

A Comparison of Immunohistochemical Assays and FISH in Detecting the *ALK* Translocation in Diagnostic Histological and Cytological Lung Tumor Material

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Introduction: Detection of the *ALK* rearrangement in a solid tumor gives these patients the option of crizotinib as an oral form of anti-cancer treatment. The current test of choice is fluorescence in situ hybridization (FISH), but various cheaper and more convenient immunohistochemical (IHC) assays have been proposed as alternatives.

Methods: Fifteen FISH-positive cases from patients, seven with data on crizotinib therapy and clinical response, were evaluated for the presence of *ALK* protein using three different commercially available antibodies: D5F3, using the proprietary automated system (Ventana), *ALK1* (Dako), and 5A4 (Abcam). A further 14 FISH-negative and three uncertain (<15% rearrangement detected) cases were also retrieved. Of the total 32 specimens, 17 were excisions and 15 were computed tomography-guided biopsies or cytological specimens. All three antibodies were applied to all cases. Antibodies were semiquantitatively scored on intensity, and the proportion of malignant cells stained was documented. Cutoffs were set by receiver operating curve analysis for positivity to optimize correct classification.

Results: All three IHC assays were 100% specific but sensitivity did vary: D5F3 86%, *ALK* 79%, 5A4 71%. Intensity was the most discriminating measure overall, with a combination of proportion and intensity not improving the test. No FISH-negative IHC-positive cases were seen. Two FISH-positive cases were negative with all three IHC assays. One of these had been treated with crizotinib and had failed to show clinical response. The other harbored a second driving mutation in the *EGFR* gene.

Conclusions: IHC with all three antibodies is especially highly specific (100%) although variably sensitive (71%-86%), specifically in cases with scanty material. D5F3 assay was most sensitive in these latter cases. Occasional cases are IHC-positive but FISH-negative, suggesting either inaccuracy of one assay or occasional tumors with *ALK* rearrangement that do not express high levels of *ALK* protein.

Key Words: Pulmonary adenocarcinoma, *ALK*, Immunohistochemistry, Fluorescence in situ hybridization, Crizotinib

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Rearrangements of the anaplastic lymphoma kinase (*ALK*) gene drive the malignant phenotype in 3% to 7% of primary lung adenocarcinomas.^{1–5} The resulting fusion protein, most often a fusion with echinoderm microtubule-associated protein-like 4 (*ELM4*), has a constitutively active tyrosine kinase domain. The small molecule drug crizotinib is a specific inhibitor of this kinase,⁶ and cases with the rearrangement respond to crizotinib treatment.⁷ Therefore, accurate, rapid, and inexpensive identification of tumors growing under the influence of translocated *ALK* is needed.

Currently the only test approved by the FDA is fluorescence in situ hybridization (FISH) using “break-apart” probes (Vysis, Abbott Molecular, Abbott Park, IL). This test is regarded as the “gold standard” for detection of re-arrangements and is recommended by CAP/International Association for the Study of Lung Cancer/AMP⁸ However, FISH is technically demanding, expensive, and many diagnostic laboratories lack either the expertise or the facilities to perform the test. Even in ideal circumstances, the results are often difficult to interpret, requiring the scrutiny of large numbers of individual cells by a highly experienced diagnostician. Furthermore, there are rare circumstances (such as small intrachromosomal inversion) in which the FISH test is negative but the tumor nevertheless expresses *EML4-ALK* fusion protein.^{5,9–11}

A cheaper and potentially more widely applicable method is immunohistochemistry (IHC); indeed, overexpression of *ALK* protein has been used in the diagnosis of anaplastic large-cell lymphoma for many years. Although

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early studies in lung cancer lacked sensitivity,^{4,5} more recent studies have shown greater specificity and sensitivity,^{8–11} and recent international guidelines (CAP/International Association for the Study of Lung Cancer/AMP) have recommended that, if clinically validated, IHC may be used as a screening test for FISH testing.⁸ However, there have been few comparative studies on the most appropriate antibody to use.

