Fluorescence In Situ Hybridization, Immunohistochemistry, and Next-Generation Sequencing for Detection of EML4-ALK Rearrangement in Lung Cancer

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Disclosures of potential conflicts of interest may be found at the end of this article.

Abstract

Background. The U.S. Food and Drug Administration-approved method for detecting EML4-ALK rearrangement is fluorescence in situ hybridization (FISH); however, data supporting the use of immunohistochemistry (IHC) for that purpose are accumulating. Previous studies that compared FISH and IHC considered FISH the gold standard, but none compared data with the results of next-generation sequencing (NGS) analysis.

Materials and Methods. We studied FISH and IHC (D5F3 antibody) systematically for EML4-ALK rearrangement in 51 lung adenocarcinoma patients, followed by NGS in case of discordance.

Results. Of 51 patients, 4 were positive with FISH (7.8%), and 8 were positive with IHC (15.7%). Three were positive with both. NGS confirmed that four of the five patients who were positive with IHC and negative with FISH were positive for ALK. Two were treated by crizotinib, with progression-free survival of 18 and 6 months. Considering NGS as the most accurate test, the sensitivity and specificity were 42.9% and 97.7%, respectively, for FISH and 100% and 97.7%, respectively, for IHC.

Conclusion. The FISH-based method of detecting EML4-ALK rearrangement in lung cancer may miss a significant number of patients who could benefit from targeted ALK therapy. Screening for EML4-ALK rearrangement by IHC should be strongly considered, and NGS is recommended in borderline cases. Two patients who were negative with FISH and positive with IHC were treated with crizotinib and responded to therapy. *The Oncologist* 2015;20:1–7

Implications for Practice: ALK-related therapies are the most effective therapies available for advanced lung cancer; therefore, accurate diagnosis for ALK rearrangement is crucial. Previous studies compared fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) and considered FISH the reference method. For the first time, discordant cases have been further investigated by next-generation sequencing (NGS), indicating that FISH may miss a significant number of cases that were positive with IHC and that may benefit from ALK-related therapies. Previous comparisons wrongly considered cases negative with FISH and positive with IHC to be false positive with IHC. We recommend reassessment of the current algorithm for ALK testing and suggest screening by IHC followed by NGS for borderline cases.

Introduction

Lung cancer remains the top killer among all cancers. Although many of the risk factors have been identified in the past, there were 228,190 new cases of lung cancer in 2012 in the U.S., leading to 159,480 deaths [1].

The treatment regimen for lung cancer has changed significantly over the past decade from histology-driven to molecularly driven therapy. More than 20% of patients with lung adenocarcinoma are treated with targeted therapy

based on their mutational status. The most common targets are EGFR (10%–30%), EML4-ALK (3%–7%), HER2 (2%–5%), BRAF (1%–3%), ROS1 (1%), and RET (1%) [2, 3].

The second most common gene abnormality associated with targeted therapy in adenocarcinoma of the lung is EML4-ALK rearrangement. *ALK* (anaplastic lymphoma kinase) encodes a tyrosine kinase receptor that is expressed only in neuronal cells and that does not exist in noncancerous cells; however, the mutated protein was found in a number of cancer cells, including neuroblastomas, anaplastic large cell lymphomas, and inflammatory myofibroblastic tumors [4]. Soda et al. demonstrated that 6.7% of patients suffering from adenocarcinoma of the lung bear this unique gene rearrangement [5–7]. EML4-ALK is a chimeric protein that arises from EML4 (echinoderm microtubule-associated protein-like 4) and ALK. The fused gene product demonstrates constitutive kinase activity, and cancer cells with the EML4-ALK rearrangement are dependent on the activity of this kinase for uncontrolled growth and survival [5–7]. Patients harboring this rearrangement were younger, and most were nonsmokers or light smokers in the past [6, 8–11]. Analysis of the Israeli cohort shows that this rearrangement is most common in young men, and the chances for finding the fusion are reduced by 7% with each additional year in a lung cancer patient aged >52 years [12]. Even with the proposed characteristics, the current International Association for the Study of Lung Cancer (IASLC) guidelines state, "ALK molecular testing should be used to select patients for ALK-targeted tyrosine kinase inhibitor (TKI) therapy and patients with lung adenocarcinoma should not be excluded from testing on the basis of clinical characteristics" [13–15]. The prevalence of EML4-ALK rearrangement in an unselected non-small cell lung cancer (NSCLC) population is 3.4% (range: 1.6%–11.7%), whereas in the adenocarcinoma subset of NSCLC, the prevalence of EML4-ALK rearrangement is 4.5% (range: 2.4%–16.1%) [16].

