Histopathology

Histopathology 2015, **67**, 654–663. DOI: 10.1111/his.12708

Equivocal *ALK* fluorescence *in-situ* hybridization (FISH) cases may benefit from ancillary *ALK* FISH probe testing

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Date of submission 20 January 2015
Accepted for publication 29 March 2015
Published online *Article Accepted* 4 April 2015

Selinger C, Cooper W, Lum T, McNeil C, Morey A, Waring P, Amanuel B, Millward M, Peverall J, Van Vliet C, Christie M, Tran Y, Diakos C, Pavlakis N, Gill A J & O'Toole S
(2015) *Histopathology* **67**, 654–663. DOI: 10.1111/his.12708

Equivocal *ALK* fluorescence *in-situ* hybridization (FISH) cases may benefit from ancillary *ALK* FISH probe testing

Aims: Accurate assessment of anaplastic lymphoma kinase (ALK) gene rearrangement in non-small-cell lung cancers (NSCLCs) is critical to identify patients who are likely to respond to crizotinib. The aim of this study was to evaluate the *ALK/EML4* TriCheck FISH probe in a series of NSCLCs enriched for tumours with equivocal *ALK* status.
Methods and results: *ALK* FISH was prospectively performed on 45 NSCLCs with the *ALK/EML4* TriCheck probe (ZytoVision) and the Vysis *ALK* break-apart probe (Abbott Molecular). ALK immunohistochemistry was performed with 5A4 and D5F3 antibodies. Fourteen cases had equivocal *ALK* status, based on borderline or focal FISH positivity, an atypical FISH pattern, or discrepancy between *ALK* FISH and immunohistochemistry. Four of the 14 equivocal cases showed discordance between the two FISH probes. All other cases were concordant. The TriCheck probe showed that, of 31 unequivocal cases, 15 were *ALK*-rearranged, and 60% of these had *EML4* as the translocation partner. Within the group of 14 equivocal cases, 12 showed rearrangement with the Tricheck probe; only one of these showed *EML4* rearrangement. Of the six equivocal cases that received crizotinib, four showed clinical benefit.
Conclusions: The *ALK/EML4* TriCheck FISH probe may be useful for the detection of *ALK* rearrangements, especially in borderline or atypical cases, where an additional unique *ALK* FISH probe may provide further confirmation of rearrangement.

Keywords: anaplastic lymphoma kinase, fluorescence *in-situ* hybridization, lung adenocarcinoma, non-small-cell lung cancer

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Introduction

Many non-small-cell lung cancer (NSCLC) patients whose tumours show anaplastic lymphoma kinase (ALK) gene rearrangement, confirmed by fluorescence *in-situ* hybridization (FISH), have shown a rapid and pronounced response to the ALK and MET tyrosine kinase inhibitor crizotinib.[1,2] Accurate assessment of *ALK* rearrangements in NSCLCs is critical for determining eligibility for crizotinib therapy. Currently, the most widely established accurate method for identifying *ALK* rearrangements is FISH with an *ALK* break-apart probe. The Abbott Molecular *ALK* FISH test is Food and Drug Administration (FDA)-approved for detecting *ALK* rearrangement-positive NSCLC patients for treatment with the ALK inhibitor crizotinib.[3,4] The criterion for categorizing lung tumours as *ALK*-positive is ≥15% of at least 50 tumour cells showing a positive signal pattern that includes split signals at least two signal distances apart or an isolated 3′ red signal,[5] according toAbbott Molecular recommendations and the IASLC Atlas of *ALK* testing in lung cancer guidelines.

Not every cell in an *ALK*-positive tumour will show visible rearrangement; this is thought to be a result of sectioning truncation, aberrant probe hybridization, random loss or gain of signals, probe signal overlap in a three-dimensional nucleus, or chromatin folding. In addition, *ALK*-negative tumours can show cells with false positivity. Camidge *et al.*[3] described up to 11% 'background noise' of *ALK* positivity in non-tumour cells and *ALK*-negative tumours. Reports of intratumoral heterogeneity,[6–8] although somewhat disregarded as technical false positivity,[3,9] raise questions about the potential for clonal positivity or borderline positive cases that could be missed. Moreover, there have been rare reports of false-negative ALK immunohistochemistry (IHC) findings,[10,11] despite appropriate methodology, which presumably could be a result of pre-analytical factors or mutational variant proteins that inhibit antibody binding.

It is widely believed that *ALK* rearrangement represents a driver mutation in lung cancer, providing a selective advantage and therefore constituting a dominant mutation in most tumour cells. Although the majority of NSCLCs with an *ALK* rearrangement are diffusely positive throughout the tumour, in our experience rare cases may show only focal areas of rearranged signals, making determination of *ALK* positivity status with a strict criterion of ≥15% difficult. We have also observed rare atypical *ALK* FISH signal patterns of unknown clinical significance in our routine diagnostic practice. In addition, on occasion, ALK IHC staining does not correlate with the *ALK* FISH result. In these equivocal cases, where accurate determination of *ALK* rearrangement status may have a significant impact on the patient's treatment, ancillary testing may be an effective solution.

