ORIGINAL ARTICLE

ALK Protein Analysis by IHC Staining after Recent Regulatory Changes: A Comparison of Two Widely Used Approaches, Revision of the Literature, and a New Testing Algorithm

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ABSTRACT

Introduction: Recent regulatory changes have allowed the diagnostic use of immunohistochemical (IHC) analysis for the identification of patients with non–small cell lung cancer who are eligible for treatment with anaplastic lymphoma receptor tyrosine kinase (ALK) inhibitors. The U.S. Food and Drug Administration has approved the VENTANA ALK (D5F3) CDx Assay (Ventana Medical Systems, Tucson, AZ) as companion diagnostics, and the Italian Medicines Agency has recognized IHC analysis as a diagnostic test indicating an algorithm for patient selection.

Methods: On the basis of the new regulations, we compared two commonly used IHC assays on 1031 lung adenocarcinomas: the VENTANA ALK (D5F3) CDx Assay with the OptiView Amplification Kit (Ventana Medical Systems) and a standard IHC test with the clone 5A4 (Novocastra, Leica Biosystems, Newcastle Upon Tyne, United Kingdom) along with their interpretative algorithms. Fluorescence in situ hybridization (FISH) was performed in all cases. Next-generation sequencing was performed in FISH/IHC analysis–discordant samples.

Results: FISH gave positive results in 33 (3.2%) cases. When FISH was used as a reference, the VENTANA ALK (D5F3) CDx assay had a sensitivity of 90.9% ± 2.6%, a specificity of 99.8% ± 0.6%, and positive and negative predictive values of 93.8% ± 2.1% and 99.7% ± 0.6%, respectively. The clone 5A4–based IHC test showed a sensitivity of 90.9% ± 2.6%, a specificity of 98.3% ± 1.3%, and positive and negative predictive values of 63.8% ± 4.2% and 99.7% ± 0.6%, respectively. Five cases with IHC analysis/FISH-discordant results in our series were analyzed together with those previously reported in the literature. Overall, data from 35 patients indicate a response rate to ALK inhibitors in 100% of FISH-negative/IHC analysis–positive cases (seven of seven) and 46% of FISH-positive/IHC analysis–negative cases (13 of 28), respectively.

Conclusions: Our results confirm the difficulty in managing an IHC test without amplification in the absence of confirmatory FISH analysis, as well as the possibility of performing a direct diagnosis in approximately 90% of patients by the VENTANA ALK (D5F3) CDx Assay. On the basis of the recent regulatory changes, the data that have emerged from the literature, and the results of the present study, a new algorithm for ALK assessment in non–small cell lung cancer has been devised.

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Introduction

Anaplastic lymphoma receptor tyrosine kinase gene (*ALK*) rearrangements have been described in 3% to 5% of cases of non–small cell lung cancer (NSCLC), and their identification is mandatory to select patients for treatment with anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors.[1,2]

Different technologies are available to assess *ALK* gene rearrangements. Fluorescence in situ hybridization (FISH) is the accepted standard because it has been used as a reference method in clinical trials; however, it is an expensive, time-consuming, and labor-intensive assay. In addition, result interpretation is often operator dependent.[3,4] An alternative diagnostic method based on the detection of ALK fusion protein expression is immunohistochemical (IHC) analysis. This method is widely used in pathology laboratories, faster, cheaper, and particularly useful in patients with advanced-stage carcinoma, for whom small biopsy specimens with a limited number of neoplastic cells are often available.[5,6]

Different monoclonal antibodies for the detection of ALK protein expression are commercially available, including the clone ALK1 (Dako, Glostrup, Denmark), the clone 5A4 (Novocastra, Leica Biosystems, Newcastle Upon Tyne, United Kingdom), and the clone D5F3 (Cell Signaling Technology, Danvers, MA). At the moment, the clones D5F3 and 5A4 are the most widely used for the detection of ALK expression in patients with NSCLC.[5,7,8]

The clone 5A4 has been utilized on different platforms, including the BOND-MAX immunostainer (Leica Microsystems, Wetzlar, Germany). In this case, a scoring system based on four levels of ALK expression (0, 1+, 2+, and 3+) has been adopted in most previous reports.[4,7,9,10] In a large multicenter study, 1+ tumors were found to be positive by FISH analysis in 4% of cases and 2+ tumors were found in 60% of cases.[11] Therefore, 1+ or 2+ samples should be considered equivocal and should be validated by FISH. This leads to a marked cost increase and delayed medical reports.

