ALK Immunocytochemistry on Cell-Transferred Cytologic Smears of Lung Adenocarcinoma

Chen Zhang, MD, PhD1, Melissa L. Randolph, BS, SCT (ASCP)2, Kelly J. Jones, BS, CT (ASCP)2, Harvey M. Cramer, MD1, Liang Cheng, MD1, and Howard H. Wu, MD1.

Running title: ALK immunocytochemistry on cell-transferred smears

Key words: ALK, immunocytochemistry, cell-transfer

Author affiliations:

1. Department of Pathology and Laboratory Medicine, Indiana University

2. Cytopathology, Indiana University Health

Correspondence to: Howard H. Wu, MD

Department of Pathology and Laboratory Medicine
Indiana University School of Medicine
350 W 11th ST, Indianapolis, IN 46202

Tel: 317-491-6417

Email: hhwu@indiana.edu

This work had no specific funding.

There are no financial disclosures from any authors.

Abstract

**Background.** ALK immunohistochemical staining (IHC) on formalin fixed paraffin embedded tissue or cellblock (CB) has been reported as an effective alternative of fluorescence hybridization in situ (FISH) in the detection of *ALK* gene rearrangement. However, CBs frequently lack adequate cellularity even when the direct smears are cellular. This study is aimed to assess the utility of ALK immunocytochemical staining (ICC) on direct smears using the cell transfer (CT) technique in the detection of *ALK* rearrangement.

**Methods.** Fine needle aspiration (FNA) cases of lung adenocarcinoma in which ALK status had been determined by FISH on CB or concurrent biopsy were identified. ICC staining for ALK was performed on alcohol-fixed Papanicolaou-stained direct smears using the CT technique. ALK immunoreactivity was evaluated in a modified semiquantitative scale. Results were compared with those of FISH.

**Results:** A total of 47 FNA specimens were included. Five of 7 FISH positive cases showed positive ALK ICC staining (71.4%) and 39 of 40 FISH negative cases were negative on ALK ICC staining (97.5%). The overall correlation between ALK ICC and FISH was 93.6%.

**Conclusion:** ICC performed on the FNA smears using the CT technique is an alternative method for assessment of *ALK* rearrangement, especially when cellblock lacks adequate cellularity.
Anaplastic lymphoma kinase (ALK) gene rearrangements represent the primary oncogenic driver in 3-5% of patients with non-small cell lung cancers (NSCLCs) especially adenocarcinomas, with echinoderm microtubule-associated protein-like 4 (EML4) gene being the most frequent translocation partner. ALK inhibitor crizotinib has been associated with over 60% response rate in ALK-rearranged advanced NSCLCs, as shown by multi-center clinical trials. ALK status is now a required study for NSCLCs, recommended by multiple societies including the College of American Pathologists (CAP). In previous clinical trials, ALK status was determined by fluorescence in situ hybridization (FISH) using the Vysis break-apart probe set (Abbott Molecular, Des Plaines, Ill), which to date is the only diagnostic assay licensed for this purpose by the US Food and Drug Administration (FDA).

The main advantage of the FISH method is the possibility of detecting all types of ALK rearrangements known to date. However, specialized techniques and training are required for the implementation and interpretation of ALK FISH testing. Significant inter-observer variability was shown even in the hands of experienced specialists. Additionally, FISH as a screening assay is relatively expensive for most clinical settings. In contrast, immunohistochemistry (IHC) is relatively inexpensive, fast, and familiar to most pathologists. Zhou et al. studied ALK IHC on 410 resected lung adenocarcinomas and compared the results to corresponding FISH assays. They found that all 333 IHC negative cases were negative by FISH, and all 28 FISH positive cases were positive on IHC. A similar study using 465 resected NSCLCs by Paik et al. also showed that the sensitivity and specificity of IHC compared to FISH was 100% and 95.8%, respectively.
CAP guidelines recommend using a sensitive ALK IHC as screening test for ALK rearrangement on formalin-fixed paraffin-embedded (FFPE) tissue.5

With the advance of imaging-assisted biopsy techniques and fine-needle aspiration (FNA) cytology, the pathological diagnosis of lung cancer is more commonly done preoperatively on FNA material. The ALK FISH and IHC protocols used for FFPE tissue specimens can be used on cell blocks (CB) for immunocytochemistry (ICC) assays. However, although we perform rapid on-site evaluations on all FNAs performed at our institution and typically collect multiple dedicated passes for CB in lung cancer patients, the CBs still sometimes lack adequate cellularity for ALK testing even when the direct smears are cellular. As an alternative approach, if conventional CBs lack adequate cellularity, ICC can be performed on cell-transferred (CT) direct smears and multiple immunostains can be applied to a single direct smear using this technique.10-13 The aim of this study is to assess the reliability of the cell transfer (CT) technique for the immunocytochemical assessment of ALK status of aspirates of primary and metastatic lung adenocarcinomas.

