC (H-score >120) and FISH (see Supplementary Table 5, which shows the sensitivity and specificity scores of the four methods). This corresponded to 70.0% and 85.0% sensitivity for RT-PCR/NGS, with 87.1% and 79.0% specificity, respectively. IHC combined with either RT-PCR or NGS resulted in the same sensitivity, whereas the specificity increased to 88.7% and 83.9%, respectively. When only the subset of 60 cases with available results in all four methods were used, NGS had higher sensitivity and lower specificity than RT-PCR (100.0% versus 92.3% and 93.6% versus 95.7%, respectively) (see Supplementary Table 6, showing sensitivity and specificity scores of the four methods).

Discussion

We previously investigated the prevalence of ALK positivity by using IHC confirmed by FISH and compared IHC with FISH for detection of *ALK* positivity in resected lung adenocarcinoma by using the European Thoracic Oncology Platform Lungscape iBiobank.[9] The current study is, to the best of our knowledge, the first and largest study to evaluate *ALK* status with the four most common methodologies currently in use, namely IHC, FISH, RT-PCR, and NGS in predominantly European patients with resected stage I to III NSCLC.

We used any degree of ALK IHC positivity as the primary selection criterion for further parallel FISH, RT-PCR, and NGS analysis, uniquely allowing us to investigate all four techniques on the same samples, allowing direct comparisons.

If concordance of any two techniques is considered true positivity instead of the concordance of IHC and FISH being defined as the criterion standard, our study con- firms that none of the techniques exhibits 100% sensitivity and specificity. This finding affects the accuracy of treatment strategy decisions and quality of clinical man agement of individual patients if only one assay is used.

IHC can result in both false-positive and false-negative results. The two main explanations for falsenegativity are a failed staining technique—this should be identified in the analytical process, provided a validated external control is used—or preanalytical tissue fixation problems resulting in epitope alteration. Those cases can go unnoticed in the absence of ALK expression in normal tissues as an internal control. IHC results may vary by the clone and technique used, although they are usually considered equivalent when appropriately validated and scored. In a recent large series comparing the two most common antibodies, clones 5A4 and D5F3, by using FISH, sensitivity and specificity were 87% and 92% and 89 and 76% respectively.[21] On the basis of our previous work,[9] we had proposed an H-score cutoff higher than 120 as giving the highest probability for concor- dance with matched *ALK* FISH positivity. False IHC– positive cases are rarely encountered if one uses an H-score higher than 120. False positivity can also be attributed to technical/interpretative issues (back- ground being interpreted as positivity) or ALK amplification, as recently suggested.[22] Among the 1772 cases tested, 104 showed at least focal IHC positivity (maximum IHC score 1). Only one IHC-positive case was FISH negative and is discussed in the following paragraph. With the inclusion criteria for this study being any degree of ALK IHC positivity, true sensitivity cannot be determined, as the remaining IHC (0) cases were not tested by NGS or RT-PCR.

FISH can also provide both false-positive and false- negative results.[22] *ALK* FISH assay reading is subject to misinterpretation, requiring an experienced pathologist for accurate evaluation. Positivity versus negativity is determined by the presence of split signals in more or less than 15% of cells.[23] Most of the false- negative/false-positive interpretations appear to occur when the count is around this threshold and in up to 20% of cells.[21,22] Inadequate material or failed technique can be easily identified when FISH results are read by using internal

validation of hybridization quality and presence of appropriate fluorescent probes signals. FISH remains a robust technique, with a success rate reported in up to 98% to 99% of cases.[21] Two cases in our series were suggestive of FISH positivity, with an H-score of 120 or lower (one case with RT-PCR and NGS also positive and one case with a FISH split signal in 16% of cells but RT- PCR and NGS negativity). Another case was FISH nega- tive (split signal in 13% of cells) with an H-score higher than 120 and RT-PCR and NGS positivity. For the last twocases described (with FISH split signals just above and below 15%), the other three techniques (IHC, RT-PCR, and NGS) were concordant among them but contradictory toFISH results, illustrating the 15% threshold limitation.

In the break-apart designed FISH assay, a positiveresult does not imply that the fusion partner gene is*EML4*, and virtually any type of *ALK* rearrangement/ partner could be identified by this technique, leading to or preventing *ALK* gene transcription. A positive FISH result as a predictive marker for targeted therapy is thus theoretically not always guaranteed.