A D5F3-based immunoassay, the VENTANA ALK (D5F3) CDx Assay (Ventana Medical Systems, Tucson, AZ), has been developed and standardized on the automated immunostaining platform BenchMark XT (Ventana) combined with the OptiView Amplification Kit (Ventana). The interpretation of results is based on a dichotomic algorithm described in the product data sheet. Cases are defined as positive or negative according to the presence or absence of a specific immunoreaction in tumor cells.[12]

In June 2015, the U.S. Food and Drug Administration (FDA) approved the VENTANA ALK (D5F3) CDx Assay as a companion diagnostic to aid in the identification of patients eligible for treatment with the ALK inhibitor crizotinib.[13] The Italian Medicines Agency (AIFA), in line with the FDA, has recognized IHC analysis as a diagnostic test, suggesting an algorithm for patients selection that is based on a definitive IHC testing result (positive or negative) regardless of the antibody used. Equivocal cases must be confirmed by FISH (Supplementary Fig. 1, Supplementary Digital Content 1).[14]

On the basis of the new recommendation for the IHC analysis of ALK in NSCLC, we decided to compare two commonly used IHC assays on a large series of lung adenocarcinomas: the ALK (D5F3) CDx Assay on the BenchMark XT platform with the Optiview Amplification Kit along with its related interpretative algorithm and an assay based on the use of the clone 5A4 on the BOND-MAX platform with its own algorithm. The main objective of this study was to compare the performances of these two diagnostic approaches for the selection of patients to be enrolled for treatment with anti-ALK drugs.

Materials and Methods

Tumor Samples

The study was conducted on a retrospective series of 1031 lung adenocarcinoma samples obtained from as many patients as underwent a radical resection of a primary NSCLC at the Department of Thoracic Surgery, University of Chieti (Chieti, Italy). Tumor samples were fixed in formalin, embedded in paraffin, and histologically classified as adenocarcinomas on the basis of hematoxylin and eosin and IHC staining according to the WHO classification of lung tumors.[15] Representative tumor areas were identified and tissue microarrays (TMAs) were built using two large (2-mm-diameter) cores for each case. Informed consent was obtained from all patients under study. The study was approved by the local human investigations committee and was conducted in accordance with the precepts of the Helsinki Declaration.

ALK IHC Analysis

TMA samples were cut to a thickness of 4 μm and stained using two different ALK IHC assays: the Novocastra mouse monoclonal antibody p80 ALK (Clone 5A4, Leica Biosystems, Newcastle Upon Tyne, United Kingdom) and the Ventana anti-ALK rabbit monoclonal primary antibody (Clone D5F3, Cell Signaling Technology).

The monoclonal antibody 5A4 was used in a 1:20 dilution and incubated for 2 hours at room temperature. The immunostaining was performed with the BOND Polymer Refine Detection Kit on the BOND-MAX immunostainer. The staining intensity was scored as 0 (negative), 1+, 2+, or 3+, and only strong staining (3+) was defined as an ALK-positive result. Cases scored as 1+ and 2+ were considered equivocal. The monoclonal antibody D5F3 was applied on the BenchMark XT Immunostainer (Ventana) with the OptiView DAB IHC Detection Kit and OptiView Amplification Kit. The staining results were evaluated using a binary scoring system: positive or negative following the manufacturer's instructions. The ALK IHC staining data were compared with results obtained by FISH.