ALK tyrosine kinase inhibitors yield a spectacular objective response rate of >60% [17, 18]. Consequently, it is imperative to perform appropriate molecular tissue investigation. Currently, the approved method for selecting patients with EML4-ALK is the fluorescence in situ hybridization (FISH) assay, using dual-labeled break-apart probes; however, ALK rearrangement also can be identified through immunohistochemistry (IHC) after proper validation of the method [12, 14].

The recent IASLC guidelines advocate the use of ALK FISH assay with dual-labeled break-apart probes for selecting patients for ALK TKI therapy. The guidelines also mention that ALK IHC, if carefully validated, may be considered as a screening method to select specimens for ALK FISH testing [14]. Several studies have compared IHC and FISH [19] and indicated a wide range of accuracy, partly related to the IHC antibody used. In all studies, FISH was considered the gold standard. Because our clinical experience has shown that patients who were positive with IHC and negative with FISH may also benefit from ALK-related therapy [20], we wished to investigate whether FISH is indeed the appropriate gold standard. In this study, we tested 51 patients consecutively for ALK rearrangement by FISH and IHC and further

sequenced any discordant specimens by next-generation sequencing (NGS).

METHODS

Ethics Committee
The institutional review board of Sheba Medical Center in Tel Hashomer, Israel, reviewed this study and issued approval on April 22, 2014 (protocol number 9480-12-SMC).

Patients
This retrospective, cross-sectional study included 57 patients suffering from adenocarcinoma of the lung who were assessed for EML4-ALK rearrangement between the years 2011 and 2013. Sufficient material for analysis was obtained from 51 patients. Pathological staging at the time of surgery was done using the 7th edition of the American Joint Committee on Cancer staging system (TNM-7). The epidemiological and clinical data on all patients were collected from their clinical charts.

FISH Analysis
Formalin-fixed paraffin-embedded (FFPE) specimens from the patients were sent by Oncotest-Teva (Teva Pharmaceutical Industries Ltd., Petah Tikva, Israel, http://www.oncotest.co.il/?lang=en) to Clarient Diagnostic Services Inc. (GE Healthcare, Little Chalfont, U.K., http://www3.gehealthcare.com) for FISH using the Vysis ALK Break Apart FISH Kit (Abbott Molecular, Abbott Park, IL, https://www.abbottmolecular.com). The identification probes for the LSI ALK 5′ probe (SpectrumGreen) and the LSI ALK 3′ probe (SpectrumOrange) were applied, hybridized, and assessed along with standard controls. At least 50 nonoverlapping nuclei were analyzed, and the localization of the LSI ALK 5′ probe (green) and LSI ALK 3′ probe (orange) signals were recorded and interpreted according to the manufacturer's guidelines.

IHC Analysis
IHC was done at the University of Colorado Anschutz Medical Campus. The slides were stained with the D5F3 antibody from Cell Signaling Technology (Beverly, MA, http://www.cellsignal.com) on a BenchMark XT autostainer with the UltraView DAB detection kit (Ventana Medical Systems Inc., Oro Valley, AZ, http://www.ventana.com). The antibodies are sensitive to all EML4-ALK variants. The intensity of stain depends on the degree of expression.