RT-PCR provides mainly false-negative results in discordant cases. The limitation of this very specific tech- nique resides in its ability to detect only known trans- locations and, more specifically, those that the assay was designed to detect (variants V1 to V8 [as defined by the PCRprimer]), thus conferring to this technique very high spec ificity but lower sensitivity. Another limitation is linked tothe fact that this is an RNA-based assay. Its success relies onRNA quality and thus depends on preanalytical factors. Prolonged ischemia or inadequate fixation can result in RNA degradation. In our series two cases were classified as RT- PCR–negative with FISH and IHC-positive, potentially illustrating this issue. One of these cases failed NGS (un- certain imbalance evidence) and the other was NGS positive(strong imbalance evidence), meaning they represent probable translocations not covered by both assays. *NGS*, also an RNA-based assay, has limitations similarto those of RT-PCR regarding primer design and RNA quality; nevertheless, it covers almost all variants described to date, including partners other than EML-4 (see Supplementary Table 2). Our data show that itssuccess rate for passing quality control assessment with the same RNA is approximately equal (81% versus 80% success rate [see Supplementary Table 3, which shows RNA concentration by RT-PCR and NGS result]) to that with RT-PCR. The limitation of detecting only known translocations can be overcome by using imbalance score as described earlier in this article and appropriately validating high probability imbalance scores by using an orthogonal assessment. This orthogonal vali- dation is of great importance, as shown in one of our cases with a “positive” imbalance score but classified as negative with the three other techniques.

Assessing the sensitivity and specificity of each assayis challenging because defining a criterion standard for ALK status is problematic, as exceptional cases occur. Our reasoned approach was that the best approximationof a criterion standard would be concordance of the most widely used techniques, IHC (H-score cutoff >120) and FISH, allowing the most robust comparison with RT-PCR and NGS. On that basis, 22 cases were considered ascriterion standard ALK-positive/rearranged.

Whereas the sensitivities of NGS as a stand-alone method or used in combination with IHC in compari-son with the criterion standard (H-score >120 and FISH-positiveity) are high 85% for both, specificities are lowerat 79% and 84%, respectively. However, the true sensitivity of NGS as a primary ALK screening method- ology cannot be inferred from these data because the analysis was limited to cases preselected from the entireLungscape cohort by IHC. Limitations to this approach are discussed later in this article.

In our study we did not find superiority of RT-PCR over FISH as reported by other authors[24,25] or NGS over FISH[26] as suggested in a recent study with a limitednumber of centers. If any of the assays are successful, their sensitivity and specificities appear to be almostequivalent if taken individually.

Variants Characterization

One of the added values of RT-PCR and NGS is their ability to characterize the type/variant of *ALK* trans- location. Interestingly, 10 of 21 NGS-positive cases corresponded to Variant 3a/b, representing 66.7% of the 15 characterized ALK-positive cases. According to the data available in the literature, this variant is usually found in around 30% of *ALK*-rearranged cases.[27–29] Despite the limited number of cases in our study, this finding might suggest that this variant is more frequently linked with lower stage (stage I–III) tumors and/or the European population than with the Japanese population.[30] Variantswere concordant between NGS and RT-PCR.

Treatment outcome and duration of response vary widely in patients treated with crizotinib. The significance of the *ALK* variants in clinical practice is not clear yet andremains controversial, although in vitro studies suggest variable degrees of crizotinib efficacy depending on the variant present.[31,32] Moreover, a differential duration of response to crizotinib was suggested according to the *ALK* translocation variants, with V1/2 being characterized by a higher objective response rate and longer progression- free survival.[30,33] Characterizing the type of trans- location

is necessary for the investigation of its relevance as a better predictor of response and might become standard in the future in clinical practice,[34,35] giving an advantage to NGS or RT-PCR over IHC and FISH.

The multiplex method for fusion detection presented takes the approach of using a literature-based PCR library design, meaning that all fusion partners are identified before assay design and the assay is appropriately focused on these most common variants. The advantage of this approach is that it will function with the small amounts of RNA typical of clinical lung cancer samples. Hybridization-based methods, which can be designed to detect novel fusion partners, typically require higher RNA input volumes and are assumed to be less likely to return a result when presented with a small sample. A more recent method known as anchored multiplex PCR would allow the identification of novel fusion partners and function with a relatively small input RNA volume.[36] Whereas the Ampliseq-based method does not specifically identify novel fusion partners, the 3'/5' imbalance score functions as a fail-safe to highlight cases in which a novel fusion might be present in the sample.