ALK FISH

FISH was performed on unstained 3- to 4-μm formalin-fixed, paraffin embedded tumor tissue sections using the Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular, Des Plaines, IL) according to the manufacturer's protocol. The ALK FISH was performed with a slide scanning system under an 63× oil immersion objective with a fluorescence microscope (Olympus BX61, Olympus Corporation, Tokyo, Japan) equipped with appropriate filters, a charge-coupled device camera, and the FISH imaging and capturing software SoloTouch (Bioview Duet, BioView, Ltd, Rehovat, Israel). More than 50 cancer cells per case were scored and signals were evaluated with the imaging system. Tumor samples were considered ALK FISH positive (ALK rearranged) if more than 15% of the tumor cells showed split red and green signals (signals separated by one or more signal diameters) and/or single red 3' signals (deleted green signal) in addition to fused and/or broken-apart signals. Otherwise, the samples were considered FISH negative.

NGS

Next-generation sequencing (NGS) analysis of *ALK* rearrangement was performed by the Archer Universal RNA Reagent Kit v2 (Archer DX, Boulder, CO) in conjunction with Archer FusionPlex assays and molecular bar code adapters on the Illumina MiSeq system (Illumina Inc., San Diego, CA). Data analysis was generated from the Archer Analysis DEMO site.[16]

Statistical Methods

The variables measured in the study were investigated for association by contingency tables or the Fisher's exact test, as appropriate. A p value less than 0.05 was considered significant. Statistical analyses were performed using SPSS version 22 (SPSS Inc., Chicago, IL).

Results

A consecutive, retrospective series of 1031 resected lung adenocarcinomas was investigated. A set of 84 TMAs containing two large (2-mm) closely assembled cores taken in the areas with the greatest percentage of neoplastic cells for each of the 1031 cases was analyzed by FISH and IHC analysis by using the clone D5F3 coupled with the OptiView Amplification Kit on the BenchMark XT platform and the clone 5A4 on the BOND-MAX platform without an amplification system. All cores were found to be evaluable for IHC and FISH tests. IHC assessment on selected cores with a high percentage of tumor cells was found to give data corresponding exactly to those obtained on matching whole sections of 50 samples from this series of tumors (comprising 22 FISH/IHC analysis–positive cases and 28 negative cases). Samples were evaluated blindly by three pathologists (A.M., F.B., and A.D.L.) (data not shown).

FISH analysis gave positive results in 33 of the 1031 cases investigated (3.2%) (see Supplementary Table 1, Supplementary Digital Content 2). On average, 83 nuclei (range 56–233) were examined. The mean percentage of rearrangements was 42% (range 22%–90%) (30% single red, 60% split, and 10% both signals).

IHC analysis with the VENTANA ALK (D5F3) CDx Assay was positive in 32 cases (3.1%). Three of the 33 FISH-positive cases (9.1%) were found to be negative by IHC assessment with the Ventana system. Two of the 32 cases (6.2%) that were positive with the VENTANA ALK (D5F3) CDx Assay gave negative results when tested by FISH analysis. No equivocal cases were found by the Ventana IHC assay when the criteria described in the product data sheet were strictly followed, so the results obtained could be directly used to define the ALK status of patients according to the AIFA criteria. When the FISH method was used as the accepted standard, the VENTANA ALK (D5F3) CDx Assay had a sensitivity of 90.9% ± 2.6%, a specificity of 99.8% ± 0.6%, a positive predictive value (PPV) of 93.8% ± 2.1%, and a negative predictive value (NPV) of 99.7% ± 0.6% (Table 1).

IHC analysis with the clone 5A4 gave positive results (1+, 2+, or 3+) for ALK expression in 47 of the cases examined (4.6%) (Fig. 1). In three of the 33 FISH-positive cases (9.1%), the clone 5A4 provided negative results. Definitely positive results (3+), to be directly used for the selection of patients eligible for treatment, according to AIFA prescriptions were obtained in 16 cases (34%), and equivocal results of 1+ or 2+ were present in 17 cases (36.2%) and 14 cases (29.8%), respectively. When the FISH method was taken as the accepted standard, the clone 5A4–based IHC test showed a sensitivity of 90.9% ± 2.6% and a specificity

Table 1. Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive Value of Immunohistochemical Analyses Performed with D5F3 Clones versus FISH Data as Reference Standard

	FISH+	FISH−	Total	
D5F3+	30	2	32	PPV = 93.8% ± 2.1%
D5F3−	3	996	999	NPV = 99.7% ± 0.6%
Total	33	998	1031	

