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Reflex ALK immunohistochemistry is feasible and highly specific for *ALK* gene rearrangements in lung cancer

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Summary

Fluorescence *in situ* hybridisation (FISH) is considered the gold standard for the detection of *ALK* gene rearrangements in lung adenocarcinoma. The presence of *ALK* gene rearrangement predicts response to specific targeted therapy, but these rearrangements are relatively rare and FISH studies are expensive, not widely available, potentially challenging to interpret and therefore difficult to undertake in all patients with non-small cell lung cancer. We developed and then deployed into the routine clinical setting a screening program for *ALK* gene rearrangement in all non-small cell lung cancer patients based on immunohistochemistry (IHC) with a mouse monoclonal antibody (clone 5A4). ALK IHC was strongly positive in 12 (4%) of 307 tumours from consecutive patients. Only 10 of these cancers were initially thought to be rearranged by diagnostic FISH studies. The two tumours which were IHC positive but initially interpreted as FISH negative underwent repeat FISH testing because of the discrepancy. Repeat FISH testing confirmed the presence of *ALK* gene rearrangement with the discrepancy being attributable to an atypical FISH pattern. Therefore, in our experienced hands, IHC for ALK performed on initial diagnosis of lung cancer is 100% specific for the presence of *ALK* gene rearrangement. When ALK IHC and FISH studies are discrepant, IHC may outperform FISH. Although our study was not intended to formally assess the sensitivity of ALK IHC, the 4% rate of gene rearrangements identified by this approach is consistent with the expected incidence in our population. We conclude that reflex ALK IHC followed by confirmatory FISH testing can be readily integrated into the routine clinical setting and represents a cost effective and practical approach to screening for these clinically significant gene rearrangements.

Key words: ALK gene rearrangement, ALK immunohistochemistry, crizotinib, *EML4-ALK* fusion, lung cancer.

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INTRODUCTION

The presence of activating gene rearrangements involving anaplastic lymphoma kinase (ALK) in lung cancer predicts response to targeted therapy with crizotinib and other ALK inhibitors.[1] Currently fluorescence *in situ* hybridisation (FISH) studies are considered the 'gold standard' for detecting these gene rearrangements and for recruiting patients for clinical trials.[2] However, FISH studies are expensive, labour intensive, not widely available and, due to the subtlety of intrachromosomal inversion events, can be difficult to interpret, particularly in small biopsies. Therefore, although reflex *ALK* FISH testing of all lung cancer patients for this actionable gene rearrangement would be desirable, it is difficult and expensive to deploy this strategy in the routine clinical setting. This has prevented widespread uptake of reflex *ALK* testing in diagnostic surgical pathology laboratories.

Recently, several retrospective studies have suggested that immunohistochemistry (IHC) for ALK can be used as a screening test for *ALK* gene rearrangements in lung cancer.[3–6] We developed and tested an algorithm for universal testing of all lung cancers for *ALK* gene rearrangements based on reflex immunohistochemistry followed by confirmatory FISH studies in IHC positive cases. We then deployed this testing prospectively in the routine clinical setting for all lung cancer patients encountered in the department of Anatomical Pathology at the Royal North Shore Hospital, Sydney, Australia.

We report our experience with reflex ALK IHC testing of 383 eligible specimens from 307 consecutive lung cancer patients encountered from 1 January 2012 to 30 September 2013. We demonstrate that reflex ALK IHC testing is both cost efficient and readily deployable into the routine clinical setting and suggest that reflex ALK IHC at time of diagnosis for all non-squamous non-small cell lung cancers (NSCLC) followed by confirmatory FISH testing in positive cases may represent a new standard of care in lung cancer diagnosis.