Limitations

Sensitivity analysis of ALK IHC positivity is precluded by the use of IHC positivity as an inclusion criterion, but previously published data[37] suggest that it is very high. In our series, the proportion of cases with failed technique is high compared with the literature, probably because of the use of archival material from various centers using variable fixation protocols.[38] According to the literature, the variability of discrepancies is higher when pooling data and materials from multiple sites,[21] probably because of the use of archival material without standardized fixation protocols.[38] We also observed such an effect in this study, with the RT-PCR failure rate being higher than 87% for material originating from specific centres, representing more than 35% of failed assays (data not shown). The failure rates (19.8% and 18.9% for RT-PCR and NGS, respectively, in this study) might be much lower in a controlled setting, when adequate fixation is used as shown in the literature[38]. Thus, RT-PCR and mostly NGS are routinely possible and have the advantage that failed assays because of inap- propriate RNA quality can be identified.

Costs

Although not formally assessed in this study, cost is clearly relevant to this discussion. Molecular testing, including at least EGFR and ALK analysis, is now routinely undertaken in advanced nonsquamous NSCLC.[6,39] Health system structures, testing recommendations, and resources vary from country to country, as do the costs of each test. Thus, the best testing algorithm, as defined by the highest sensitivity and specificity for ALK status, might not be the most cost-effective, and consequently, it might be unaffordable in specific coun- tries. Among the four techniques discussed, IHC is the least complex and least expensive, followed by RT-PCR and FISH.[40] The costs of NGS-based assays remain higher and require sophisticated instrumentation and interpretive expertise but generate more genetic data than any other assay. Besides providing the variant and imbalance score, they also provide information about other relevant and potentially targetable molecular alterations (ret proto-oncogene gene [*RET*], *ROS1*, and neurotrophic receptor tyrosine kinase 1 gene [*NTRK1*] translocation and *EGFR*, *BRAF*, and erb-b2 receptor tyrosine kinase 2 gene [*HER2*] mutations, depending on the assay used). A single *RET*-rearranged case was identified among the patients tested.

Conclusion

Our study confirms high sensitivity and specificity for FISH, RT-PCR, and NGS with use of the Oncomine Solid Tumor Fusion transcript kit for evaluation of ALK status. These findings support the feasibility of NGS-based (and RT-PCR–based) approaches on FFPE samples in diagnostic routine for ALK detection, as long as standardized and adequate preanalalytic handling of the samples is performed to guarantee good RNA quality.

IHC is currently approved for selecting therapy without orthogonal testing. It is an inexpensive, easy to set up and interpret technique and is therefore usually recommended/used for initial screening to eliminate negative cases. However, it seems intuitive that as multiplex testing become more widely used, and infor- mation about fusion variants proves to be relevant, NGS will become the primary test and any fusion gene posi- tivity will need confirmation by an orthogonal test.

Every technique has limitations; thus, if maximal sensitivity and specificity for detection of *ALK* rearrange- ment is sought, confirmation of ALK status by using at least two techniques is mandatory, adding a third one if cases are discordant (Supplementary Table 7 for reclassified cases). Nevertheless, depending on local skills, equipment access, and costs, a variety of validated approaches will be developed as proposed in our algorithm (Fig. 3). This

significantly influences existing testing paradigms and hasclear clinical and economic impact.