The aim of this study was therefore to compare three different immunohistochemical assays, two being routine methods using antibodies widely used in the diagnosis of lymphoma, with the third being a proprietary system, including signal amplification, that is currently being promoted as an alternative to FISH (Ventana). We also evaluated the relationships between ALK rearrangement as detected by FISH, IHC, and patient response to therapy.

MATERIALS AND METHODS

Clinical Samples

The diagnostic archives from the Royal Brompton and Harefield NHS Foundation Trust and Royal Marsden hospitals from 2007 onwards were reviewed to identify cases with a diagnosis of lung adenocarcinoma that tested positive for an ALK rearrangement (>15% positive cells), and a randomly selected complementary group of cases with a normal ALK locus for comparison. We had been testing all primary lung tumors regardless of stage as part of a feasibility study, which led to a large number of early stage cases being included. More recently, our current policy is only to test advanced cases of non-squamous non-small-cell carcinoma using IHC screening with confirmatory FISH as per recently published guidelines.⁸ The cases under study are summarized in Table 1.

TABLE 1. Summary Data of All Cases Included in the Study

Tumor Type	Specimen	Sex	Age	Stage	Smoking History?	ALK FISH	TKI	Clinical Response
aCa	Excision	F	61	pT1a(2)/T3N0pM1b	Not known	Pos	Yes	Yes
aCa	Biopsy	F	47	T4	Non-smoker	Pos	Yes	Yes
NSCLC fav aCa	Cytology	M	35	T4 (pN3)	Not known	Pos	Yes	Yes
aCa	Cytology	F	50	T4B	Non-smoker	Pos	Yes	Yes
aCa	Cytology	F	50	T2N0M1B	Non-smoker	Pos	Yes	No
aCa	Biopsy	F	38	T4B	Non-smoker	Pos	Yes	Mixed
aCa	Cytology	M	35	T4B	Non-smoker	Pos	Yes	Yes
Carcinosarcoma	Cytology	M	77	T4N2M0	Ex-smoker	Uncertain	No	
aCa	Biopsy	F	78	T4N0M0	Ex-smoker	Uncertain	No	
SCC	Biopsy	M	64	T4N3M1B	Ex-smoker	Uncertain	No	
NSCLC fav aCa	Cytology	F	73	T1apN3	Non-smoker	Pos	No	
aCa	Excision	F	66	pT2bN2	Not known	Pos	No	
aCa	Excision	M	63	pT1aN0	Smoker	Pos	No	
aCa	Excision	M	54	Recurrence (unknown)	Smoker	Pos	No	
aCa	Excision	M	45	M1b	Not known	Pos	No	
NSCLC fav aCa	Cytology	F	41	pN2	Not known	Pos	No	
aCa	Excision	F	64	pT1bN0	Non-smoker	Pos	No	
aCa	Cytology	F	28	TXN0M1A	Non-smoker	Pos	No	
SCLC	Biopsy	M	30	T4B	Ex-smoker	Neg		
aCa	Excision	M	74	pT1bN0	Not known	Neg		
NSCLC fav aCa	Biopsy	F	66	pT2apN0	Not known	Neg		
aCa	Excision	M	71	pT1a N0	Ex-smoker	Neg		
aCa	Excision	M	79	pT2a N0	Not known	Neg		
aCa	Excision	M	63	pT2aN0	Smoker	Neg		
aCa	Biopsy	M	61	pT1a N0	Not known	Neg		
aCa	Excision	F	54	pT1a NX	Non-smoker	Neg		
aCa	Excision	F	48	pT1b N0	Smoker	Neg		
aCa	Excision	F	66	pT2b N0	Non-smoker	Neg		
aCa	Excision	F	82	pT2b N0	Ex-smoker	Neg		
aCa	Excision	M	78	pT2a N2	Not known	Neg		
aCa	Excision	M	53	pT2a N2	Ex-smoker	Neg		
aCa	Excision	M	68	pT1a N0	Smoker	Neg		

aCa, adenocarcinoma; SCC, sodium citrate; NSCLC, non-small-cell lung cancer; TKI, tyrosine kinase inhibitor treatment (crizotinib); NSCLC fav aCa, NSCLC favoring adenocarcinoma on immunohistochemistry; FISH, fluorescence in situ hybridization.