**Materials and Methods**

This study was approved by the Institutional Review Board of Indiana University. A computerized search of the cytopathology and surgical pathology archives was performed. FNA cases diagnosed as primary or metastatic lung adenocarcinoma in which ALK status had been previously evaluated by FISH on a CB or concurrent surgical biopsy were identified over a period of 33 months (September 2011 through May 2014). The search yielded a total of 47 FNA specimens including FNAs from 26 primary lung
lesions and 21 metastatic lesions (lymph node 15, bone 2, liver 2, chest wall 1, and adrenal gland 1). Of these, formalin-fixed core biopsies were used for FISH in 8 cases and formalin-fixed CBs were used in 39 FNA cases.

The ethanol-fixed, Papanicolaou-stained direct smears from each case were reviewed, and the most cellular slide was chosen for CT. CT technique was performed as described previously\textsuperscript{13}. Briefly, after the removal of coverslip using xylene, a thin layer of Mount-Quick media (Daido Sangyo Co Ltd, Japan) was spread uniformly over the wet slide to entirely cover the cellular material. The slide was then placed in a 60°C heated oven for 3 hours or more until the media hardened. The cellular areas (at least 200 tumor cells) to be transferred were then marked using a permanent marker. After incubating in a 50°C water bath for 2 hours, the mounting media with the embedded cells was slowly peeled off the slide using forceps and a scalpel. The marked area was cut off and placed on a charged glass slide. The slide was dried in a 60°C oven for at least 2 hours. The dried slide was washed in four exchanges of xylene (15 min each) to remove the mounting media, and then was rehydrated with two exchanges of absolute alcohol, two exchanges of 95% alcohol, and two exchanges of deionized water (15 min each). The slide was stored in deionized water until ICC was performed.

ICC for ALK was performed on the cell-transfer slides using primary rabbit monoclonal anti-ALK antibody D5F3 (Cell Signaling Technology, Billerica, MA) with Dako EnVision detection kit. No modifications were made in the immune-staining process on the cell-transferred slides compared with formalin-fixed tissue. The results were scored by two independent observers (C.Z. and H.H.W.) using a modified semi-quantitative
graded criteria described previously \(^8\) (Table 1). ICC results were excluded from the study if the CT slide contained less than 200 cells. ICC results were compared with those of FISH performed on the correlating CB or core biopsy.

**Results**

The results of the ICC on the CT smears and results of FISH on correlating formalin-fixed CB or core biopsy were summarized in Table 2.

All 47 FNA specimens were adequately cellular for evaluation. The correlating FISH studies showed that 40 were negative and 7 were positive for \(\text{ALK} \) rearrangement.

Five of 7 FISH positive cases (71.4%) showed positive ALK ICC staining, with 4 cases showing 3+ staining (Figure 1) and one case showing 2+ staining. Two FISH positive cases were negative on ICC (score 1+, Figure 2), and the concurrent core biopsy and cell block of the two cases were also negative for D5F3 immunostaining (score 0). Both false negative cases showed borderline cellularity (approximately 200 tumor cells) and relatively low percentage of tumor cells with ALK rearrangements on FISH test (20% and 22%).

Thirty-nine of 40 FISH negative cases (97.5%) were negative on ALK ICC staining. All 39 ICC negative cases showed no staining (score 0). One FISH negative case showed strong ICC staining (score 3+) on the cell transferred smear (Figure 3) and the section cut from the cell block also demonstrating 3+ expression for D5F3 immunostaining. ALK FISH test of this case showed 0% tumor cells with ALK rearrangements and no sign of ALK gene copy number gain due to polysomy or amplification.
The negative predictive value of ALK ICC is 95%. The overall correlation between ALK ICC and FISH was 93.6%.