MATERIALS AND METHODS

We first sought to validate immunohistochemistry for ALK in a retrospective cohort of NSCLCs undergoing surgery with curative intent. The demographic, pathological and outcome data from this cohort has been previously reported.[7] Briefly, it comprised all patients identified from the pathology database of the Royal North Shore Hospital, Sydney, Australia, who underwent surgical resection with curative intent for NSCLC between 2000 and 2010. The original pathology slides and blocks were retrieved and reviewed independently by two

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pathologists to reclassify the tumours according to the International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS) and European Respiratory Society (ERS) system[8] and to restage according to the 7th edition 2009 Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) TNM staging system.[9] Areas containing tumour were identified and a tissue microarray (TMA) constructed including two 1 mm cores from all tumours. Tumours from this TMA cohort which showed positive IHC staining for ALK then underwent confirmatory IHC and FISH testing on whole sections. As this arm of the study was only intended to assess the specificity rather than sensitivity of ALK IHC, tumours that did not show positive staining for ALK did not undergo FISH testing. This study was approved by the Northern Sydney and Central Coast Human Research and Ethics Committee.

Following validation that ALK IHC was highly specific for the presence of *ALK* gene rearrangements in this retrospective cohort, reflex ALK IHC was then deployed prospectively in the routine diagnostic laboratory from 1 January 2012. Our algorithm for testing is presented in Fig. 1. Briefly, ALK IHC was performed in all patients with non-squamous, non-small cell lung carcinoma in which sufficient material was available including fine needle aspiration biopsies with cell block preparations, transbronchial biopsies, percutaneous biopsies and resection specimens. All ALK IHC positive cases underwent FISH testing. ALK IHC negative cases did not undergo FISH testing unless there was a high clinical suspicion of *ALK* gene rearrangement identified by the treating oncologist; for example, lung adenocarcinoma wild type for *EGFR* and occurring in non-smokers. All cases which underwent ALK IHC from 1 January 2012 to 30 September 2013 were audited as part of this study.

ALK immunohistochemistry

Immunohistochemistry was performed on formalin fixed, paraffin embedded tissue using previously described methods.[10] Briefly, we used a mouse monoclonal antibody to ALK at high concentration (dilution 1:10, clone 5A4; Novocastra, Leica Biosystems, United Kingdom). All slides were processed with an automated staining system, the Leica BondIII autostainer (Leica Biosystems) used according to the manufacturer's protocol and with the manufacturer's retrieval solutions. Heat induced epitope retrieval was performed for 30 min in the manufacturer's alkaline retrieval solution ER2 (VBS part no: AR9640). Staining was interpreted as positive if there was strong cytoplasmic staining in tumour cells. Staining in any percentage of tumour cells was considered a positive result (however, it was noted that in positive cases all or nearly all tumour cells showed ALK expression).

Fluorescence *in situ* hybridisation (FISH)

FISH was performed at a separate institution on formalin fixed, paraffin embedded (FFPE) tumour tissue using the Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe (Abbott Molecular, USA) performed and interpreted according to the manufacturer's instructions. This probe set includes two DNA probes labelled with Spectrum Orange and Spectrum Green. Each slide was scored by an observer who was not blinded to the IHC result based on 50 representative nuclei which could be readily identified. A case was regarded as positive for *ALK* gene rearrangement when >15% of tumour cells showed ≥2 signal diameters between the red and green signals (red 3', green 5') or if there was an isolated red signal. When ALK IHC and FISH studies were discordant (that is ALK IHC was positive but FISH studies were negative for gene rearrangement), then repeat FISH testing was performed at a third institution and with a second probe (Zytolight SPEC ALK/EML4 TriCheck Probe; ZytoVision, Germany).

RESULTS

From the retrospective cohort, 256 cases of non-small cell lung cancer which underwent surgery with curative intent between 2000 and 2010 were available for assessment in the TMA. Of these, 152 (59%) were classified as adenocarcinoma using the IASLC/ATS/ERS system.[8]

Four cases demonstrated positive staining for ALK, all of which were classified as adenocarcinoma. All four of these cases also demonstrated diffuse strong staining for ALK when IHC was performed on whole sections and were confirmed to be rearranged by FISH performed on whole sections (Fig. 2). That is, the specificity for ALK staining in this cohort was 100%. Although this study was not designed to assess the sensitivity of ALK staining for *ALK* gene rearrangement, as not all cases underwent FISH, the minimum rate of gene rearrangement in this surgically treated cohort was 1.5% of all histologies and 2.6% of all lung adenocarcinomas which is within the range reported in similar cohorts of patients undergoing surgery with curative intent.[11,12]