Note: Sensitivity = 90.9% ± 2.6%; specificity = 99.8% ± 0.6%.
FISH, fluorescence in situ hybridization; PPV, positive predictive value; NPV, negative predictive value.

of 98.3% ± 1.3% with a PPV and NPV of 63.8% ± 4.2% and 99.7% ± 0.6%, respectively (Table 2). When FISH was used as the accepted standard and only the definitely positive results (3+) were considered, the clone 5A4–based IHC test had a sensitivity of only 48.5%. The percentage of "equivocal" cases to be submitted for FISH confirmation was 0% with the Ventana assay and 3% (66% of positive cases) with the clone 5A4–based IHC assay. This latter test, in combination with FISH analysis, provided results similar to those obtained using the Ventana IHC assay alone, with two exceptions (cases 34 and 35, which were positive with both IHC approaches and negative by FISH).

Five FISH/IHC analysis (both clones)–discordant cases emerged in this study (Table 3). These particular cases were investigated by NGS using the Archer Universal RNA Reagent Kit v2. The NGS assay confirmed the FISH data, clearly indicating that there are cases with ALK fusion detected at a genomic level that are negative for ALK expression and vice versa. For three of the five discordant cases, follow-up data after treatment with crizotinib were obtained, and a partial response was observed in two patients (see Table 3). These results pushed us to review the literature for FISH/IHC-discordant cases and the responses obtained in these particular patients. Table 4 includes a number of recent studies reporting (1) FISH-positive/IHC analysis–negative cases (IHC analysis performed by any test) and (2) FISH-negative/IHC analysis–positive cases (IHC analysis performed with the VENTANA ALK [D5F3] CDx Assay). Overall, 123 of 10,388 tumors (1.2%) showed discordant results when investigated by FISH and IHC analysis. For 35 cases, follow-up data after treatment with anti-ALK therapy were available. The response