Unstained paraffin tissue sections were dried in a 60°C drying oven for 1 hour. Slides were labeled with a bar-coded standardized antibody-specific protocol and loaded into the BenchMark XT autostainer. Slides were treated with standard cell conditioning 1 for 60 minutes. Primary antibody (clone D5F3; Cell Signaling Technology) was manually applied at 1:75 dilution (~13 μg/mL), and the slides were incubated inside the Benchmark XT autostainer at 37°C for 1 hour. The UltraView DAB detection kit was used with the amplification kit. Slides were counterstained with hematoxylin for 4 minutes and postcounterstained with bluing agent for 4 minutes. Slides were washed with mild soapy water and
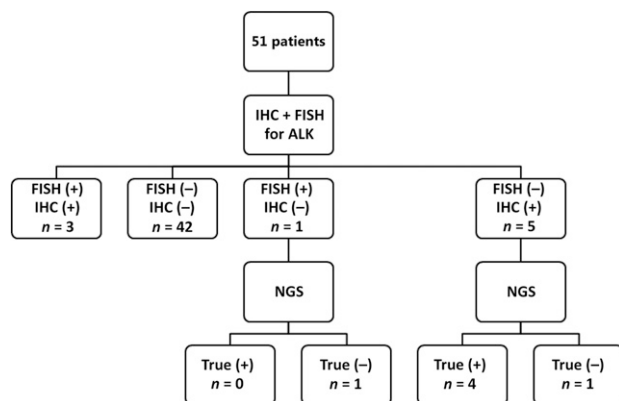
Figure 1. Study algorithm for ALK detection using immunohisto-chemistry and fluorescence in situ hybridization. Mismatched cases: FISH (+) IHC (−) and FISH (−) IHC (+) were sent to FoundationOne next-generation sequencing assay sequencing.

Abbreviations: (−), negative; (+), positive; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NGS, next-generation sequencing.

then dehydrated in 70%–100% reagent alcohol baths followed by Xylene baths before applying coverslips. Positive control ALK (D5F3) antibody was applied as the primary antibody (1:75 dilutions) to ALK-positive cell lines H2228 and H3122 for positive control.

A positive result was defined as any positive staining considered to be specific on an intensity scale from 0 to ≥3, although an H-score system (0–300) was used for the final report. Although any staining is abnormal, we defined an H-score of 40 as the cutoff value to avoid any background effect. The H-score was calculated by multiplying the staining intensity (0 to ≥3) by the percentage of cells that presented this intensity.

NGS Analysis

Cases that had discordant results were sent by Oncotest-Teva (Teva Pharmaceutical Industries Ltd) to Foundation Medicine (Cambridge, MA, http://www.foundationmedicine.com) for FoundationOne NGS assay using the HiSEquation 2000 (Illumina, San Diego, CA, http://www.illumina.com). This assay was conducted on hybridization-captured, adaptor ligation-based libraries using DNA extracted from four FFPE sections cut at 10 μm. The pathological diagnosis of each case was confirmed on routine hematoxylin and eosin-stained slides, and all samples containing a minimum of 20% tumor cells were forwarded for DNA extraction. DNA sequencing was conducted for 3,769 exons of 236 cancer-related genes and 47 introns of 19 genes frequently re-arranged in cancer (a total of 1.14 million base pairs [bp]) on indexed, adaptor-ligated, hybridization-captured libraries (SureSelect Custom Kit; Agilent Technologies, Santa Clara, CA, http://www.agilent.com). Full sequencing was performed using 49-bp paired reads on the Illumina HiSEquation 2000 to an average depth of 843 times and assessed for genomic alterations including base substitutions, insertions, deletions, copy number alterations (amplifications and homozygous deletions), and selected gene fusions or rearrangements, as described previously [21]. The study algorithm is shown in Figure 1.

Table 1. Clinical characteristics of the study cohort

Characteristic	Patients (*N* = 51)
Age, years, average (range)	63.5 (41–90)
Sex	
Male	28
Female	23
Histology	Adenocarcinoma
Stage	IV
Smoking status	
Never smoker	26
Previous or current smoker	21
Unknown	4
IHC TTF1 staining	
Positive	32
Negative[a]	10
Unknown[a]	9

[a]TTF1-stained samples were all defined as adenocarcinoma by morphology. Of the 10 samples that were negative with TTF1, 7 were positive with cytokeratin 7.
Abbreviations: IHC, immunohistochemistry; TTF1, thyroid transcription factor 1.