Having determined that ALK IHC was highly specific for *ALK* gene rearrangement, we then undertook prospective IHC testing of all non-squamous NSCLC encountered in our department as part of the routine diagnostic work up. Between 1 January 2012 and 30 September 2013 a total of 383 specimens from 307 patients with non-squamous NSCLC were encountered. The patient and biopsy details are summarised in Table 1. Briefly, of the 383 specimens, 51% ($n = 196$) were fine needle aspirations, 29% ($n = 110$) were core biopsies and 20% ($n = 77$) were excision specimens. Of these 307 patients, 345 specimens from 293 patients (95.4% of all patients encountered) underwent ALK IHC testing. Of the 345 specimens which underwent ALK IHC, 52% ($n = 178$) were cell block preparations from fine needle aspiration biopsies, 29% ($n = 102$) were core biopsies and 19% ($n = 65$) were excision specimens. Of the 14 patients that did not undergo testing on at least one sample, five had insufficient material available. The remaining nine patients did not have ALK IHC testing due to pathologist preference and/or lack of adherence to the protocol.

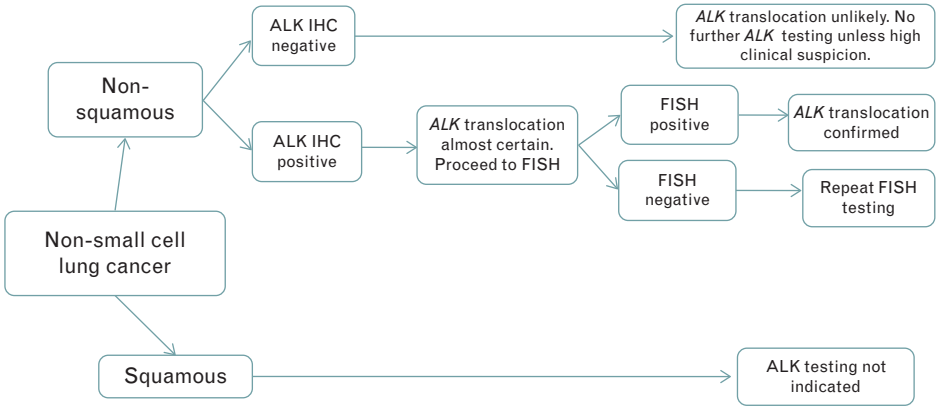

Fig. 1 Algorithm for screening for *ALK* rearrangements in non-small cell lung cancer (NSCLC). All patients with non-squamous NSCLC underwent screening IHC for ALK. Confirmatory FISH studies were performed on all IHC positive cases. If ALK IHC was positive but FISH was negative, FISH studies were repeated. ALK IHC negative cases did not undergo FISH studies unless there was a high clinical suspicion of *ALK* gene rearrangements.

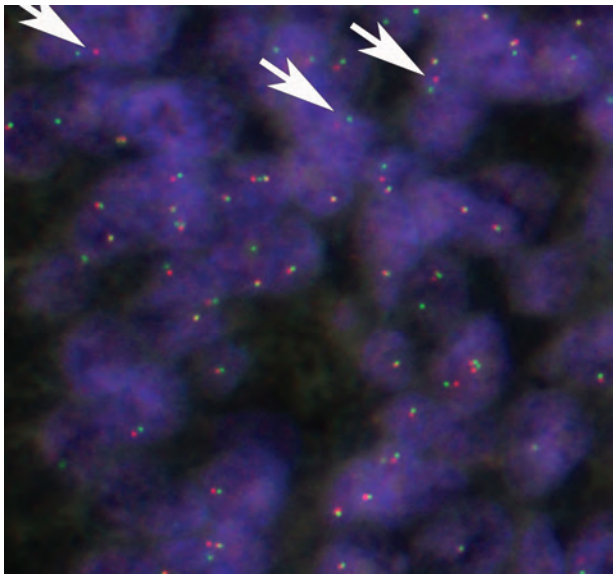

Fig. 2 Dual colour break apart FISH assay in an *ALK* rearranged lung adenocarcinoma. Split signals indicated by red and green signals which are separated by greater than two signal diameters (arrows) are readily identified.