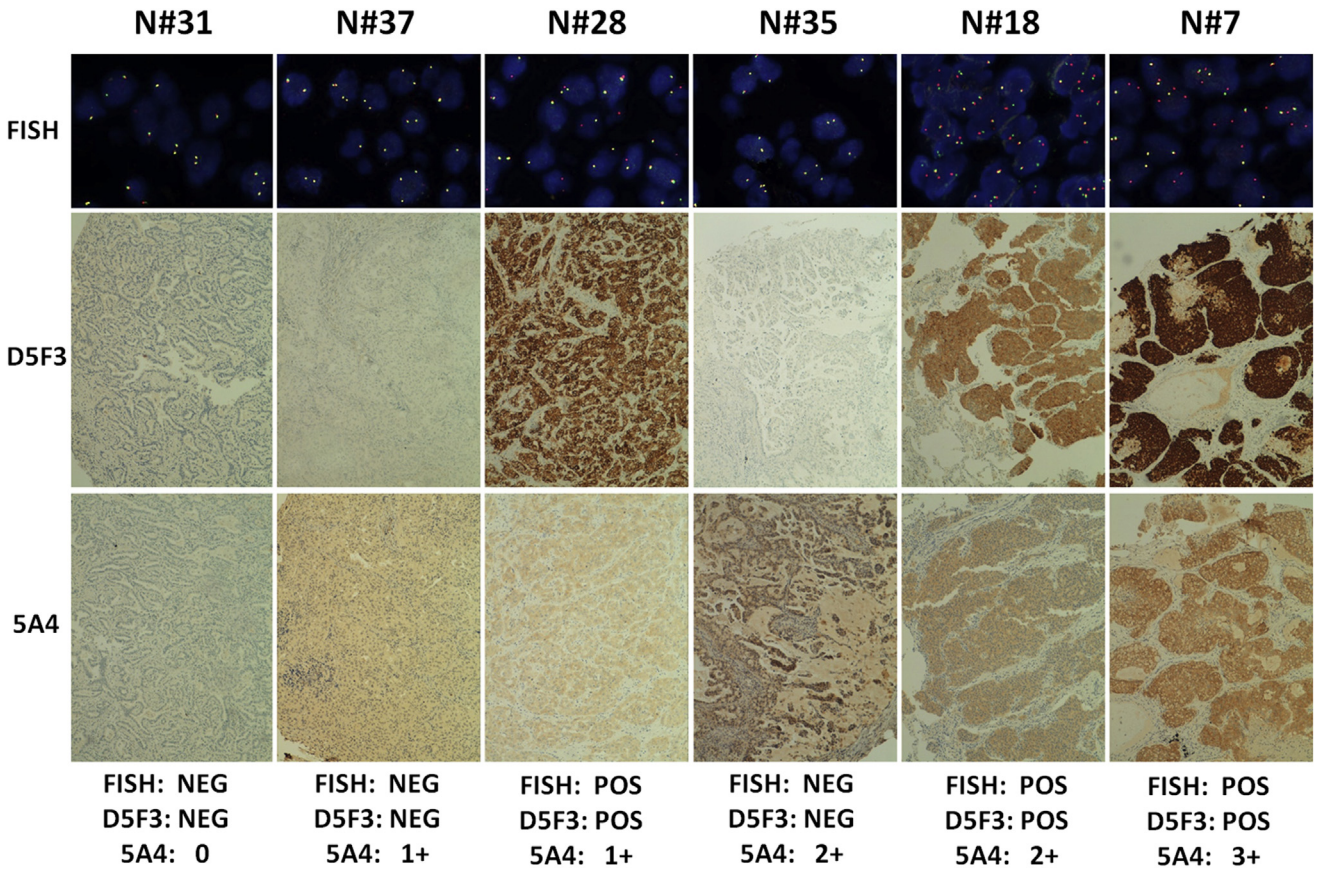

Figure 1. Comparison of fluorescence in situ hybridization (FISH), immunohistochemical assay with the Ventana anti-ALK(D5F3)CDx assay (D5F3), and a standard immunohistochemical assay with the clone 5A4 (Novocastra) (5A4) with their scores on selected cases. NEG, negative; POS, positive.

Table 2. Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive Value of Immunohistochemical Analyses Performed with 5A4 Clones versus FISH Data as Reference Standard

	FISH+	FISH−	Total	
5A4+	30	17	47	PPV = 63.8% ± 4.2%
5A4−	3	981	984	NPV = 99.7% ± 0.6%
Total	33	998	1031	

Note: Sensitivity = 90.9% ± 2.6%; specificity = 98.3% ± 1.3%.
FISH, fluorescence in situ hybridization; PPV, positive predictive value; NPV, negative predictive value.

rates in FISH-negative/IHC analysis–positive and FISH-positive/IHC analysis–negative patients were 100% and 46%, respectively, $p = 0.03$ (Fisher's exact test).

On the bases of the recent regulatory changes, the data that have emerged from the literature, and the results of the present study, a new algorithm for ALK assessment in NSCLC has been devised (Fig. 2).

Discussion

In normal tissues, the ALK protein is undetectable by IHC testing, except in nerve tissue.[17] In NSCLC, the *ALK* gene rearrangement induces a remarkable increase in protein expression that can be detected by IHC analysis. This method has been clinically proved to be an effective diagnostic tool to select patients for ALK-targeted therapies. Among the different anti-ALK antibodies to assess *ALK* gene status by IHC analysis, the clone D5F3 and the clone 5A4 are currently the most widely used.[18]

In this study we compared the VENTANA ALK (D5F3) CDx Assay on the BenchMark XT immunostainer with an IHC assay based on the use of the clone 5A4 on the BOND-MAX immunostainer.