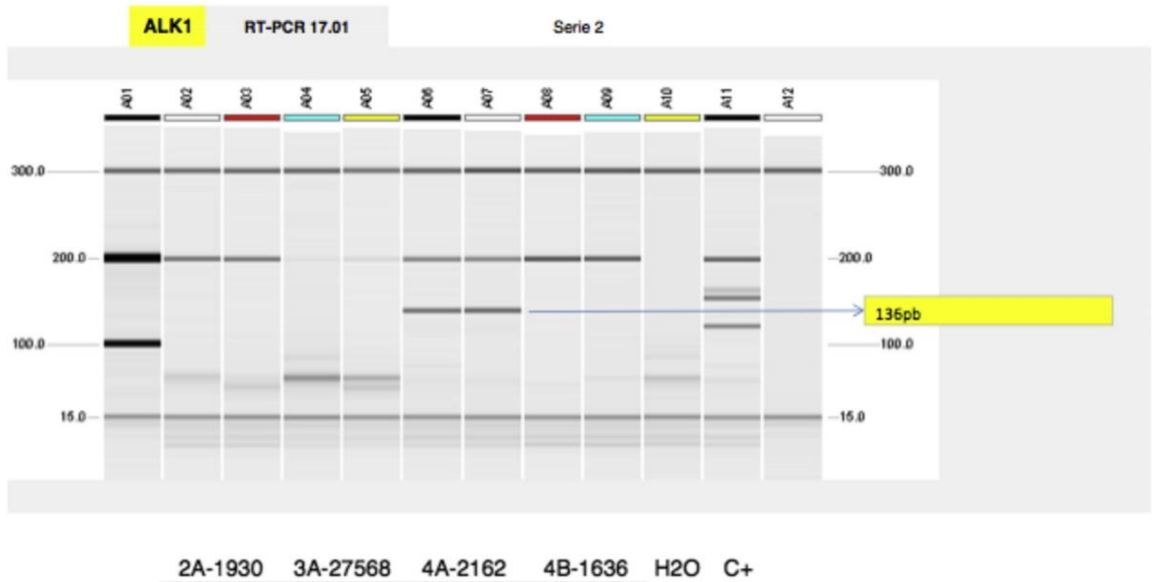
References

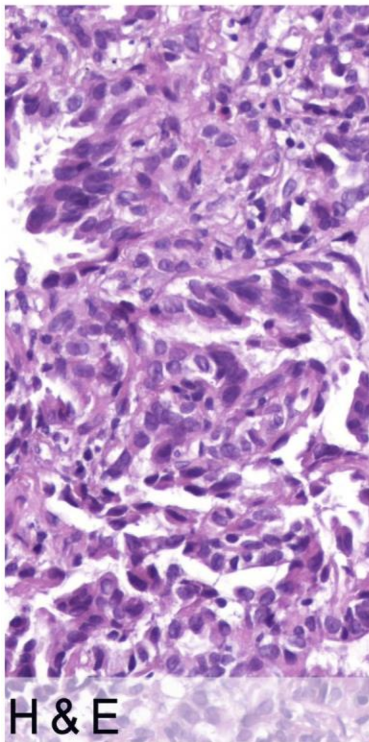
1. Hirsch FR, Suda K, Wiens J, Bunn PA Jr. New andemerging targeted treatments in advanced non-small- cell lung cancer. *Lancet*. 2016;388:1012–1024.
2. Tan DS, Yom SS, Tsao MS, et al. The International Association for the Study of Lung Cancer consensus statement on optimizing management of EGFR mutation- positive non-small cell lung cancer: status in 2016.*J Thorac Oncol*. 2016;11:946–963.
3. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010;363:1693–1703.
4. Shaw AT, Kim DW, Nakagawa K, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N Engl J Med*. 2013;368:2385–2394.
5. Solomon BJ, Mok T, Kim DW, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. *N EnglJ Med*. 2014;371:2167–2177.
6. Novello S, Barlesi F, Califano R, et al. Metastatic non-small-cell lung cancer: ESMO clinical practice guide- lines for diagnosis, treatment and follow-up. *Ann Oncol*.2016;7:v1–v27.
7. Peters S, Camidge DR, Shaw AT, et al. Alectinib versus crizotinib in untreated ALK-positive non-small-cell lungcancer. *N Engl J Med*. 2017;377:829–838.
8. Soda M, Choi YL, Enomoto M, et al. Identification of thetransforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*. 2007;448:561–566.
9. Blackhall FH, Peters S, Bubendorf L, et al. Prevalence and clinical outcomes for patients with ALK-positive resected stage I to III adenocarcinoma: results fromthe European Thoracic Oncology Platform Lungscape Project. *J Clin Oncol*. 2014;32:2780–2787.
10. Camidge DR, Kono SA, Flacco A, et al. Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. *Clin Cancer Res*. 2010;6:5581–5590.
11. Shaw AT, Yeap BY, Mino-Kenudson M, et al. Clinical features and outcome of patients with non-small-cell lungcancer who harbor EML4-ALK. *J Clin Oncol*. 2009;27: 424–453.
12. Solomon B, Varella-Garcia M, Camidge DR. ALK gene rearrangements: a new therapeutic target in a molecu-larly defined subset of non-small cell lung cancer. *J Thorac Oncol*. 2009;4:1450–1454.
13. Yi ES, Boland JM, Maleszewski JJ, et al. Correlation of IHC and FISH for ALK gene rearrangement in non-small cell lung carcinoma: IHC score algorithm for FISH. *J Thorac Oncol*. 2011;6:459–465.
14. McLeer-Florin A, Moro-Sibilot D, Melis A, et al. Dual IHC and FISH testing for ALK gene rearrangement in lung adenocarcinomas in a routine practice: a French study.*J Thorac Oncol*. 2012;7:348–354.
15. Jokoji R, Yamasaki T, Minami S, et al. Combination of morphological feature analysis and immunohisto- chemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. *J Clin Pathol*. 2010;63: 1066–1070.
16. Mino-Kenudson M, Chirieac LR, Law K, et al. A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry. *Clin Cancer Res*. 2010;6: 1561–1571.
17. FDA: Approval Application (PMA) Number for VENTANA ALK (D5F3) CDx Assay. Summary data sheet, 2015.https://www.accessdata.fda.gov/cdrh_docs/pdf14/p140025b.pdf. Accessed January 9, 2018.
18. McShane LM, Altman DG, Sauerbrei W, et al. Reporting recommendations for tumor marker prognostic studies.*J Clin Oncol*. 2005;23:9067–9072.
19. Peters S, Weder W, Dafni U, et al. Lungscape: resected non-small-cell lung cancer outcome by clinical and pathological parameters. *J Thorac Oncol*. 2014;9: 1675–1684.
20. R Development Core Team. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing; 2015.
21. Lantuejoul S, Rouquette I, Blons H, et al. French multi-centric validation of ALK rearrangement diagnostic in 547 lung adenocarcinomas. *Eur Respir J*. 2015;46:207–218.
22. Ilie MI, Bence C, Hofman V, et al. Discrepancies between FISH and immunohistochemistry for assessment of the ALK status are associated with ALK 'borderline'-positive rearrangements or a high copy number: a potential major issue for anti-ALK therapeutic strategies. *Ann Oncol*. 2015;6:238–244.
23. Lindeman NI, Cagle PT, Beasley MB, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline fromthe College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. *J Thorac Oncol*. 2013;8: 823–859.
24. Teixidó C, Karachaliou N, Peg V, Gimenez-Capitan A,