ALK IHC was strongly positive in 12 patients (4% of all patients tested). In all cases there was diffuse strong cytoplasmic staining in virtually all malignant cells (Fig. 3). The details of the positive cases are presented in Table 2. Briefly, six of the positively staining cases were from fine needle aspiration biopsies, five from small biopsies (core biopsies or transbronchial biopsies) and one from an excision specimen. The one case which was positive in the excision specimen did not undergo preoperative fine needle aspiration in our department. Only one of 11 (9%) of the other patients who were ALK IHC positive on biopsy specimens subsequently underwent surgery with curative intent, with the other 10 (83%) being metastatic or otherwise inoperable at presentation. When ALK IHC was performed on more than one sample from the same tumour it showed concordant results.

Table 1 Demographic and biopsy details of 307 consecutive non-squamous NSCLC patients

	Total
Patients, *n* (%)	307 (100%)
Age, years (all patients)	
Range	21–94
Median	68
Age, years (*ALK* rearranged)	
Range	29–85
Median	58
Sex, *n* (%)	
Female	141 (46.0%)
Male	166 (54.0%)
Procedure, *n* (%)	383 (100%)
FNA	196 (51.2%)
Core biopsy	110 (28.7%)
Excision	77 (20.1%)
ALK testing	
ALK IHC	293 (95.4%)
Positive, *n* (%)	12 (3.9%)
Negative, *n* (%)	281 (91.5%)
ALK FISH	12 (3.9%)
Positive, *n* (%)	12 (3.9%)
Negative, *n* (%)	0 (0%)

FISH studies were performed on the specimens from the 12 ALK IHC positive cases. Ten of these patients were confirmed to be rearranged on initial FISH studies. Two ALK IHC positive cases were reported as FISH negative. Because of the discrepancy between ALK IHC and FISH both of these cases underwent repeat FISH testing with a second probe and at a different institution. On repeat testing both cases were thought to be FISH positive, although both showed variant FISH patterns. That is, after repeat FISH testing all 12 (100%) of ALK IHC positive cases were interpreted as having *ALK* gene rearrangement and ALK IHC was positive in two cases with atypical FISH patterns which were initially reported as FISH negative but confirmed to be rearranged upon review.

In addition to the 12 ALK IHC positive cases, three cases which were IHC negative also underwent FISH testing at the request of the treating oncologist due to a high clinical suspicion of gene rearrangement; that is, the occurrence of lung adenocarcinoma in young, never or light smokers, wild type for *EGFR* mutation. FISH testing on these three patients was negative. One further case did show weak focal non-specific staining in a fine needle aspiration specimen (mostly in macrophages rather than neoplastic cells) and was favoured as being ALK IHC negative by the reporting pathologist. However, because of the clinical significance of a potential gene rearrangement and some diagnostic uncertainty this also underwent FISH testing and was also negative.

DISCUSSION

Currently crizotinib therapy is indicated only in *ALK* rearranged NSCLC patients who are either inoperable or have residual or recurrent disease after surgery. However, the majority of patients with lung cancer will die of disease and at the time of initial tissue biopsy many patients have not been fully staged or assessed for surgery. Furthermore, the potential role of ALK inhibitors in the adjuvant setting for operable patients remains to be explored. Therefore the guidelines for molecular testing in lung cancer recently published by the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology, which currently recommend performing *ALK* FISH testing at the time of diagnosis for patients presenting with advanced-stage NSCLC who are suitable for medical therapy or at a time of recurrence also 'encourages' *ALK* FISH for early stage disease.[13] That is, reflex screening of all lung cancer patients for *ALK* gene rearrangements would be valuable and is encouraged but is only possible if it can be delivered in a cost effective and timely manner.