Paraffin blocks from a total of 32 diagnostic cases were retrieved; 15 of these had tested positive for the ALK rearrangement by FISH, three were uncertain (with <15% of cells showing rearrangement), and the remaining 14 cases were negative. All but two blocks dated from 2011 or later. Seventeen cases were blocks from tumor excisions (six of these were FISH positive) and the remainder were cytological or core biopsy/endobronchial ultrasound samples.

Data on treatment with crizotinib and response were retrieved from patient records. Cases with at least partial response to treatment defined according to the Response Evaluation Criteria in Solid Tumors criteria¹² (i.e., at least 30% decrease in the sum of the longest diameters of target lesions) were designated as “responsive”.

The study was evaluated and classified as a service evaluation by the Imperial College Heads of Consortia and as such was exempt from Research Ethics Committee review.

Fluorescent In Situ Hybridization

Unstained 2 μm FFPE sections were put through deparaffinization and protease pretreatment steps before being denatured and hybridized overnight with the commercially available Vysis ALK dual color break apart probe (Abbott Molecular). Tissue sections then underwent SSC washes and were mounted in 4',6'-diamidino-2-phenylindole for nuclei counterstaining. Results were analyzed and interpreted in accordance with probe manufacturer's instructions.

Non-rearranged ALK showed as fused (yellow) signals. Rearranged ALK appeared as split 3' (red) and 5' (green) signals or an isolated 3' (red) signal. The recommended cutoff of 15% was used to interpret samples as positive or negative for ALK rearrangements in 200 nuclei.

Immunohistochemistry

An additional five sections were cut per case. Three were used for the immunohistochemical assays and the remaining two for negative controls.

Immunohistochemical assays were optimized using the monoclonal antibodies D5F3 (Ventana), ALK1 (Dako) and 5A4 (Abcam). The D5F3 assay was performed using the Ventana autostainer and a tyramide amplification step, as specified in the manufacturer's protocol. The other assays were performed using a Dako autostainer with conventional polymer-based diaminobenzidine staining (no tyramide amplification). Details of the antibodies and conditions employed are given in Table 2.

Scoring

Immunohistochemically stained sections were examined without knowledge of FISH status by two pathologists independently. Scores for proportion and intensity of immunohistochemical staining were assigned by consensus.

The predominant intensity of staining was recorded on a scale of 0–3 (0 = negative, 1 = weak, 2 = moderate, 3 = strong). As the Ventana stain was more intense, due at least partly to the signal amplification step, the visual cutoffs for intensity scoring with this antibody were different (e.g., a “moderate” degree of intensity seen with the Ventana stain would usually be interpreted as “strong” on a section stained with 5A4). The proportion of malignant cells staining positive was recorded as per “Allred” estrogen receptor scoring in breast cancer, on a scale of 0–5 (0 = 0%, 1 ≤ 1%, 2 = 1–10%, 3 = 11–33%, 4 = 34–66% and 5 ≥ 66%). A composite score (intensity + proportion) was also derived.

Statistical Analysis

Statistical analyses were performed using the STATA/IC package.

RESULTS

Fluorescence In Situ Hybridization

Slides were scored according to the manufacturer's recommendations. Representative FISH images are shown in Figure 1A. The 15 positive cases all showed greater than 15% cells with rearranged ALK genes. Three cases were classified as “indeterminate”; these were all scanty biopsy or cytological samples with 10% to 15% of positively rearranged FISH signals. Seventeen further cases were FISH negative.

Immunohistochemistry

No signal was observed in negative controls. The intensity of staining between the three antibodies varied (Fig. 1B). IHC was impossible to assess in three cases with very scanty material (two FISH negative and one FISH positive). The Ventana assay using the D5F3 antibody gave the most intense cytoplasmic signal, but this was accompanied by higher background staining, which was especially noticeable in macrophages. The ALK1 and 5A4 antibody assays produced weaker staining but with less background. Of the two, 5A4 had marginally more background staining, especially in macrophages.