The first system includes an amplification step (OptiView) that by increasing the signal difference between the specific immunoreaction and the background signal, can reduce or eliminate equivocal results, allowing for identification of patients as positive or negative and therefore immediately eligible or not eligible for anti-ALK treatment. This system has recently been approved as a companion diagnostic by the FDA. Currently, the second clone is not in the market in association with an amplification system. Therefore, it is widely used in standard IHC assays with a diagnostic algorithm that was adopted in most of the previous studies and provides a four-level score, with two of the levels (3+ and 0) clearly indicating a positive or negative result, respectively, and the other two (1+ and 2+) indicating a greater or lesser level of uncertainty. Uncertain cases must be subjected to FISH analysis for a definitive diagnosis and clinical decision. These different systems and algorithms have been recently recognized by the AIFA, which included them in the eligibility data sheet for crizotinib treatment (see Supplementary Fig. 1, Supplementary Digital Content 1).[14]

Table 3. Evaluation of FISH/IHC Analysis-Discordant Cases with Next-Generation Sequencing by the Archer Assay and Comparison with Response to Crizotinib

Case	IHC	FISH	NGS	Response to Crizotinib
31	Negative	Positive	*EML4-ALK* fusion	NA
32	Negative	Positive	*EML4-ALK* fusion	Yes
33	Negative	Positive	*EML4-ALK* fusion	No
34	Positive	Negative	Negative	Yes
35	Positive	Negative	Negative	NA

FISH, fluorescence in situ hybridization; IHC, immunohistochemical analysis; NGS, next-generation sequencing; *EML4*, echinoderm microtubule associated protein like 4 gene; *ALK*, anaplastic lymphoma receptor tyrosine kinase gene; NA, not applicable.

At the moment, however, there is a little knowledge regarding comparison of these two different approaches in diagnostics. Therefore, we decided to compare the two systems in a large series of consecutive lung adenocarcinomas by constructing TMAs. These were constructed with large cores (two per patient) to be representative of the lesion. The TMAs were first assessed by FISH, then subjected to the two IHC assays on adjacent sections.

The results obtained indicate that the two IHC tests had the same level of sensitivity, whereas the specificity was higher with the Ventana system (99.8% versus 98.3%). In the same way, the NPV was similar for the two tests, whereas a significant difference was observed for the PPV (93.7% with the Ventana system versus 63.8% with the Novocastra system). This latter difference was mainly due to the presence of a large amount (82.3%) of false positives among cases with a score of 1+. The large consecutive series examined in this study, unlike the limited numbers of cases selected by FISH that were examined in several other studies, highlighted this "Achilles heel" of the standard IHC test with the antibody 5A4 and showed the possibility of overcoming the problem with the Ventana system, which completely ruled out these false-positive results. Our data are consistent with those reported in a large multicenter series previously examined by using the clone 5A4 in a Lungscape European project.[11] Among the cases scored 2+, we observed a lower level of false positives: in 3 of 14 cases (21.4%).

The application of the 5A4 algorithm suggested by AIFA (Supplementary Fig. 1, Supplementary Digital Content 1) in our series of cases made it possible to obtain a specificity and PPV of 100% by using the FISH assay as a reference and performing 31 FISH tests (17 cases scored 1+ and 14 cases scored 2+) in a cohort of 1031 patients (thus testing 3% of the cohort). This can be considered efficient and not very laborious. However, two cases (34 and 35) found to be positive by the Ventana test

Table 4. FISH/IHC-Discordant Results Reported in the Literature and in This Study: Comparison with Response to Anti-ALK Therapy

			Discordant Cases (with Follow-Up)		Response to Anti-ALK Therapy	
Studies	No. Patients	Methods	FISH−/IHC+	FISH+/IHC−	FISH−/IHC+	FISH+/IHC−
Alì et al.[23]	523	BA Vysis; D5F3 Ventana		2 (2)		0
Cabillic et al.[10]	2714	BA Vysis/DAKO; D5F3 Ventana/ 5A4 Bond Leica	19 (2)	36 (4)	2	3
Gao et al.[24]	1614	BA Vysis; D5F3 Ventana		3 (2)		0
Ilie et al.[21]	176	BA Vysis; D5F3 Ventana	2	5 (3)		3
Mitsudomi et al.[20]	2337	BA Vysis; 5A4 amplification		21 (15)		6
Pekar-Zlotin[25]	51	BA Vysis; D5F3 Ventana	5 (2)		2	
Peled et al.[26]	1	BA Vysis; D5F3 Ventana	1 (1)		1	
Ren et al.[27]	1	BA Vysis; D5F3 Ventana	1 (1)		1	
Savic et al.[7]	375	BA Vysis; D5F3 Ventana/5A4		4		
Sholl et al.[4]	186	BA Vysis; 5A4	1	3		
Von Laffert et al.[28]	753	BA Vysis; D5F3 Ventana	1	5		
Wang et al.[29]	430	BA Vysis; D5F3 Ventana	7			
Ying et al.[30]	196	BA Vysis; D5F3 Ventana	2			
Marchetti et al., (present study)	1031	BA Vysis; D5F3 Ventana/5A4	2 (1)	3 (2)	1	1
Total	10388	—	41 (7)	82 (28)	7 (100%)[a]	13 (46%)[a]