We aimed to evaluate the *ALK*/*EML4* TriCheck FISH probe for both effectiveness in confirmation of *ALK* rearrangements and simultaneous detection of *ALK*–*EML4* inversions by employing the third single-colour break-apart probe for *EML4*. The *ALK*/*EML4* TriCheck FISH probe has the potential to confirm an *EML4* translocation partner in *ALK*-rearranged cases, which represent ~80% of *ALK* translocations in lung cancer.[12] If *EML4* rearrangement is present, this provides further evidence confirming true *ALK* rearrangement. This is particularly useful for cases showing variant 1 *ALK*–*EML4* rearrangements, where the break-apart split can be quite subtle.[13]

Materials and methods

PATIENT COHORT

Over a 2-year period from July 2012 to July 2014, we identified 14 cases with equivocal ALK status from a total of 153 cases referred for *ALK* rearrangement analysis at Royal Prince Alfred Hospital (RPAH) in Sydney, Australia in a diagnostic laboratory with National Association of Testing Authorities (NATA) accreditation, which is equivalent to National Quality Assurance Advisory Panel-type certification. From the 153 cases tested with *ALK* FISH (Abbott Molecular, Abbott Park, IL, USA), *ALK* TriCheck FISH (ZytoVision, Bremerhaven, Germany) was also undertaken in all 14 equivocal cases, as well as in an additional control group of 31 cases with unequivocal *ALK* status (15 *ALK*-positive and 16 *ALK*-negative). Equivocal *ALK* status was defined as follows: (i) an *ALK* break-apart score close to 15% (10–20%); (ii) an aberrant signal pattern (other than split signals or isolated red signals); or (ii) discordant ALK IHC and *ALK* FISH results (FISH-positive and IHC-negative, or FISH-negative and IHC-positive). Ethics approval was granted by the Sydney Local Health District Ethics Review Committee (X12-0313 and HREC/12/RPAH/479).

The cohort consisted of predominantly epidermal growth factor receptor (EGFR) wild-type cases referred for *ALK* rearrangement testing from multiple hospitals and laboratories. Sixteen cases were mutation-tested at the RPAH in parallel with diagnostic *ALK* testing. Mutation testing was performed with the Oncocarta v1.0 kit on the Sequenom MassARRAY platform (Sequenom, San Diego, CA, USA). IHC or FISH testing for *ROS1* was performed in all cases where appropriate, except for six of 45 cases.

All cases were adenocarcinomas, except for one case that was NSCLC, not otherwise specified (NOS).

In the cases discordant for IHC and FISH or FISH probe status between the Abbott and ZytoVision *ALK* FISH probes, clinical follow-up was sought from the treating oncologists, where possible, for patients who received crizotinib. Tumour assessments were made by computed tomography scan or chest X-ray, with response (complete response, partial response, stable disease, and progressive disease) being determined according to Response Evaluation Criteria in Solid Tumours version 1.1.[14] The length of follow-up ranged from 2 to 17 months, with average of 9 months.

FLUORESCENCE *IN-SITU* HYBRIDIZATION FOR *ALK*

Interphase FISH for *ALK* rearrangement was performed in a NATA-accredited diagnostic laboratory with two *ALK* break-apart FISH probes, according to the manufacturers' guidelines. The first probe was the Vysis LSI *ALK* Dual Colour, Break Apart Rearrangement Probe (Abbott Molecular), henceforth referred to as the Abbott probe. The Abbott probe detects rearrangements in 2p23 encompassing the *ALK* gene, and includes a SpectrumOrange-labelled 250-kb DNA fragment telomeric to *ALK* (3′ end) and a SpectrumGreen-labelled 300-kb DNA fragment centromeric to *ALK* (5′ end). The probe set does not identify the specific rearrangement gene partner. Non-overlapping nuclei were scored; cells showing only a single signal or weak or diffuse signals were ignored. According to Abbott Molecular scoring criteria and the IASLC ALK Atlas,[5] FISH for *ALK* locus rearrangement was considered to be positive if at least 15% of cells analysed showed either a split of one set of red and green signals >2 signal widths apart, and/or an isolated red signal (3′ probe) in at least 50 tumour nuclei. For cases in which the percentage of cells positive for rearrangement was 10–15%, a further 50–100 tumour nuclei were counted and reviewed by an independent scorer.

The second probe employed was the ZytoLight SPEC *ALK*/*EML4* TriCheck Probe (ZytoVision), henceforth referred to as the TriCheck probe. The TriCheck probe detects rearrangements in 2p23 encompassing the *ALK* gene, and includes a ZyOrange-labelled 800-kb DNA fragment telomeric to *ALK* (3′ end) and a ZyGreen-labelled 650-kb DNA fragment centromeric to *ALK* (5′ end). In addition, a single ZyBlue-labelled 1.8-Mb DNA fragment spanning the *EML4* gene is included in the probe set. FISH for *ALK* locus rearrangement was considered to be positive if at least 15% of cells analysed showed either a split of one set of red and green signals >2 signal widths apart and/or an isolated red signal (3′ probe) in at least 50 tumour nuclei, according to ZytoVision scoring criteria. *EML4* break-apart signal patterns showing an additional aqua signal were classed as *EML4*-rearranged (that is, in cells diploid for *ALK*, three *EML4* signals indicate a break-apart). Co-localization of an aqua signal with an isolated red signal and a co-localization of an aqua signal with an isolated green signal indicate *ALK–EML4* translocation, according to ZytoVision scoring criteria. For cases where the percentage of cells positive for rearrangement was 10–15%, a further 50–100 tumour nuclei were counted and reviewed by an independent scorer.

Finally, cases that showed 10–20% *ALK*-positive tumour nuclei were grouped into a category of 'equivocal', and were re-scored and re-reviewed by three independent scorers blinded to the other scorers' result. All FISH slides for equivocal cases were reviewed by a senior FISH scientist and two lung specialist pathologists with extensive experience in evaluating FISH and ALK IHC (S.O. and W.C.). A final consensus decision was made after review of histology, ALK IHC, and all FISH slides.