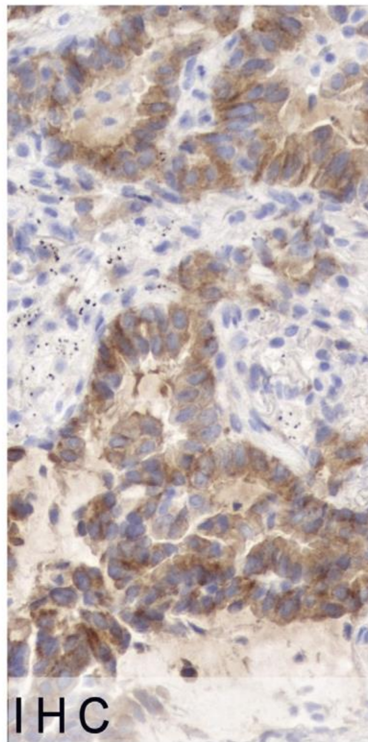
Rosell R. Concordance of IHC, FISH and RT-PCR for EML4-ALK rearrangements. *Transl Lung Cancer Res*.2014;3:70–74.

25. Wallander ML, Geiersbach KB, Tripp SR, Layfield LJ. Comparison of reverse transcription-polymerase chain reaction, immunohistochemistry, and fluorescence in situ hybridization methodologies for detection of echi- noderm microtubule-associated proteinlike 4-anaplastic lymphoma kinase fusion-positive non-small cell lungcarcinoma: implications for optimal clinical testing. *Arch Pathol Lab Med*. 2012;136:796–803.
26. Rogers TM, Arnau GM, Ryland GL, et al. Multiplexed transcriptome analysis to detect ALK, ROS1 and RET rearrangements in lung cancer. *Sci Rep*. 2017;7: 42259.
27. Forbes SA, Beare D, Bindal N, et al. COSMIC: High- resolution cancer genetics using the Catalogue of Somatic Mutations in Cancer. *Curr Protoc Hum Genet*. 2016;91:10.11.1–10.11.37.
28. Peters S, Taron M, Bubendorf L, Blackhall F, Stahel R. Treatment and detection of ALK-rearranged NSCLC. *Lung Cancer*. 2013;81:145–154.
29. Moran T, Quiroga V, Gil Mde L, et al. Targeting EML4-ALK driven non-small cell lung cancer (NSCLC). *Transl LungCancer Res*. 2013;2:128–141.
30. Yoshida T, Oya Y, Tanaka K, et al. Differential crizotinib response duration among ALK fusion variants in ALK- positive non-small-cell lung cancer. *J Clin Oncol*. 2016;34:3383–3389.
31. Tanizaki J, Okamoto I, Takezawa K, et al. Combined effect of ALK and MEK inhibitors in EML4-ALK-positive non-small-cell lung cancer cells. *Br J Cancer*. 2012;106:763–767.
32. Heuckmann JM, Balke-Want H, Malchers F, et al. Differential protein stability and ALK inhibitor sensitivityof EML4-ALK fusion variants. *Clin Cancer Res*. 2012;18:4682–4690.
33. Woo CG, Seo S, Kim SW, et al. Differential protein stability and clinical responses of EML4-ALK fusion variants to various ALK inhibitors in advanced ALK- rearranged non-small cell lung cancer. *Ann Oncol*. 2017;28:791–797.
34. Lin JJ, Shaw AT. Differential sensitivity to crizotinib: does EML4-ALK fusion variant matter? *J Clin Oncol*. 2016;34:3363–3365.
35. Sabir SR, Yeoh S, Jackson G, et al. EML4-ALK variants: biological and molecular properties, and the implica- tions for patients. *Cancers (Basel)*. 2017;9:piii:E118.
36. Zheng Z, Liebers M, Zhelyazkova B, et al. Anchored multiplex PCR for targeted next-generation sequencing.*Nat Med*. 2014;20:1479–1484.
37. Tsao MS, Hirsch FR, Yatabe Y. *IASLC Atlas of ALK and ROS1 Testing in Lung Cancer*. 2nd ed, North Fort Myers,FL: Editorial Rx Press; 2016.
38. Guillou L, Coindre J, Gallagher G, et al. Detection of thesynovial sarcoma translocation t(X;18) (SYT;SSX) in paraffin-embedded tissues using reverse transcriptase- polymerase chain reaction: a reliable and powerfuldiagnostic tool for pathologists. A molecular analysis of
    1. mesenchymal tumors fixed in different fixatives.