The value of both intensity and proportion scores was assessed. Cutoffs for positivity were set using ROC analysis to optimize correct classification of ALK status, using FISH

TABLE 2. Immunohistochemical Assay Conditions Used

Antibody	Supplier	Species	Antigen Retrieval	Dilution	Primary Incubation	Tyramide Amplification?
D5F3	Ventana	Rabbit	92 minutes, 100°C, Cell conditioning 1	1:1	16 minutes, 37°C	Yes
ALK1	Dako	Mouse	60 minutes, 97°C, high pH TRS	1:20	30 minutes, ambient temp	No
5A4	Abcam/Novocastra	Mouse	60 minutes, 97°C, low pH TRS	1:50	30 minutes, ambient temp	No

The D5F3 assay was performed using the proprietary Ventana immunostaining system. ALK1 and 5A4 assays were performed using a Dako autostainer. TRS, target retrieval solution (Dako).

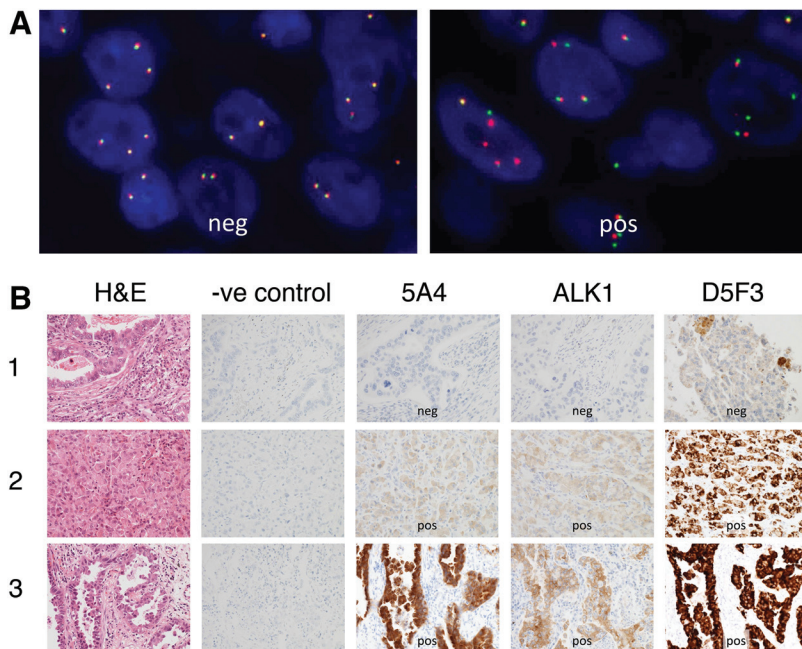


FIGURE 1. (A) Representative fluorescence in situ hybridization images showing normal fused signals (neg) and nuclei with multiple separated red signals (pos). (B) Three representative excision specimens of adenocarcinoma. Case 1 is negative with all three immunohistochemical assays; the D5F3 assay shows relatively high background, presumably because of the tyramide signal amplification (TSA) step. Cases 2 and 3 are positive with all three immunohistochemical assays, with clear cytoplasmic staining. The markedly reduced signal seen with the 5A4 and ALK1 assays in case 2 was typical and again probably related to the absence of tyramide amplification. Case 3 demonstrates that occasional cases show strong staining using the non-TSA assays.

results as the standard (Table 3). The intensity score alone was seen to outperform both proportion and the aggregate score. Therefore intensity scoring using the optimized cutoff was used in subsequent analyses.

Concordance between FISH and IHC is shown in Table 4. No cases with negative FISH results and positive IHC were identified (100% specificity of IHC in these data). Sensitivity was the same (83%) for all three assays in excision specimens. In small biopsies and cytological specimens, however, the D5F3 antibody was the most sensitive. The ALK1

and 5A4 assays failed to identify a further one or two FISH-positive cases, respectively. All three assays failed to stain the same two cases which contain rearranged ALK genes detectable by FISH.

Response to Therapy

Seven cases with ALK rearrangements detected by FISH went on to receive crizotinib therapy. All but one showed at least a partial response. This crizotinib-refractory case showed no detectable ALK expression by all three IHC assays.