Table 2. FISH and IHC summary

Technique, *n*	IHC positive	IHC negative	Total
FISH positive	3	1	4 (7.8%)
FISH negative	5	42	47
Total	8 (15.7%)	43	51

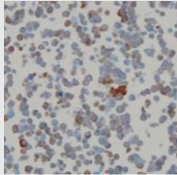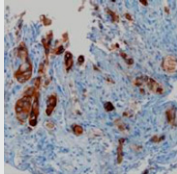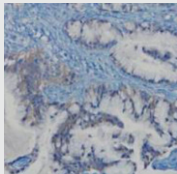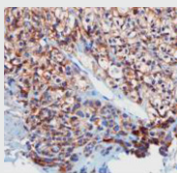Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.
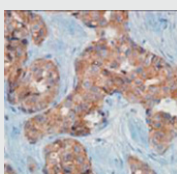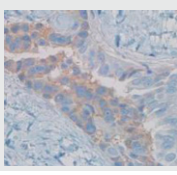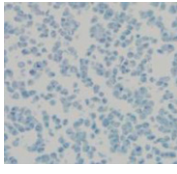
RESULTS

Epidemiological and Histological Criteria of NSCLC Patients

Fifty-seven consecutive advanced NSCLC patients (stage IV) with a histologic subtype of adenocarcinoma and referred for EML4-ALK FISH testing under our routine clinical practice were included in the study. Fifty-one specimens were included in the final analysis. The average age was 63.5 years (range: 41–90 years). All tumors originated in the lung and were diagnosed histologically as adenocarcinoma through morphological appearance and standard IHC markers (thyroid transcription factor 1, cytokeratin 20, and cytokeratin 7) (Table 1).

FISH and IHC Analysis

The FISH and IHC results are summarized in Table 2. Fifty-one patients had sufficient tissue for ALK FISH and IHC testing. Of those, four were positive with FISH and eight were positive with IHC. Three had concordant results (i.e., positive with both FISH and IHC). One sample was positive with FISH and negative with IHC, and five were positive with IHC and negative with FISH. The six cases with discordant results were sequenced by the FoundationOne assay.

Table 3. FISH, IHC, and NGS data for all mismatched cases (IHC positive, FISH negative)

Patient	IHC score	FISH	NGS	Clinical response
IHC (+) and FISH (−)				
IHC positive control				
Patient 1	300	Negative	ALK fusion ALK intron 19, HSPB1 intron 3	N/A
Patient 2	150	Negative	ALK EML4-ALK fusion, CDKN2A/B loss, TP53 r209fs*6	N/A
Patient 3	300	Negative	EML4-ALK fusion; NF2 E 404*; CDKN2A/B loss; MCL1 amplification; TP53 Q144fs*26; ARID1A d1850fs*4	N/A
Patient 4	230	Negative	ALK − rearrangement, intron 19	Complete response (18 months)
Patient 5	40	Negative	Negative	Stable disease (6 months)
IHC (−) and FISH (+)				
IHC negative control				IHC negative control
Patient 1	0	Positive	NF1 splice 205-1delAG; PIK3R1 truncation, exon 7	

Abbreviations: (−), negative; (+), positive; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; N/A, not available; NGS, next-generation sequencing.

NGS Analysis

Four of the five cases that were negative with FISH and positive with IHC harbored ALK rearrangements, as demonstrated by clinical-grade NGS. The one case that was positive with FISH and negative with IHC did not harbor an ALK rearrangement (Table 3). Consequently, the final incidence of EML4-ALK rearrangement in our cohort was 13.7% and not 7.8%, as was defined previously by FISH (Table 2).

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Table 4. Accuracy of FISH and IHC for EML4-ALK based on 51 cases with next-generation sequencing as the gold standard

Method	Sensitivity	Specificity	PPV	NPV
IHC, %	100	97.7	87.5	100
FISH, %	42.9	97.7	75	91.4

Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NPV, negative predictive value; PPV, positive predictive value.