    Hum Pathol. 2001;32:105–112.
39. Travis WD, Brambilla E, Noguchi M, et al. Interna- tional Association for the Study of Lung Cancer/ American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol. 2011;6: 244–285.
40. Doshi S, Ray D, Stein K, et al. Economic analysis of alternative strategies for detection of ALK rearrange- ments in non small cell lung cancer. Diagnostics (Basel). 2016;6:pii.







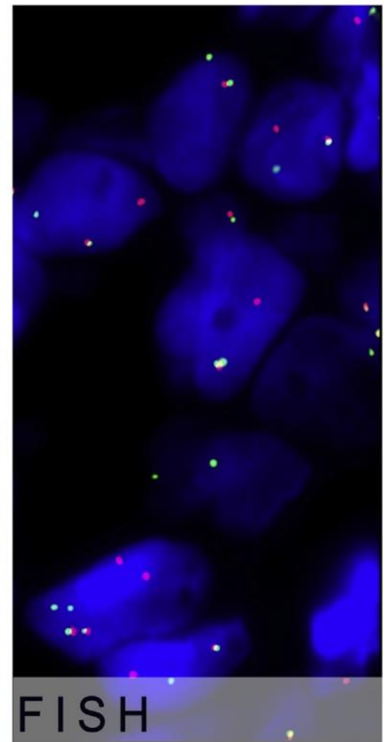


Figure 1. Illustration of three methodologies: reverse-transcriptase polymerase chain reaction (RT-PCR), anaplastic lymphoma kinase immunohistochemistry (ALK IHC), and ALK receptor tyrosine kinase gene fluorescence in situ hybridization (*ALK* FISH) in a rearranged case. H&E, hematoxylin and eosin.