TABLE 3. Optimised Cutoff Values for Immunohistochemical Tests

		Intensity		Proportion		Combined	
		Cutoff	% Correctly Classified	Cutoff	% Correctly Classified	Cutoff	% Correctly Classified
DF3	Ventana	>1	93	>4	80	>5	86
ALK1	Dako	>1	90	>2	90	>3	90
5A4	Abcam	>0	86	>0	86	>0	86

TABLE 4. Concordance of Immunohistochemistry (IHC) and Fluorescence In Situ Hybridization (FISH) Assays

IHC			FISH		Specificity (%)	Sensitivity (%)		
			Neg/Indeterminate	Pos		All cases	Excisions	Biopsies/Cytology
DF3	Ventana	Neg	15	2	100	86	83	88
		Pos	0	12				
ALK1	Dako	Neg	15	3	100	79	83	75
		Pos	0	11				
5A4	Abcam	Neg	15	4	100	71	83	63
		Pos	0	10				

DISCUSSION

We have compared three different antibody assays for the ALK kinase domain to the current standard FISH assay in a set of archival tumors, including 15 FISH-positive cases. We found all three assays to be specific (100%) and sensitive (up to 86%), especially when a signal amplification technique is employed. Furthermore, data on response to crizotinib therapy in seven treated cases showed all but one case responded. The case that failed to respond to therapy was negative by all three IHC assays.

Of the three antibody methods compared, the D5F3 antibody using a Ventana proprietary assay performed the best, especially in scanty samples, which is likely to be a consequence of the tyramide signal amplification step incorporated into the Ventana assay. It is possible that the other two antibodies would perform as well, if a suitable signal amplification step were introduced. However, an assay using the 5A4 without tyramide amplification has been successfully applied by To *et al* in a recent comparable assessment of IHC as a test for ALK rearrangement.⁵ In a set of 373 tumors that included 20 ALK rearrangements as detected by FISH, their IHC assay was 99% specific and 100% sensitive.

In contrast to this, we find 100% specificity and (at best) 86% sensitivity; that is to say, we identified rare FISH-positive IHC-negative cases, whereas To *et al* found occasional FISH-negative IHC-positive cases, which were proved to harbor EML4-ALK rearrangements by reverse transcription polymerase chain reaction (RT-PCR).⁵ It is unsurprising that we do not identify FISH-negative IHC-positive cases as we only examined 17 FISH-negative or indeterminate cases in comparison to the 356 examined by To *et al*.⁵ It is more notable that To *et al*.⁵ do not identify FISH-positive, IHC-negative cases. This might be explained by their use of tissue microarrays for FISH, which is even more technically demanding and hard to interpret than FISH using whole sections.

This possible shortcoming of tissue microarray methods might be apparent in two other recent studies using tissue microarrays for a comparison of FISH with ALK1, 5A4 and D5F3 antibodies.^{13,14} Selinger *et al*.¹³ describe 100% sensitivity for all three antibody assays. Conklin *et al*.¹⁴ also find 100% sensitivity and a maximum specificity of 88% (again using the 5A4 antibody). Again, it may be that in both these additional studies the approach used hampered the identification of FISH-positive, IHC-negative cases because of the difficulty of applying FISH to TMAs, especially when the primary test has been IHC and the FISH test is not “blind” to the IHC result.

Other recent studies compare various immunohistochemical assays and FISH for the detection of ALK rearrangements.^{15,16} As in this study, Sholl *et al*.¹⁵ identify occasional FISH-positive, IHC-negative cases. They explain two cases by identifying co-existent mutations in other driving oncogenes (presumably thereby relieving the tumor “addiction” to ALK) and one by insufficient tumor material for accurate IHC assessment. Savic *et al*.¹⁶ compared an immunocytochemical assay using the 5A4 antibody to FISH in cytological specimens and achieved a sensitivity of 93% and specificity of 96%, which is comparable with our findings in cytological and small biopsy cases (sensitivity 88%, specificity 100%).