[a]Percentage values in parentheses indicate response rates for IHC-positive (Ventana test)/FISH-negative or IHC-negative (all tests)/FISH-positive patients.
FISH, fluorescence in situ hybridization; IHC, immunohistochemical analysis; ALK, anaplastic lymphoma receptor tyrosine kinase; BA, break-apart probe.

and scored 2+ with the Novocastra assay were negative by FISH. In these two cases, the FISH reaction was technically faultless and the number of neoplastic cells on the two cores was much higher than required for the diagnostic definition. Moreover, the test was repeated on matching donor blocks, leading to comparable results. In addition, an NGS analysis by the Archer test revealed no evidence of ALK fusions. Recent regulations from AIFA, reported in the eligibility data sheet for crizotinib, could be problematic in rare cases such as those that we reported in our series (34 and 35), which were equivocal with the Novocastra system, positive by the Ventana assay, and negative by FISH and NGS. In fact, one can witness the paradox of a case being found positive by the Ventana assay and negative by the Novocastra test (2+) after the confirmatory FISH analysis. In other words, in such cases, on the basis of the AIFA criteria, the patients would paradoxically have been considered eligible for treatment by using the Ventana ALK (D5F3) CDx Assay and not eligible by the Novocastra assay after the outcome of the FISH test. So, in these cases, the FISH confirmation test could be detrimental for the selection of patients to be treated with anti-ALK drugs. Although we are aware that we are speaking about rare situations, this point needs further consideration.

To address the criticisms related to a standard IHC assay with the Novocastra 5A4 clone, a dichotomic algorithm (+/−) that defines as negative the cases 0 and 1+ and positive the cases 2+ and 3+ has recently been suggested.[19] In our series, however, this algorithm would have excluded from treatment three cases (28, 29, and 30) that were positive by the Ventana ALK (D5F3) CDx Assay, confirmed by FISH, and scored 1+ by the clone 5A4, and it would have included one case (36) that was found to be negative by the Ventana System and by FISH but scored 2+ using the clone 5A4. Overall, four cases would have been misdiagnosed (three false-negative and one false-positive results). To overcome