In this study we demonstrate that screening IHC is feasible to deploy in the routine clinical setting as a reflex test for all patients with non-squamous NSCLC. In our practice we recommend that the need for advanced molecular testing for both *EGFR* mutation and *ALK* gene rearrangement be considered when choosing biopsy approaches for patients presenting with presumed lung cancer. Therefore, the great majority of patients in our unit with lung cancer have tissue available in cell block preparations or conventional paraffin blocks. Furthermore, most non-squamous NSCLC already undergo immunohistochemistry to either confirm glandular differentiation in equivocal or small biopsy specimens as recommended by the IASLC/ATS/ERS classification system[8] or to confirm primary pulmonary origin in cases with definite adenocarcinoma differentiation. Therefore, the only significant additional cost

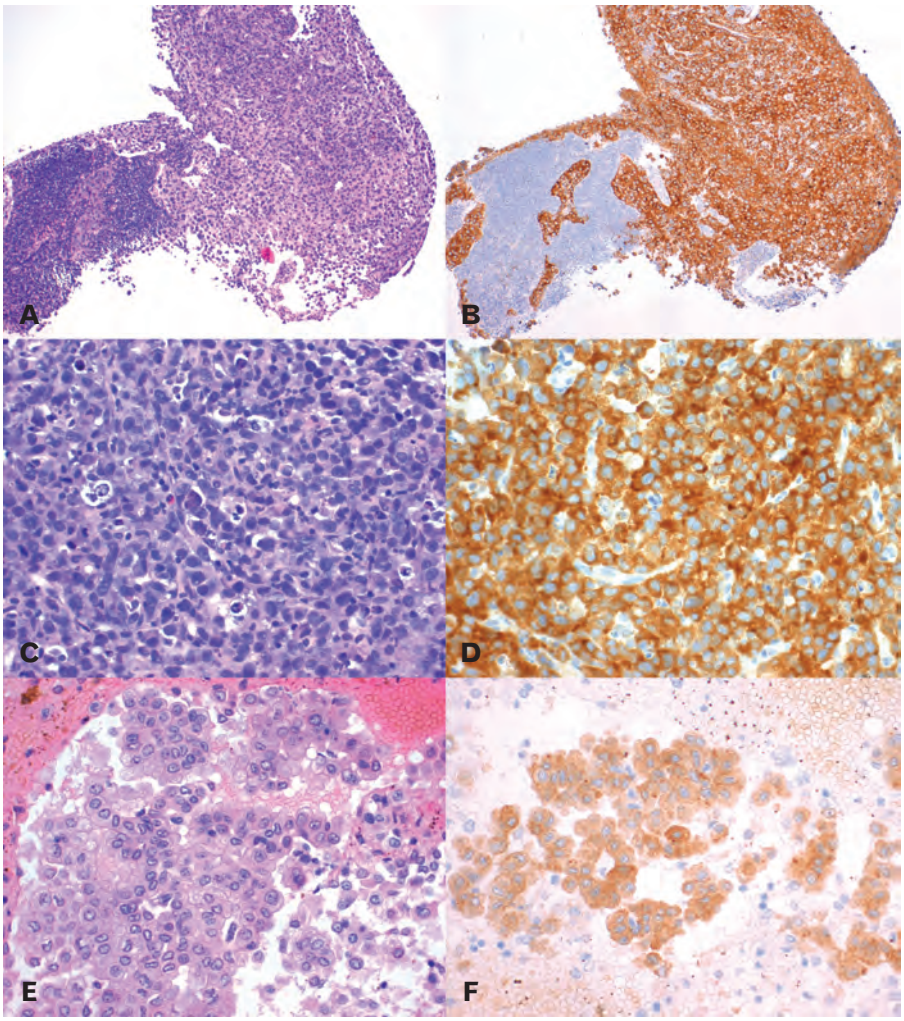

Fig. 3 (A–D) Serial sections stained with H&E and ALK IHC from a core biopsy and (E, F) cell block preparation from two patients. There is diffuse intense cytoplasmic staining for ALK in essentially all malignant cells. In both of these cases the tumours demonstrate a solid adenocarcinoma growth pattern, one of the morphologies more commonly associated with *ALK* rearrangement.

involved in reflex ALK IHC is the disposables required (both primary antibody and secondary detection systems) with minimal increased labour expenses and no change in turnaround time if IHC is already being prepared and interpreted.