A validation set of 20 non-lesional clinically 'normal' lung samples were used to evaluate the reference range (normal cut-off) by calculating the background level of false-positive signal patterns in both the Abbott and ZytoVision probe sets with the binomial method calculation.

Tissue sections cut at 4 μm were stained for FISH. Signals were counted with an epifluorescence microscope (Zeiss, Oberkochen, Germany).

IHC FOR ALK

IHC was performed on sections cut at 4 μm, with either the Novocastra mouse monoclonal antibody p80 ALK (Clone 5A4, NCL-ALK; Leica, Wetzlar, Germany), the Cell Signalling Technology ALK XP rabbit monoclonal antibody (Clone D5F3, #3633; Cell Signalling Technology, Danvers, MA, USA), or the Ventana anti-ALK (D5F3) rabbit monoclonal primary antibody (Roche, Basel, Switzerland), sold under licence from Cell Signalling Technology. 5A4 ALK IHC was performed with an antibody dilution of 1:25 and 15 min of incubation. Staining was performed with the Leica Bond Polymer Refine DAB detection kit (Leica Microsystems, Basel, Switzerland), including Bond DAB Enhancer (Leica Microsystems), and retrieval with ER2 (Leica Microsystems) for 30 min, and was performed on a Leica Bond Max autostainer (Leica Microsystems). The anti-ALK (D5F3) rabbit monoclonal antibody (Cell Signalling Technology) was used

at a dilution of 1:100 for 24 min, or the Ventana ALK IHC assay was utilized (~14 μg/ml; Roche) with 16 min of incubation. Staining was performed with the OptiView DAB IHC Detection kit (Roche), with an amplification kit (Roche) and CC1 (Roche) retrieval for 92 min on a Ventana Benchmark GX (Roche). Positive controls included lung tumour confirmed by FISH to be positive for *ALK* rearrangement. Cases with 1+, 2+ or 3+ staining of tumour cells were regarded as positive, and cases with no staining were regarded as negative.

Results

Mutation testing was performed at the RPAH for 16 of the cases in conjunction with diagnostic *ALK* testing. Two cases had a G12C *KRAS* mutation, one case had a *BRAF* exon 11 G469V mutation, and one case had an E709K *EGFR* mutation. The other cases were wild-type for *EGFR*. Three of the mutation-positive cases were negative for *ALK* rearrangement, and the case that was positive for an unusual *BRAF* exon 11 mutation (case 11) was *ALK*-positive. One *ALK*-negative case was positive for *ROS1* rearrangement (case 35).

Despite using two different antibody clones to evaluate ALK IHC staining, we previously confirmed that both clones were able to correctly identify all *ALK*-positive cases in a cohort of 594 NSCLCs;[8] also, numerous studies reported in the literature have also established both clones as suitable for detecting *ALK* rearrangements when used with appropriate lung protocols and controls.[8,10,15,16]

ALK rearrangement was assessed prospectively with ALK IHC and two *ALK* FISH probes in 45 diagnostic cases (Figure 1). All 31 unequivocal cases showed 100% concordance for both FISH probes, whereas 10 of the 14 equivocal cases were concordant for both FISH probes (71%).

Of the 31 unequivocal cases, 15 showed *ALK* rearrangement with both *ALK* FISH probes, and nine of the 15 *ALK*-rearranged cases also showed *EML4* rearrangement (60%).

Interestingly, and perhaps concerningly, the two FISH probes performed slightly differently, with the TriCheck probe showing a higher percentage of *ALK*-positive cells in cases within the equivocal category ($P = 0.02$) but not in the overall group ($P = 0.18$). This result is difficult to explain, and could be the result of a difference in probe design or simply the low number of samples in this study. Observer bias is possible, although unlikely, owing to the intense scrutiny by three experienced and independent scorers, who reached a consensus following evaluation of >100 tumour nuclei. The TriCheck FISH probe has