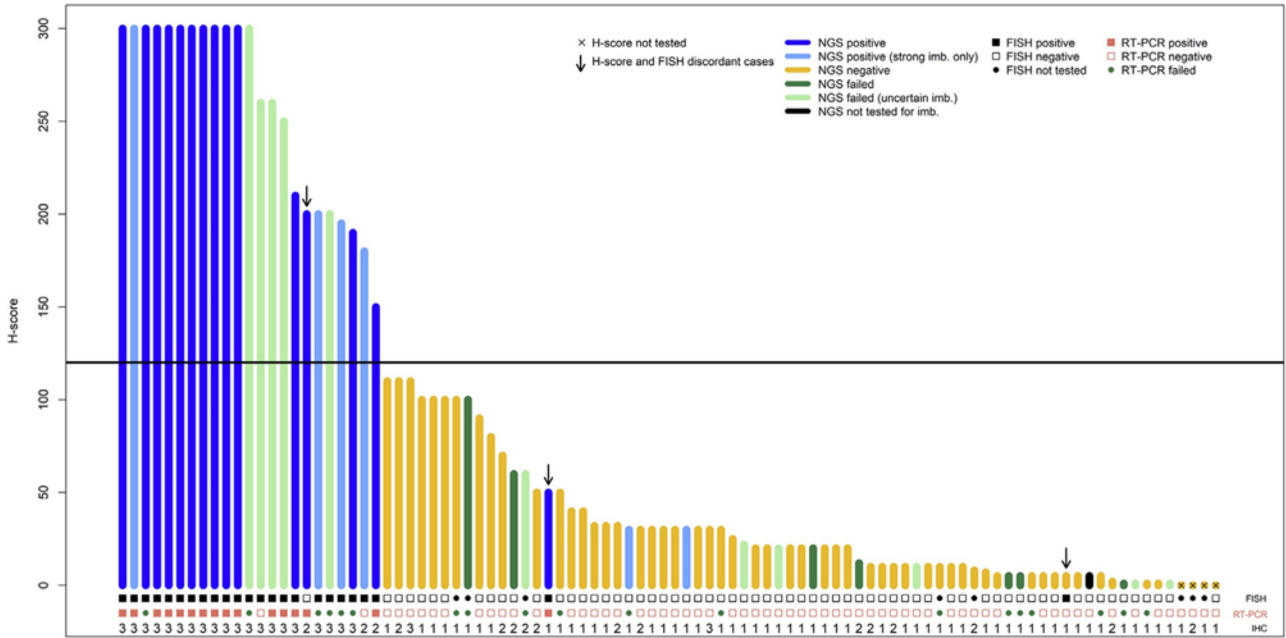


Figure 2. Waterfall plot for the concordance of H-score, next-generation sequencing (NGS), fluorescence in situ hybridization (FISH), reverse-transcriptase polymerase chain reaction (RT-PCR), and immunohistochemistry (IHC) testing for anaplastic lymphoma kinase (*ALK*) assessment (N ¼96). Each bar represents one case of the total of 96 cases. The different colors in the bars depict the result of use of the NGS method, whereas the height of each bar corresponds to the exact H-score (*y* axis). The horizontal black line represents the threshold (in H-score 120) below ¼which the cases are IHC negative. The different colors below the bars represent the results of FISH and RT-PCR for each case (fi*lled squares* indicate a positive result, *clear squares* indicate a negative result, *green circles* indicate that the method failed, and *black circles* indicate that the method was not tested). The last row below the bars corresponds to the respective maximum IHC intensity (1þ, 2þ, or 3þ).

Table 1. Paired Concordance of Available Results of RT-PCR, NGS with IHC, H-Score, or FISH results

	RT-PCR Status				NGS Status			
Test Result	Positive (n ¼ 16)	Negative (n ¼ 61)	All Patients (N ¼ 77)	k (*p* Value)	Positive (n ¼ 21)	Negative (n ¼ 56)	All Patients (N ¼ 77)	k (*p* Value)
ALK IHC, n (%)								
IHC 3þ	13 (81.3)	3 (4.9)	16 (20.8)	0.50[a] (<0.001)	15 (71.4)	2 (3.6)	17 (22.1)	0.57[a] (<0.001)
IHC 2þ	2 (12.5)	13 (21.3)	15 (19.5)		3 (14.3)	10 (17.9)	13 (16.9)	
IHC 1þ	1 (6.3)	45 (73.8)	46 (59.7)		3 (14.3)	44 (78.6)	47 (61.0)	
Total	16 (100.0)	61 (100.0)	77 (100.0)		21 (100.0)	56 (100.0)	77 (100.0)	
H-score, n (%)								
>120	15 (93.8)	2 (3.5)	17 (23.3)	0.88 (<0.001)	18 (85.7)	0 (0.0)	18 (24.7)	0.90 (<0.001)
≤120	1 (6.3)	55 (96.5)	56 (76.7)		3 (14.3)	52 (100.0)	55 (75.3)	
Total	16 (100.0)	57 (100.0)	73 (100.0)		21 (100.0)	52 (100.0)	73 (100.0)	
ALK FISH result, n (%)								
Positive	15 (93.8)	3 (5.3)	18 (24.7)	0.85 (<0.001)	18 (85.7)	1 (2.0)	19 (26.8)	0.86 (<0.001)
Negative	1 (6.3)	54 (94.7)	55 (75.3)		3 (14.3)	49 (98.0)	52 (73.2)	
Total	16 (100.0)	57 (100.0)	73 (100.0)		21 (100.0)	50 (100.0)	71 (100.0)	
RT-PCR result, n (%)								
Positive	—[b]	—	—	—	14 (87.5)	0 (0.0)	14 (21.5)	0.91 (<0.001)
Negative	—	—	—		2 (12.5)	49 (100.0)	51 (78.5)	
Total	—	—	—		16 (100.0)	49 (100.0)	65 (100.0)	

Note: Boldface indicates statistical significance.
[a]Categories IHC 2þand IHC 3 þare combined as strong positive for the k coefficient test.
[b]Not applicable.
RT-PCR, reverse-transcriptase polymerase chain reaction; NGS, next-generation sequencing; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; ALK, anaplastic lymphoma kinase.