We have estimated the disposables cost for routine ALK IHC as AU$25 per case. This cost is partially offset by the time and cost savings in not having to add ALK testing on to archived cases when they are found to be inoperable or recurrent. Furthermore, because *ALK* gene rearrangement and *EGFR* mutations are virtually always mutually exclusive,[14] tumours suspected to harbour *ALK* gene rearrangements do not need to undergo routine *EGFR* mutation testing which is currently rebated at AU$397.35 per test, more than completely offsetting the cost of confirmatory FISH testing in ALK IHC positive cases. Put another way, we were able to screen 293 patients with IHC for an additional cost of $7325 without significantly affecting laboratory workflow or turnaround time and with the benefit of identifying 12 patients with confirmed *ALK* gene rearrangement (10 of whom were inoperable and would immediately benefit from crizotinib). Since *ALK* gene rearrangement is reported to present at a higher stage and is considered an unfavourable prognostic factor,[15,16] even the two operable patients may benefit from crizotinib therapy in the future if they recur.

Table 2 Details of 12 cases demonstrating positive staining for ALK

Patient no.	Age	Sex	Specimen type	ALK IHC	ALK FISH
1	29	F	FNA	Positive	Positive
2	50	F	Core biopsy	Positive	Positive*
3	55	F	Core biopsy	Positive	Positive
4	60	F	FNA	Positive	Positive
5	85	F	FNA	Positive	Positive
6	33	M	Core biopsy	Positive	Positive*
7	52	M	Core biopsy	Positive	Positive
8	54	M	FNA	Positive	Positive
9	62	M	FNA	Positive	Positive
10	63	M	FNA	Positive	Positive
11	64	M	Excision	Positive	Positive
12	78	M	Core biopsy	Positive	Positive

* These cases demonstrated variant FISH patterns. They were initially interpreted as FISH negative, but this was revised on repeat testing and review.

Our findings demonstrate that positive ALK IHC is clearly highly specific for the presence of *ALK* gene rearrangement as all IHC positive cases were ultimately confirmed to be rearranged with FISH studies. This is in line with other studies which have found that ALK IHC has high sensitivity and specificity when compared to FISH.[5,12,17,18] One of the

potential disadvantages of ALK IHC is that staining can be affected by pre-analysis factors such as differences in fixation or processing which may vary between laboratories.[19] Another interpretive challenge is heterogeneous staining of ALK IHC in small biopsy or cytology specimens. Although this may be an issue when performing testing on small samples, interpreting IHC is significantly less challenging than interpreting FISH studies in small biopsies, and in fact IHC and ALK IHC is particularly useful when FISH is unable to be performed due to insufficient material. Neither the retrospective study nor audit of our prospective implementation of IHC was intended or designed to formally assess the sensitivity of ALK IHC. However in our prospective cohort, we found a rate of ALK gene rearrangement of 4% which is well within the range of 2–7% of lung adenocarcinomas reported in similar cohorts including both surgically resectable and metastatic NSCLC.[2,20–23] A lower occurrence in our retrospective cohort is to be expected given that *ALK* rearranged lung cancer is more likely to present with inoperable disease.[15,16] We found a rate of *ALK* gene rearrangements of 1.5% in this cohort which is very similar to the reported incidence of 1% in two separate studies performed in similar populations of surgically resected tumours.[11,12] None of three additional patients who underwent FISH studies due to a high clinical suspicion of *ALK* gene rearrangement despite negative IHC staining were found to be rearranged in the small number of IHC negative cases studied. Furthermore, Selinger *et al.* [12] also reported no false negative IHC cases in their retrospective series of 594 NSCLC cases. For all these reasons we suspect that ALK immunohistochemistry when deployed in the routine clinical setting is highly sensitive for *ALK* gene rearrangement and identifies the overwhelming majority of *ALK* rearranged cases as has been reported by retrospective series.[3–6,12] However, until the sensitivity of ALK immunohistochemistry is confirmed, we would still recommend that those patients considered to be at high risk for *ALK* gene rearrangement (for example non-smokers with adenocarcinoma histology found to be negative for *EGFR* mutation) be offered FISH testing if inoperable or upon recurrence. Of course, if further audits prove that all IHC negative cases are consistently FISH negative then this low yield approach could be ceased.