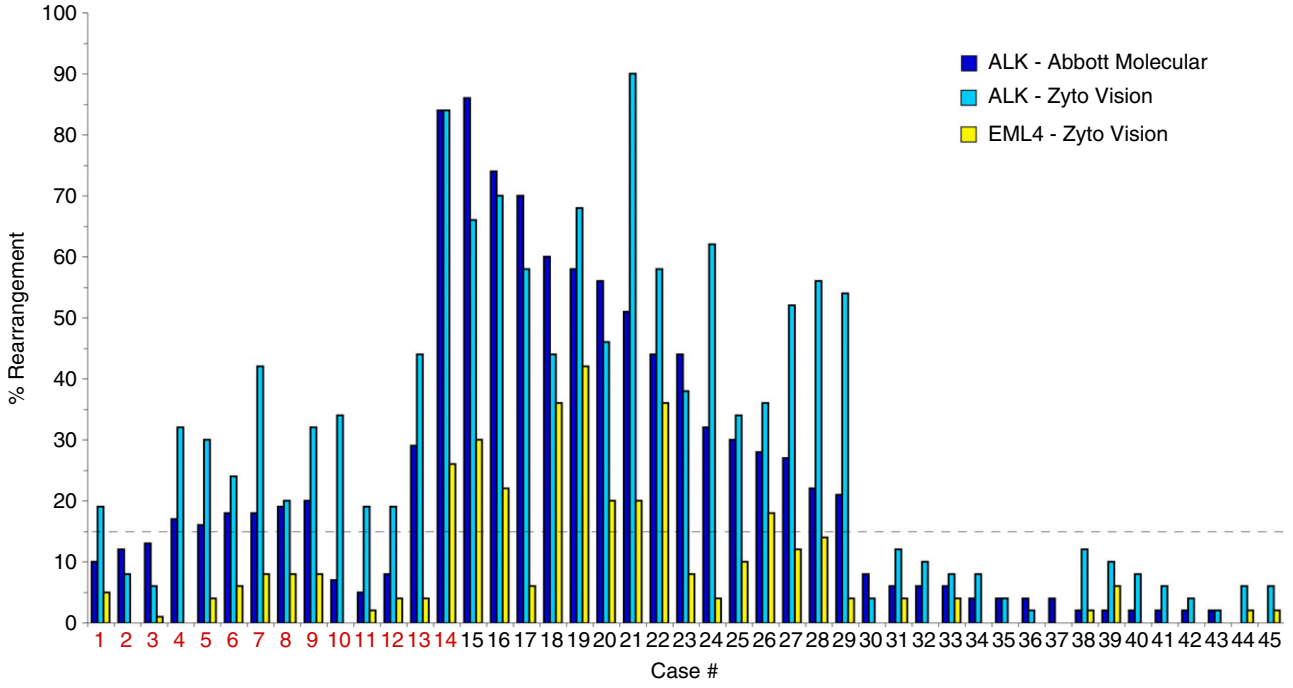

Figure 1. Cases with percentage rearrangement for *ALK* fluorescence *in-situ* hybridization (FISH) with the Abbott Molecular probe, *ALK* FISH with the ZytoVision probe, and *EML4* FISH with the ZytoVision probe. The first 14 cases were classified as equivocal, owing to an *ALK* break-apart score close to 15% (10–20%) or an aberrant FISH signal pattern, or discordant immunohistochemistry and FISH results.

been validated by the manufacturer to be used with a threshold of positivity at 15% rearrangement and signal separation of two signal distances for both the *ALK* and *EML4* break-apart probes. We analysed a set of 20 non-lesional lung samples, which showed a very low level of 'background noise' positivity, and use of the binomial method calculation[17] resulted in threshold cut-offs of 8% for the ZytoVision *ALK* probe and 2% for the *EML4* probe. The same threshold of 8% was calculated when the Abbott *ALK* probe was analysed. Precise reference ranges (normal cut-off) can be very difficult to obtain accurately when positive signal patterns are very rare in normal clinical samples used to undertake the validation. However, this illustrates that the TriCheck probe set does not systematically differ from the Abbott *ALK* FISH probe set in terms of background levels of positivity.

Only one of the 14 equivocal cases (case 7) would have been suitable for further testing using quantitative reverse transcription- polymerase chain reaction or sequencing, owing to insufficient tumour material being available in the majority of small biopsy specimens. Therefore, ancillary testing with FISH was evaluated as a potentially pragmatic solution.

The 14 equivocal cases included eight that were classified as *ALK* rearrangement-positive with the standard break-apart probe (Abbott Molecular). An additional four cases were classified as *ALK* rearrangement-positive with the *ALK–EML4* TriCheck probe (ZytoVision), giving a total of 12 *ALK*-positive cases with the second probe set. One of the 12 equivocal *ALK*-rearranged cases showed *EML4* rearrangement (8%).

Details of the 14 cases (14/45, 31%) with equivocal *ALK* status because of an equivocal or atypical *ALK* rearrangement result or discordant FISH and IHC results are shown in Table 1.

A number of cases (cases 1–3 and 5–9; Table 1) showed *ALK* rearrangement close to the 15% threshold with the standard break-apart probe, despite being ALK IHC-negative, warranting ancillary FISH testing. All cases, except for cases 2 and 3, were confirmed to be positive, with a higher percentage of *ALK* rearrangement being detected with the ZytoVision TriCheck probe. Case 5, which was confirmed to be *ALK*-positive, was histologically NSCLC, NOS. One of the negative cases, case 3, was also found to have an E709K *EGFR* mutation. Cases 1 and 8 received crizotinib therapy, with case 8 being reported to have stable disease. The treating oncologists reported that case 1 did not respond to treatment; however, owing to chemotherapy treatment immediately preceding crizotinib therapy, accurate assessment of a crizotinib-specific response was not possible. Case 4 also showed a level of *ALK* rearrangement close to the 15% threshold, but was IHC-positive. The second FISH probe confirmed rearrangement, also with a higher percentage of rearrangement being detected.

Case 10 was a male aged 34 years with lung adenocarcinoma metastatic to the ischium. The tumour was ALK IHC-positive, but showed an atypical signal pattern with the Abbott Molecular probe. This included triplets of two green signals and one red signal (Figure 2A), in addition to 6.5% of cells with a positive signal pattern (Table 1), according to the standard Abbott Molecular scoring criteria. This case showed a positive signal pattern in 34% of cells with the ZytoVision TriCheck probe and standard scoring criteria. The triplet signal pattern was also observed with the TriCheck probe set (Figure 2B). *EML4* rearrangement was not observed, suggesting that this case was not a variant 1 *ALK* rearrangement. The patient symptomatically responded to crizotinib therapy, and had stable disease with resolution of pain from the pathological fracture (Table 1).

Cases 11 and 12 showed IHC positivity discordant with the negative Abbott probe FISH result, with secondary FISH testing with the ZytoVision TriCheck probe resulting in a higher percentage of rearranged cells being detected (Table 1). Both cases received crizotinib therapy. Case 11 was reported to have stable disease and an unusual *BRAF* exon 11 mutation, whereas case 12 did not respond to crizotinib.