The calculated accuracy for FISH and IHC is summarized in Table 4 based on NGS as the final gold standard. We considered the true-positive rate to be 7 and the true-negative rate to be 44; IHC had 1 false-positive case (IHC score: 40) and no false-negative cases. In contrast, FISH had three false-negative cases and one false-positive case. Consequently, the sensitivity and specificity of IHC in our study were 100% and 97.7%, respectively, with a positive predictive value (PPV) of 87.5% and a negative predictive value (NPV) of 100%. For FISH, the sensitivity and specificity were 42.9% and 97.7%, respectively, with a PPV of 75% and an NPV of 91.4% (Table 4).

Response to Crizotinib Therapy
Among all discordance cases, only two patients were treated with crizotinib. Case 4 showed a complete response to therapy [20], with progression-free survival of 18 months, whereas case 5 presented stable disease. The other positive cases (with IHC) died before we could obtain a final conclusion regarding their tissue status (retrospective study).

DISCUSSION
This study is the first to use clinical-grade NGS as the final reference for ALK rearrangement diagnosis. This study demonstrates that IHC may play a role as a primary screening method for ALK rearrangement in lung cancer; however, it may also suggest that FISH may miss patients who might benefit from ALK-related therapy. Our data suggest that ALK IHC should be considered, rather than FISH, for the selection of patients for ALK-targeted therapy, and NGS should be applied if IHC is inconclusive. In this study, we used DF53 antibody (Cell Signaling Technology) for IHC.

FISH, as defined by Abbott Molecular and approved as companion diagnostics by the U.S. Food and Drug Administration, misses clinically important rearrangements in ALK because of the existence of numerous variants of EML4-ALK rearrangements in lung cancer and the very specific definition of the ALK-positive FISH pattern. Break-apart FISH should depict rearrangements as long as the probes are designed to accurately target the break point of the *ALK* gene, which is highly conserved, regardless of the break point of *EML4*. The rearrangements contain various regions of the *EML4* gene, although the kinase region of *ALK* is conserved in all of them [4, 22, 23]. Moreover, RNA editing abnormalities associated with intron abnormalities cannot be detected by FISH. In this study, two of the six mismatched cases had abnormalities in intron 19 of the *ALK* gene (Table 3). One of these patients was recently reported as having a complete response to crizotinib over 18 months [20].

Previous studies have compared IHC and FISH but adopted FISH as the gold standard for ALK rearrangement. Yi et al. [4]

compared IHC (Dako, Glostrup, Denmark, http://www.dako.com) and FISH (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular) in an enriched population of 101 nonsmokers with lung adenocarcinoma. They reported that all IHC(+3) cases were positive for ALK on FISH (8 of 8), whereas positive results with FISH were less frequent among IHC(+2) and IHC(+1) cases (1 of 3 and 1 of 21, respectively). None of the IHC(0) cases was positive with FISH. The authors concluded that IHC is a good screening test for ALK rearrangement; however, in their study, 21 patients were stained positive for IHC, although weakly IHC(+1). Their algorithm sensitivity was 90% with a specificity of 97.8% [4].

McLeer-Florin et al. [19] also performed dual IHC (5A4 monoclonal antibody [mAb]; Abcam, Cambridge, U.K., http://www.abcam.com) and FISH testing for ALK rearrangement in lung adenocarcinomas in 100 subjects, and IHC was performed on 441 specimens. They reported sensitivity and specificity of 95% and 100%, respectively, for the 5A4 mAb, adopting FISH as the gold standard. It should be noted that in their cohort, seven positive IHCs were not interpretable over FISH but were still included as positive cases in their summary.

Minca et al. [24] also used the D5F3 IHC antibody and concluded that IHC was more informative than FISH, including adding more ALK-positive cases. They compared 318 FFPE and 40 matched ThinPrep (Hologic, Bedford, MA, http://www.hologic.com) specimens from 296 patients diagnosed with advanced NSCLC. This study revealed that IHC demonstrated 100% sensitivity and specificity (95% confidence intervals of 0.86–1.00 and 0.97–1.00, respectively) for ALK detection using 249 dual-informative NSCLC samples. This study, like ours, emphasizes the role of IHC as the first screening method for ALK testing in NSCLC patients [24].