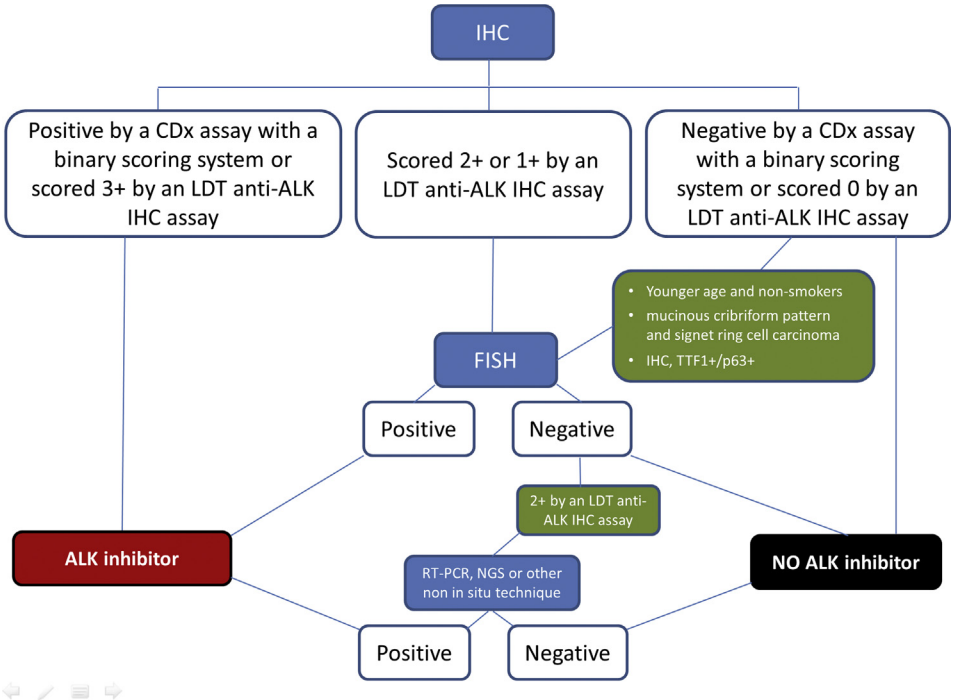

Figure 2. Comprehensive algorithm for the selection of patients with non-small cell lung cancer to be treated with anti-anaplastic lymphoma receptor tyrosine kinase (ALK) therapy. A first step by immunohistochemical (IHC) staining with a companion diagnostic (CDx) assay based on a binary scoring system or IHC with a laboratory-developed test (LDT) based on a four-level score is suggested. Fluorescence in situ hybridization (FISH) analysis is reserved for cases scored 2+ or 1+ by an LDT assay and for IHC analysis-negative cases with clinicopathological parameters more frequently reported in ALK-positive patients (*large green box*). Further confirmation by real-time polymerase chain reaction (RT-PCR), next-generation sequencing (NGS), or other non-in situ techniques is recommended in cases scored 2+ by an LDT assay and negative by FISH (*small green box*). TTF1, thyroid transcription factor 1.

these drawbacks, we have devised a new algorithm (described in Fig. 2) for the selection of patients to be treated with ALK inhibitors. This new algorithm comprises two green boxes that are related to a very limited number of potentially positive patients who need to be considered with a comprehensive technical approach so as not to leave behind patients for treatment.

Our results confirm the superiority of the Ventana ALK (D5F3) CDx Assay and the difficulty in managing a nonstandardized IHC assay without amplification in the absence of a confirmatory FISH analysis. An H-score algorithm has been reported that could help in managing data.[11] Another possibility is the use of an amplification system and a standardized protocol also with other antibodies (5A4 [Novocastra] ALK [Dako]) to obtain a dichotomic algorithm that is better suited to daily clinical practice. A recent study has shown that in the presence of an amplification protocol the two clones (D3F3 and 5A4) can perform equally well.[7]

In addition, in our large series of lung adenocarcinomas, we had three cases that were scored negative by IHC assessment with both antibodies and positive by FISH and NGS. Overall, we observed five discordant cases, three IHC analysis–negative/FISH-positive/NGS-positive cases and two IHC analysis–positive/FISH-negative/NGS-negative cases (see Table 3). These discordant data could be ascribed to different technical problems affecting FISH or IHC assays or to biological issues, including the presence of ALK-activating mutations/amplifications or posttranslational changes.[10,20–22] Three patients with discordant results were treated with crizotinib, and two of them responded (one IHC analysis–positive/FISH-negative/NGS-negative patient and one of two IHC analysis–negative/FISH-positive/NGS-positive patients). A revision of the literature on FISH/IHC analysis–discordant cases, including our series (reported in Table 4) indicates a response rate of 100% and 46% for IHC analysis–positive (the Ventana test)/FISH-negative and IHC analysis–negative (all tests)/FISH-positive patients, respectively.[4,7,10,20,21,23–30] These data indicate the important role of IHC analysis in the selection of patients for anti-ALK treatment.[31] To ensure that no ALK-positive patients are left behind, however, it would be desirable to carry out IHC and FISH analysis in all cases. Given the costs of extending a double test to all patients and the lack of widespread

diffusion of FISH technology, different algorithms,[18,32] including the one reported in this study, could be used for an accurate selection of patients to be treated with ALK inhibitors.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at http://dx.doi.org/10.1016/j.jtho.2015.12.111.

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