Cases 13 and 14 were FISH-positive with the Abbott probe, however, secondary testing with the TriCheck probe was performed, owing to a discordant ALK IHC result (Table 1). Both FISH probes confirmed *ALK* rearrangement in these cases. Case 13 received crizotinib therapy and had stable disease.

The *ALK* rearrangement status and results of cases that received treatment are summarized in a flow diagram in Figure 3.

Discussion

This is the first study describing the use of a secondary FISH probe to assist in the assessment of *ALK* rearrangement in equivocal or atypical NSCLC cases, and was performed on the types of challenging cases and limited biopsy specimens regularly observed in routine diagnostic practice. In most cases, the only ancillary testing available involves methods that require only a small amount of tissue, such as FISH or IHC. Ancillary FISH testing has the additional

Table 1. Equivocal *ALK* rearrangement status cases, comparing percentage rearrangement for *ALK* fluorescence *in-situ* hybridization (FISH) observed for the Abbott Molecular probe and the ZytoVision probe in addition to ALK immunohistochemistry (IHC)

Case no.	Equivocal *ALK* rearrangement status	*ALK* FISH—Abbott Molecular (%)	*ALK* FISH—ZytoVision (%)	*EML4* FISH—ZytoVision (%)	Concordance between FISH probes	ALK IHC result	ALK inhibitor treatment outcome	Histology
1	Close to 15% threshold	10	19	5	No	Negative	Progressive disease	Adenocarcinoma
2	Close to 15% threshold	12	8	0	Yes	Negative	Negative, not treated	Adenocarcinoma
3	Close to 15% threshold	13	6	1	Yes	Negative	Negative, not treated	Adenocarcinoma
4	Close to 15% threshold	17	32	0	Yes	Positive	Not treated	Adenocarcinoma
5	Close to 15% threshold, discordant IHC	16	30	4	Yes	Negative	Unknown	NSCLC, NOS
6	Close to 15% threshold, discordant IHC	18	24	6	Yes	Negative	Unknown	Adenocarcinoma
7	Close to 15% threshold, discordant IHC	18	42	8	Yes	Negative	Not treated	Adenocarcinoma
8	Close to 15% threshold, discordant IHC	19	20	8	Yes	Negative	Stable disease	Adenocarcinoma
9	Close to 15% threshold, discordant IHC	20	32	8	Yes	Negative	Not treated	Adenocarcinoma
10	Triplet signal pattern, IHC-positive	7	34	0	No	Positive	Stable disease*	Adenocarcinoma
11	FISH-negative, discordant IHC	5	19	2	No	Positive	Partial response	Adenocarcinoma
12	FISH-negative, discordant IHC	8	19	4	No	Positive	Progressive disease	Adenocarcinoma
13	FISH-positive, discordant IHC	29	44	4	Yes	Negative	Stable disease	Adenocarcinoma
14	FISH-positive, discordant IHC	84	84	26	Yes	Negative	Unknown	Adenocarcinoma

NSCLC, NOS, Non-small-cell lung cancer, Not otherwise specified.
*This patient also showed resolution of pain from the pathological fracture.