Table 2. Three-Way Concordance of RT-PCR and NGS with H-Score and FISH results

	RT-PCR			NGS		
FISH Status	Positive	Negative	Total	Positive	Negative	Total
FISH negative						
H >120	1 (6.3)	0 (0)	1 (1.4)	1 (4.8)	0 (0.0)	1 (1.4)
H ≤120	0 (0)	53 (94.6)[a]	53 (73.6)	2 (9.5)	48 (98.0)[b]	50 (71.4)
Total	16	56	72	21	49	70

[a]Fourteen positive plus 53 negative cases for a total of 67 cases (93.1% of 72 cases) with full matching of RT-PCR, FISH, and IHC status.
[b]Seventeen positive plus 48 negative cases for a total of 65 cases (92.9% of 70 cases) with full matching of NGS, FISH, and IHC status.
RT-PCR, reverse-transcriptase polymerase chain reaction; NGS, next-generation sequencing; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization.

Table 3. *ALK* NGS result according to RT-PCR result, *ALK* FISH status, and H-score levels utilizing the imbalance scores

	H-Score>120				H-score≤120				
	FISH: Positive		FISH: Negative		FISH: Positive		FISH: Negative		
NGS Status	RT-PCR: P	RT-PCR: N	RT-PCR: P	RT-PCR: N	RT-PCR: P	RT-PCR: N	RT-PCR: P	RT-PCR: N	All patients
Positive	12[a]	1[b]	1[c]	0	1[c]	0	0	1[d]	16
Negative	0	0	0	0	0	1[c]	0	43[a]	44
Total	12	1	1	0	1	1	0	44	60

Note: Boldface indicates the criterion standard positivity.

[a]Twelve positive plus 43 negative cases for a total of 55 cases (91.7% of 60 cases) with full matching with all four methods.

[b]One RT-PCR–negative case (1.7% of 60 cases) with RT-PCR not matching the results with the other three methods.

[c]Two NGS-positive plus one NGS-negative case for a total of three cases (5% of 60 cases) with matching NGS and RT-PCR status but different FISH status or H-score.

[d]One NGS-positive case (1.7% of 60 cases) with NGS not matching the results with the other three methods.

ALK, anaplastic lymphoma kinase gene; NGS, next-generation sequencing; RT-PCR, reverse-transcriptase polymerase chain reaction; FISH, fluorescence in situ hybridization; P, positive; N, negative.

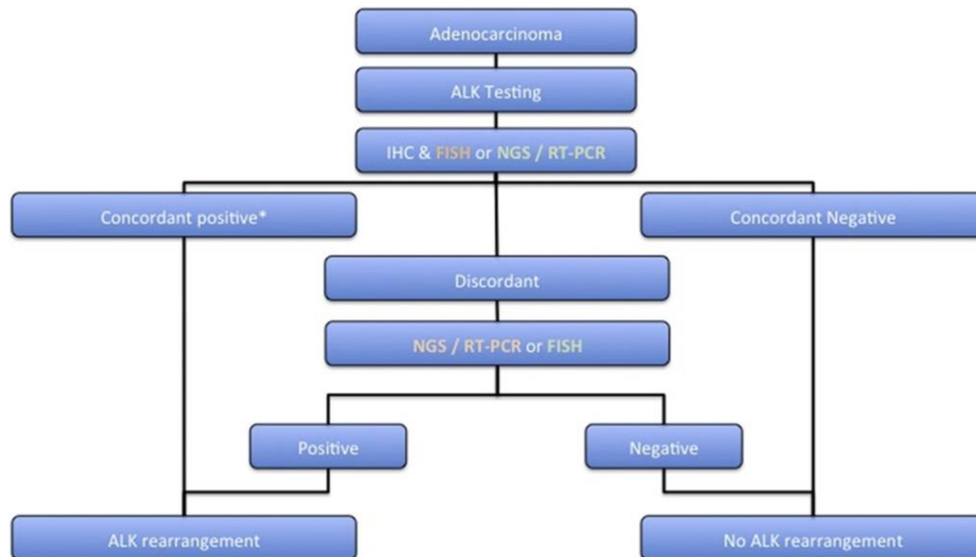


Figure 3. Proposed testing algorithm for anaplastic lymphoma kinase (*ALK*) evaluation using different techniques for maximal sensitivity and specificity, starting with a double-testing, including immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) or RNA-based assay reverse-transcriptase polymerase chain reaction (RT-PCR)/next-generation sequencing (NGS), followed by a third assay in discordant cases (RNA-based assay if FISH was performed and vice versa). *IHC positivity as defined for each antibody for the example of an H-Score higher than 120 for the clone 5A4 antibody from Novocastra.