Ali et al. recently published results of 1,070 lung carcinoma cases who underwent NGS [25]. Forty-seven patients had ALK rearrangements (4.4%). Interestingly, 28 patients were also tested by ALK FISH. Nine (32%) were negative, and 19 were positive. Five of those who were negative with FISH and positive with NGS responded to crizotinib. In the light of the responsiveness of ALK tumors positive with NGS and negative with FISH to crizotinib, the use of FISH as the gold standard for ALK detection in lung cancer warrants reinvestigation [25].

Because detection of ALK rearrangements in NSCLC is crucial for providing the appropriate therapy, particularly in view of the excellent response to crizotinib, ceritinib, or alectinib, there is a growing need for a highly sensitive, standardized, IHC-based approach. FISH testing is time-consuming and costly and is not suitable for application in the broader community. Moreover, it seems from our results and those of other researchers [20, 26] that FISH misses numerous patients that might benefit from ALK-targeted therapy. The available ALK IHC antibodies are being refined, and the current rabbit mAb D5F3 (Cell Signaling Technology) seems to provide excellent sensitivity and specificity (100% and 97.7%, respectively) (Table 4), as has also been reported previously [22]. The accuracy of the D5F3 mAb is higher in comparison with previously used antibodies, such as ALK1 clone (Dako) [4] with sensitivity of 90% and 97.8% specificity. Other antibodies such as SP8 (Abcam), ZAL4 (Life Technologies - Thermo Fisher Scientific Brand, Waltham, MA, http://www.thermofisher.com), and P80 (Nichirei Biosciences

Inc, Tokyo, Japan, https://www.nichirei.co.jp/bio/english/) had lower detection rates [27]. Interestingly, the IHC 5A4 (Novocastra; Leica Biosystems, Buffalo Grove, IL, http://www.leicabiosystems.com) has been compared with FISH on a cytological specimen and showed sensitivity, specificity, PPV, and NPV of 93.3%, 96.0%, 93.3%, and 96%, respectively [28]; however, FISH served as the gold standard in this study. Our study supports the IHC-based screening approach for detecting EML4-ALK rearrangement in lung cancer. In summary, when assessed by FISH alone, our cohort had 4 positive cases (7.8%), whereas the true incidence was 7 (13.7%). Our cohort included patients who performed ALK testing before it became routine at our institute; therefore, the cohort may not be representative of the lung cancer population. For the purposes of this study, the selected population does not affect the conclusions.

Our previous case report [20] was the first to suggest that patients with ALK positive with IHC and negative with FISH benefits from ALK-targeted therapy. A larger prospective study is required to associated IHC positivity with clinical response.

The use of IHC as a screening technique can be found in routine practices, and the technique is relatively cheap and easily applicable in a clinical setting, as in the case of HER2 overexpression found in 18%–20% of patients with breast cancer. Patients with positive results benefit from targeted therapy using, for example, trastuzumab, pertuzumab, and lapatinib. The algorithm for screening patients for HER2 using IHC is approved by the American Society of Clinical Oncology and College of American Pathologists guideline recommendations. Patients expressing IHC(+3) are considered positive and can start HER2-related treatment. IHC(0) or ICH(+1) is considered to be negative for HER2, and borderline IHC(+2) should be validated by FISH for *HER2* gene amplification [29].

Cases that were negative with both FISH and IHC were not subjected to NGS. We assumed that none of these cases would harbor an ALK fusion on NGS, but it remains possible that some of the 42 FISH/IHC double-negative cases reported may be false negative.

Our proposed algorithm for molecular testing in patients with advanced NSCLC adenocarcinoma is EGFR mutation analysis followed by IHC D5F3 for ALK. If IHC is inconclusive, sequencing with NGS should be considered. Such an algorithm will allow more patients to be treated with appropriate personalized medicine.

Conclusion

The FISH-based practice for detection of EML4-ALK rearrangement in lung cancer may miss a significant number of patients who could benefit from targeted ALK therapy. Screening for EML4-ALK rearrangements by IHC should be strongly considered, and NGS should be advised for borderline cases.

Author Contributions

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