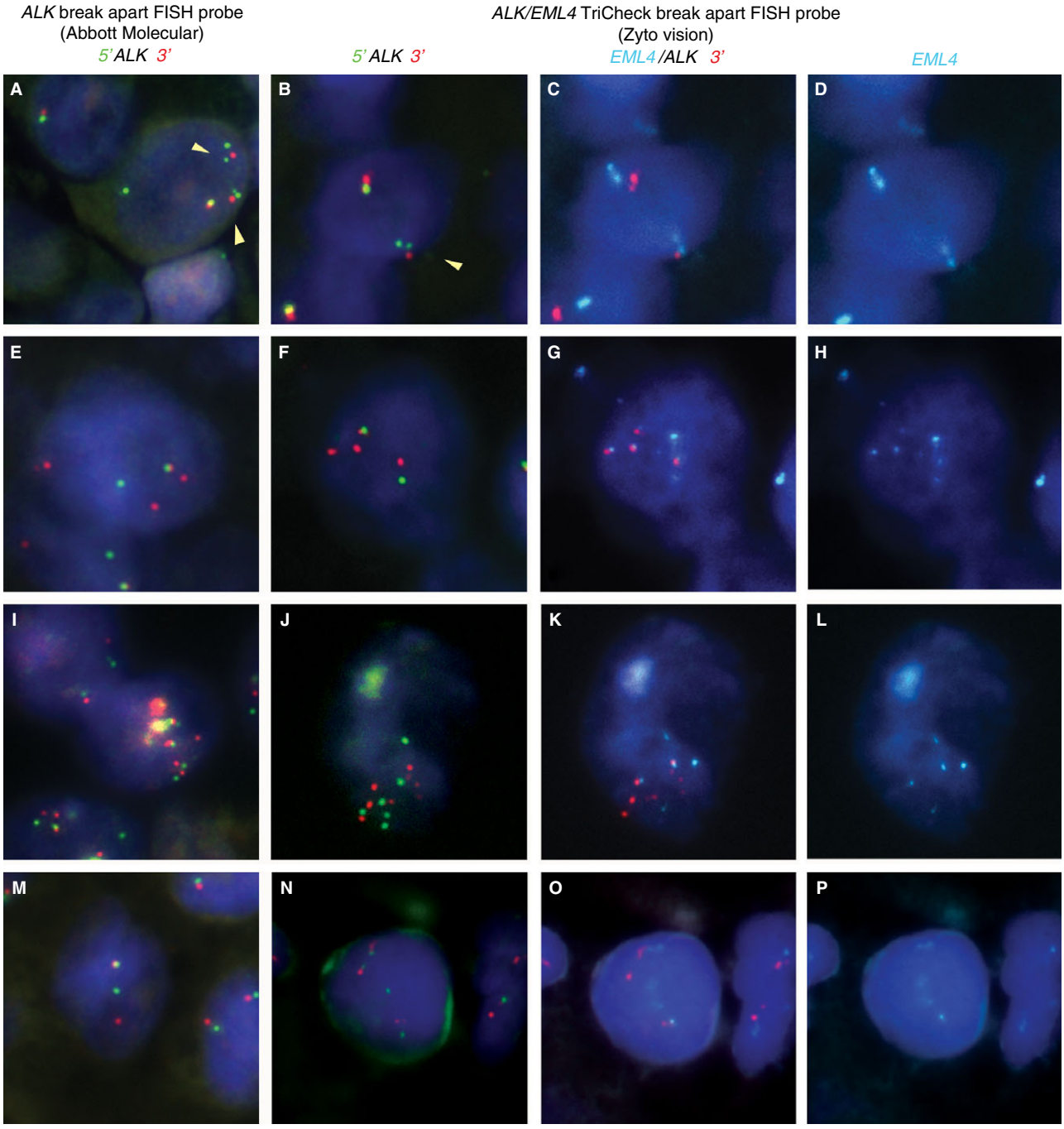

Figure 2. Example fluorescence *in-situ* hybridization (FISH) images from cases. **A–D**, Case 10, with an atypical triplet *ALK* FISH pattern (**A**) with the Abbott probe, but positive with the Tricheck *ALK* probe (**B**), although negative for *EML4* (**C**, **D**). **E–H**, A positive case (case 15) with increased red 3′ *ALK* signals and *EML4* rearrangement [confirmed by co-localization of the *EML4* aqua and red 3′ *ALK* signals (**G**) and additional aqua *EML4* signals (**H**)]. **I–L**, Case 14 with a polysomic, positive *ALK–EML4* signal pattern. **M–P**, A case with a positive *ALK* signal (case 28) with both the Abbott and Tricheck probes, but negative for *EML4* rearrangement [no co-localization of the aqua and red signals (**O**) and no additional aqua signals (**P**)]. **A**, **E**, **I**, **M**, The *ALK* break-apart FISH probe (Abbott Molecular). **B**, **F**, **J**, **N**, The 5′ green and 3′ red *ALK* break-apart FISH probe components of the *ALK*/*EML4* TriCheck break-apart FISH probe (ZytoVision). **C**, **G**, **K**, **O**, The aqua *EML4* break-apart FISH component and the 3′ red *ALK* component of the *ALK*/*EML4* TriCheck break-apart FISH probe (ZytoVision), where *ALK–EML4* rearrangement shows co-localization of the red and aqua probe signals. **D**, **H**, **L**, **P**, The aqua *EML4* probe component of the *ALK*/*EML4* TriCheck break-apart FISH probe (ZytoVision) alone.

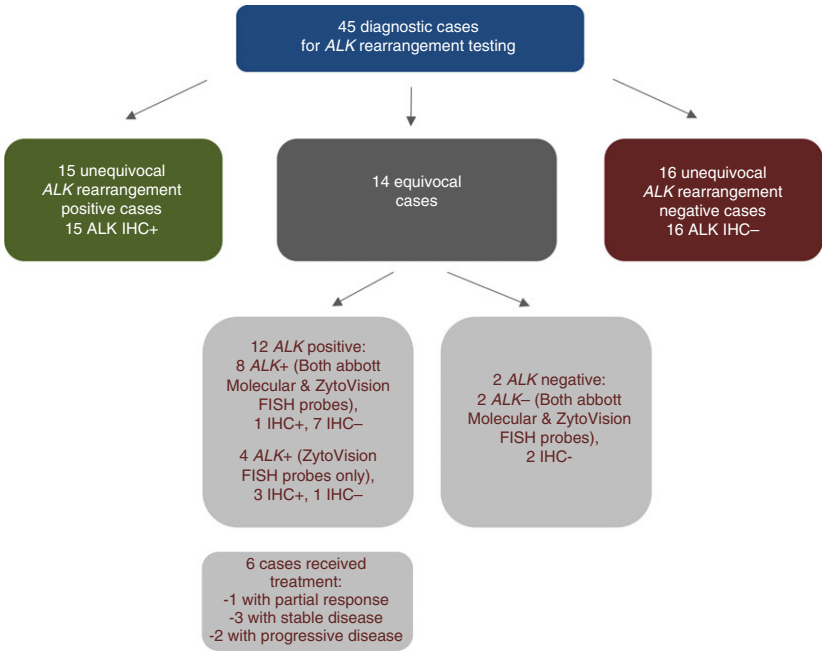

Figure 3. Flow diagram summarizing *ALK* rearrangement status in unequivocal and equivocal cases, and treatment outcomes in the equivocal subgroup.

advantage of evaluating the status of potential fusion partners, such as *EML4*.

Of the 31 unequivocal routine cases evaluated with a secondary *ALK* FISH probe, 100% concordance was observed. Fourteen equivocal cases were also evaluated, including those with borderline counts, and atypical or IHC-discordant cases, which showed 71% concordance of rearrangement status for both FISH probes. Four equivocal cases were discordant between the two FISH probes.

It has been reported that *EML4* is the most common *ALK* rearrangement partner, occurring in ~80% of cases.[12] Of the unequivocal FISH-positive cases, 60% were *EML4*-rearranged; however, only one of the equivocal cases showed *EML4* rearrangement. The low rate of *EML4* rearrangement is probably attributable to the higher proportion of atypical, borderline and difficult cases evaluated; these are distinct from a more representative general population of cases, in which the rate of *EML4* rearrangement is reported to be ~80%. In addition, the equivocal cases analysed may also be enriched for alternative rare rearrangement partners; this is worthy of further investigation.