In the current study, we detected two “false-negative” cases, which were positive by FISH and negative by all three IHC assays. This combination has two possible explanations. Firstly, they might represent tumors which are not expressing ALK protein at detectable levels, because of a false-positive FISH result, or an absence of addiction to rearranged ALK protein despite presence of recombinant ALK DNA. These tumors are unlikely to respond to crizotinib. Secondly, they might represent a failure of the IHC assay, because of poor preservation of antigen, insufficient material or another technical error. In this case, crizotinib therapy would still be likely to be effective.

The study includes seven cases with positive FISH results with data on response to crizotinib. Six showed at least a partial response to crizotinib therapy, as assessed by Response Evaluation Criteria in Solid Tumors criteria. A single case showed no response, and this was one of the two “false negatives”. Thus, in this one case, the IHC test would have correctly predicted response. This was a scanty cytological specimen in which FISH interpretation was difficult, and only 20% of 515 cells showed rearranged ALK signals (fusion plus split red/green probes and fusion plus isolated red signal). Therefore, it seems possible that this represents a technical failure of the FISH assay.

The other case, an excision specimen, showed 39% of 626 cells with rearranged signals. It was also shown to harbor a driving mutation in a second gene; PCR testing demonstrated the loss of exon 19 of endothelial growth factor receptor. This has been described in another study that characterized two such “false-negative” cases.¹⁵ Thus, these tumors may well escape “oncogene addiction” to the ALK kinase activity, which would be consistent with undetectable ALK protein expression. Again, IHC would be expected to be the best predictor of response to tyrosine kinase inhibitor therapy in such cases.

It is essential to identify and molecularly characterize other “false-negative” cases that have received crizotinib therapy. In addition, it seems likely that IHC should guide treatments in “false-positive” cases that express high levels of ALK from genetic lesions that are invisible to the current FISH assay.

Although we identified no “false positives”, *i.e.*, FISH-negative, IHC-positive cases, our sensitivity may be an overestimate (as judged by FISH) because of the small number of FISH-negative cases under study. Several studies have identified rare cases with rearrangements that are undetectable by FISH but detectable by IHC and confirmed by reverse-transcriptase PCR.^{5,9,10} Such cases would be expected to respond to crizotinib, and a recent study shows that at least one novel “FISH-undetectable” rearrangement does indeed drive the malignant phenotype.⁵

One limitation of this study is the small number of cases under study, although 15 FISH-positive cases is comparable to most other studies. The relatively small number of FISH-negative cases may have affected our ability to identify FISH-negative, IHC-positive cases. However, the study design does permit an assessment of the sensitivity of the IHC assay, which is the most important consideration for a possible screening

test. Our comparison of the immunohistochemical assays was not directly equivalent, as the D5F3 assay included a proprietary tyramide signal amplification step, whereas the ALK1 and 5A4 assays were conducted using our routine diagnostic detection system. However, our study design also has several strengths. In particular, the use of archival diagnostic paraffin blocks, and FISH testing conducted in the course of routine diagnosis make the results of the study directly relevant to clinical practice.

In summary, we find IHC to be a highly sensitive (86%) and specific (100%) test for ALK rearrangement in lung adenocarcinoma. We find a slight advantage of a proprietary amplified assay (D5F3 Ventana) over two other antibodies with conventional DAB staining (ALK1 Dako and 5A4 Abcam), but only in scanty samples. Intensity of staining was the most discriminating measure, and the proportion of cells staining did not contribute. We identified two cases that were positive for the ALK rearrangement by FISH but negative by all immunohistochemical assays and suggest that in discordant cases the IHC test result may be more predictive of treatment response than FISH. Further discordant cases need to be examined to help guide the treatment of these cases.

Immunohistochemical testing is clearly at least a useful adjunct to FISH, and we feel that it is reasonable in routine practice to use a sensitive IHC assay as a screening test. The danger of missing treatable cases using this method (i.e., FISH-positive, IHC-negative, crizotinib-sensitive tumors) appears very small, especially when specimens contain adequate material. In difficult cases, further investigations such as re-biopsy and repeated IHC/FISH may be helpful.

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