The use of routine ALK immunohistochemistry can also serve as an additional quality control measure for FISH studies which can be difficult to interpret particularly in small biopsies. In our prospective cohort we encountered two cases which were positive with screening IHC, but which were ultimately considered falsely negative on initial FISH studies due to atypical FISH patterns. Both of the cases which were falsely negative had either a variant or atypical pattern. In one case the discrepancy was due to a narrow split signal which was interpreted as being less than the recommended two signal distance required to fulfil the criteria of a positive test. In the second case the discrepancy was due to an unusual triplet pattern which was also reported as atypical by a second expert opinion. Both these cases were interpreted as positive when testing was performed at a second institution and also when testing was performed with a second probe (Zytolight SPEC ALK/EML4 TriCheck Probe; ZytoVision). Similar events have been reported by other groups;[15,24] therefore, we caution that FISH studies should be repeated if discrepant with IHC results and that a second assay (e.g., RT-PCR or an alternative FISH probe) should be performed if this discrepancy remains. If ALK IHC had not been performed in the two discrepant cases in our prospective series, these patients most likely would have been denied the potential benefits of targeted therapy.

A number of different antibodies are commercially available for ALK immunohistochemistry. The most commonly used clones are 5A4, D5F3 and ALK1. We used the 5A4 clone which can reliably detect *ALK* gene rearrangements in NSCLC,[6] but other studies have also found good sensitivities and specificities with other clones.[5,12] For example, in one study the ALK1, 5A4 and D5F3 antibodies have been reported to have specificities of 99%, 98% and 99%, respectively, with all three having sensitivities of 100%.[12] Other studies have suggested that the D5F3 and 5A4 antibodies may outperform the ALK1 clone, with sensitivities of 100% versus 66%, respectively.[5] Regardless of which clone is used to implement a screening program for *ALK* gene rearrangements in lung cancer, we caution that ALK IHC should be validated in individual laboratories before it is deployed clinically and when deployed it should be performed with appropriate lung specific protocols and controls as major management decisions are based on the results of testing. Laboratories involved in testing should ensure they are enrolled in any appropriate external quality assurance programs. FISH studies can still be performed on patients with a high clinical suspicion of *ALK* gene rearrangement even if IHC is negative or if there is uncertainty about the performance of the antibody in individual laboratories.

In conclusion, we have demonstrated that reflex ALK IHC is a reliable and cost effective screening tool for the presence of clinically significant *ALK* gene rearrangements in patients with NSCLC. It can be readily deployed into the routine clinical setting with minimal extra time and expense and appears to identify the majority of *ALK* rearrangements. It can be readily used on small biopsy specimens and cell block preparations. Based on our experience, in ALK IHC positive lung cancers with negative FISH studies consideration should be given to repeat FISH testing or testing with an alternative technique (e.g., RT-PCR or an alternative FISH probe) before a clinically significant gene rearrangement is considered to be excluded. We caution that ALK IHC should be validated in individual laboratories with lung cancer specific protocols before major management decisions are made based on this stain and that IHC positive cases should be confirmed with FISH. Because ALK IHC is a screening test, and our study was not designed to directly address the sensitivity of ALK IHC, it is still possible that a very small proportion of ALK IHC negative cases could demonstrate ALK rearrangements with FISH analysis. Therefore, at this stage consideration may still be given to performing FISH studies in ALK IHC negative patients considered at high clinical risk of *ALK* rearrangement if eligible for treatment. However, our data suggest that this would be a low yield test in this setting.

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