Eight cases showed false-negative ALK IHC results (based on the ZytoVision ALK FISH) (case 1 was true negative according to the Abbott ALK FISH result alone). This is despite the use of appropriate controls and IHC protocols optimized for identifying ALK rearrangement in lung cancer (utilizing the 5A4 and D5F3 ALK antibody clones). Although rare, false-negative ALK IHC results have been described in a number of studies.[10,11] It is difficult to know whether false negativity is attributable to pre-analytical factors, such as insufficient fixation or processing, or to a rearrangement variant that inhibits antibody binding. In addition, because of the limited data on the clinical response of this group of patients to crizotinib therapy, it is not possible to state confidently whether these equivocal cases were genuinely false-negative IHC cases.

ALK rearrangement-positive NSCLC is associated with a high response rate and prolonged progression-free survival when treated with the tyrosine kinase inhibitor crizotinib. The initial trials of crizotinib therapy in NSCLC used a threshold of >15% of cells showing *ALK* rearrangement for classification as ALK-positive.[1,3,9] This threshold was updated to include ≥15% after FDA approval of the companion diagnostic FISH test.[4] The justification for this cut-point was that the range of percentage positivity contained a gap that appeared to result in a natural split into a group of higher percentage positivity and what appeared to be the technical background noise of the assay.[3] Ongoing examination of *ALK* rearrangements in ever-increasing numbers globally has revealed a

small proportion of cases that show rearrangement close to the cut-point.[9] Camidge *et al.*[7] described 8.5% of cases showing *ALK* rearrangement in the 10–15% range. Although response to crizotinib is not correlated with the percentage *ALK* positivity in cell lines and in the clinical trial cohorts,[3,7] these cases were only selected if they met strict entry criteria of >15% *ALK* positivity, and borderline negative cases would not have received crizotinib. Further investigation with supplementary methods[5] and documentation of responses to crizotinib in these borderline and atypical cases are required.

Accurate determination of *ALK* status in NSCLC is critical, and although most cases can easily be unambiguously classified as clearly *ALK*-positive or clearly *ALK*-negative, a small proportion of cases show either a percentage of rearrangement close to the 15% threshold (10–20%) or an atypical signal pattern that warrants further careful clinical investigation.[5,18] A recent study showed that discrepancies between *ALK* FISH and IHC results were often associated with *ALK* 'borderline' positivity (15–20%), with a number of cases also overexpressing c-MET.[18] Secondary testing, in addition to ALK IHC, could include polymerase chain reaction (PCR) or sequencing. PCR-based techniques are most effective when the rearrangement is not an unknown or atypical variant. It should be noted that in the first phase 1 study of crizotinib, retrospective analysis of *ALK*-positive cases with reverse transcription PCR failed to detect transcripts in 31% of cases.[1] Nevertheless, in many cases, the limited amount of tissue material prohibits effective ancillary testing with these methods. In this study, the majority of these routine diagnostic specimens had very limited or no additional material, which prevented independent confirmation of rearrangement by additional techniques beyond IHC and FISH, such as PCR.

A secondary *ALK* FISH probe may offer a viable solution in these equivocal cases, and, in our experience, in a limited proportion of cases, can provide additional convincing evidence for determining *ALK* rearrangement status. One of the initially equivocal cases showing an atypical triplet signal pattern (case 10) was reported by the treating oncologist to show a good response to crizotinib therapy with stable disease, validating the utility of secondary FISH probe testing in this case. Three other equivocal cases (owing to discordant IHC results) that were classified as *ALK*-positive were reported to have a partial response or stable disease following crizotinib treatment. Two cases with positive *ALK* TriCheck FISH results, one with a positive IHC result and one with a negative IHC result, did not respond to treatment. Response to crizotinib therapy could not be obtained for the other equivocal cases confirmed to be *ALK*-positive, and this study was not primarily designed to assess response to therapy, as it was performed in a routine diagnostic setting, with clinical data being collected retrospectively where available. In light of the limited clinical data in this study, it seems that the equivocal group may be heterogeneous in their likely response to crizotinib. Investigation of additional instances of these rare cases may provide insights into new FISH patterns associated with sensitive *ALK* rearrangements. There is a need for data on the response to crizotinib of patients with discordant IHC and FISH results or ALK positivity close to 15%, to better understand how to interpret equivocal results.

Break-apart FISH testing remains widely regarded as the current gold standard for detecting *ALK* rearrangements. The use of a secondary FISH probe, such as the *ALK*/*EML4* TriCheck FISH probe, may be useful for the detection and secondary confirmation of *ALK* rearrangements, including those involving *EML4* as the translocation partner, especially for borderline cases or cases showing atypical signal patterns, where an additional unique *ALK* FISH probe can provide further confirmation of rearrangement and requires only one additional tissue section. Further work is needed to formally evaluate the response to therapy in patients who are negative or equivocal with the Abbott Molecular *ALK* break-apart probe, but who are positive with this second FISH probe, although, given the rarity of *ALK* rearrangement, and of atypical or equivocal patterns in particular, this may prove to be difficult.

Acknowledgements

We thank the National Foundation for Medical Research and Innovation and the Sydney Breast Cancer Foundation for their generous support and funding for this study.

Author contributions

C. Selinger performed the research and wrote the paper. W. Cooper analysed and interpreted the results. T. Lum performed the IHC work. C. McNeil contributed clinical follow-up data and interpretation. A. Morey performed FISH analysis and contributed interpretation. P. Waring contributed cases and analysis. B. Amanuel contributed cases and analysis. M. Millward contributed clinical follow-up data and

interpretation. J. Peverall contributed cases and analysis. C. Van Vliet contributed cases and analysis. M. Christie contributed cases and analysis. Y. Tran contributed cases and analysis. C. Diakos contributed clinical follow-up data and interpretation. N. Pavlakis contributed cases and analysis. A. Gill contributed cases and analysis. S. O'Toole designed the study, and analysed and interpreted the results.

Conflict of interests

W. Cooper has received honoraria from Pfizer Oncology and Lilly Oncology. P. Waring has received honoraria from Roche and Pfizer. S. O'Toole has received honoraria from Roche, Pfizer, and Lilly Oncology. A. Gill has received honoraria from Pfizer Oncology and Astra Zeneca. N. Pavlakis has participated in Lung Cancer Advisory Boards for Pfizer, and received honoraria. C. McNeil has been on Roche and Merck Sharpe and Dohme advisory boards (remuneration declined), and has given invited presentations for Novartis and Roche (remuneration declined). The other authors state that they have no conflicts of interest.

References

1. Kwak EL, Bang Y-J, Camidge DR *et al.* Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N. Engl. J. Med.* 2010; **363**; 1693–1703.
2. Camidge DR, Bang Y-J, Kwak EL, Shaw AT, Iafrate AJ. Progression-free survival (PFS) from a phase I study of crizotinib (PF-02341066) in patients with ALK-positive non-small cell lung cancer (NSCLC). *J. Clin. Oncol.* 2011; **29**(15_suppl); 2501.
3. Camidge DR, Kono SA, Flacco A *et al.* Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. *Clin. Cancer Res.* 2010; **16**; 5581–5590.
4. Weickhardt AJ, Aisner DL, Franklin WA, Varella-Garcia M, Doebele RC, Camidge DR. Diagnostic assays for identification of anaplastic lymphoma kinase-positive non-small cell lung cancer. *Cancer* 2013; **119**; 1467–1477.
5. Tsao MS, Hirsch FR, Yatabe Y eds. *IASLC atlas of ALK testing in lung cancer*. Aurora, CO: IASLC Press, 2013.
6. Perner S, Wagner PL, Demichelis F *et al.* EML4–ALK fusion lung cancer: a rare acquired event. *Neoplasia* 2008; **10**; 298–302.
7. Camidge DR, Skokan M, Kiatsimkul P *et al.* Native and rearranged ALK copy number and rearranged cell count in non-small cell lung cancer: implications for ALK inhibitor therapy. *Cancer* 2013; **22**; 3968–3975.
8. Selinger CI, Rogers T-M, Russell PA *et al.* Testing for ALK rearrangement in lung adenocarcinoma: a multicenter comparison of immunohistochemistry and fluorescent in situ hybridization. *Mod. Pathol.* 2013; **26**; 1545–1553.
9. Camidge DR, Theodoro M, Maxson DA *et al.* Correlations between the percentage of tumor cells showing an anaplastic lymphoma kinase (ALK) gene rearrangement, ALK signal copy number, and response to crizotinib therapy in ALK fluorescence in situ hybridization-positive nonsmall cell lung cancer. *Cancer* 2012; **118**; 4486–4494.
10. Conklin CMJ, Craddock KJ, Have C, Laskin J, Couture C, Ionescu DN. Immunohistochemistry is a reliable screening tool for identification of ALK rearrangement in non-small-cell lung carcinoma and is antibody dependent. *J. Thorac. Oncol.* 2013; **8**; 45–51.
11. Sholl LM, Weremowicz S, Gray SW *et al.* Combined use of ALK immunohistochemistry and FISH for optimal detection of ALK-rearranged lung adenocarcinomas. *J. Thorac. Oncol.* 2013; **8**; 322–328.
12. Sasaki T, Rodig SJ, Chirieac LR, Jänne PA. The biology and treatment of EML4–ALK non-small cell lung cancer. *Eur. J. Cancer* 2010; **46**; 1773–1780.
13. Mino-Kenudson M, Chirieac LR, Law K *et al.* A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry. *Clin. Cancer Res.* 2010; **16**; 1561–1571.
14. Eisenhauer EA, Therasse P, Bogaerts J *et al.* New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur. J. Cancer* 2009; **45**; 228–247.
15. Conde E, Suárez-Gauthier A, Benito A *et al.* Accurate identification of ALK positive lung carcinoma patients: novel FDA-cleared automated fluorescence in situ hybridization scanning system and ultrasensitive immunohistochemistry. *PLoS One* 2014; **9**; e107200.
16. Zwaenepoel K, Van Dongen A, Lambin S, Weyn C, Pauwels P. Detection of ALK expression in non-small-cell lung cancer with ALK gene rearrangements—comparison of multiple immunohistochemical methods. *Histopathology* 2014; **65**; 539–548.
17. Ciolino AL, Tang ME, Bryant R. Statistical treatment of fluorescence in situ hybridization validation data to generate normal reference ranges using Excel functions. *J. Mol. Diagn.* 2009; **11**; 330–333.
18. Ilie MI, Bence C, Hofman V *et al.* Discrepancies between FISH and immunohistochemistry for assessment of the ALK status are associated with ALK 'borderline'-positive rearrangements or a high copy number: a potential major issue for anti-ALK therapeutic strategies. *Ann. Oncol.* 2015; **26**; 238–244.