**Discussion**

Our study shows that the overall correlation between ALK FISH and ALK ICC using CT technique is fairly strong (93.6%) and the negative predictive value of ALK ICC is high (95%). ALK rearrangement is a rare phenomenon in lung cancers, with a reported incidence rate of only about 3-5%. As a result, the majority of samples received for ALK rearrangement testing will be negative. The high negative predictive value of ALK ICC makes it particularly useful for ALK rearrangement detection, since it may be used to screen out the majority of the negative samples at a relatively high confidence level. The negative result by ALK ICC is even more reliable when other mutations such as EGFR mutations are identified in the same sample, since ALK rearrangements are almost mutually exclusive with other mutations.

Although ALK ICC has a fairly high negative predictive value, false negatives do exist. Two of 41 (5%) ALK ICC negative cases were tested positive using FISH on concurrent biopsy tissue or CB. Both those cases had low percentage (20% and 22%) of tumor cells with ALK rearrangements on FISH test, which may count for a possible cause of false negative. According to the manufacturer’s recommendation, 15% or more tumor cells with split signals are considered positive on ALK FISH assays. The actual percentage of tumor cells with ALK rearrangements is usually not reported. A recent study by Ilie et al\textsuperscript{14} compared the FISH and IHC results on resected lung adenocarcinoma, and found that all 5 discordant cases with FISH+/IHC- profile had low percentage of ALK+ tumor cells.
on FISH (15-20%). It is conceivable that tumors with low percentage of ALK+ tumor cells on FISH would express ALK protein at a lower level and/or in a more focal distribution, compared to those with high percentage of ALK+ tumor cells. Focal or weak expression of ALK protein may not be detected due to the limited cellularity of the transferred cytology smears. Both of our two false negative cases had low to borderline cellularity on cell-transferred smears. Choosing more cellular smears would likely reduce or eliminate false-negative results in these cases; however, it is not always feasible. In our own practice, if the CB and the direct smears are both inadequate in cellularity, a repeat sampling is recommended.

Another possible explanation of the false negative result is rare ALK translocations that do not result in ALK protein overexpression, or result in protein expression that is not recognized by this antibody clone. The study by Ilie et al\textsuperscript{14} showed that all five FISH+/IHC- cases did not contain the most frequent EML4-ALK fusion transcripts as revealed by RT-PCR analysis.

The false negative results were not likely due to loss of antigenicity during the CT procedure, since results of ICC performed on cell-transferred cytology smear have shown high levels of agreement (97.5%) with those of corresponding FFPE tissues in our previous study\textsuperscript{12}.

One case with strong staining on ICC was negative for ALK rearrangements by FISH on concurrent CB, and IHC for ALK (D5F3) performed on the same CB also demonstrated 3+ staining. Previous studies also reported rare cases in which ALK positivity by IHC was not reproduced by FISH. False-negative results of FISH may happen due to technical
difficulties in the interpretation of results. For example, the splitting of the red and green
signals can be extremely subtle, especially in interphase chromatin since the splitting of
signals is a consequence of a paracentric inversion on the same chromosomal arm and the
splitting signals are only about 12Mb apart. The percentage of ALK+ tumor cells can be
underestimated due to admixed normal cells. Another possible explanation is ALK
protein overexpression caused by unknown types of ALK rearrangements or by events
other than ALK rearrangements. ALK gene copy number gain due to polysomy has been
reported to cause ALK protein overexpression detected by IHC\textsuperscript{14}, although the false-
positive case in our current study did not have evidence of ALK gene copy number gain
on FISH test. Some authors reported significant clinical improvement with crizotinib in
patients with tumors that were ALK-negative by FISH but were found to be ALK-
positive by IHC\textsuperscript{15}.

Our results are in agreement with those of previous studies using ICC to detect ALK on
direct smears. Savic et al\textsuperscript{16} tested ALK ICC staining using the same clone (D5F3) on 41
Papanicolaou-stained cytology slides after decolorization and found a high concordance
rate when compared with FISH results. Another smaller scale study on 18 cases by
Tanaka et al\textsuperscript{17} showed high concordance rate between ALK ICC performed on destained
direct smears and IHC performed on correlating FFPE tissue. Our study is different from
these previous studies in that CT technique is used prior to ICC. CT technique has several
advantages. The transferred smear can be divided into several parts so that multiple
immunostains as well as multiple molecular tests can be performed from a single cellular
smear. This is particularly important in the frequent situation when only a few cellular
smears are available, but there is need to perform other diagnostic immunostains, such as
TTF-1 to rule out metastatic adenocarcinoma from other primary sites. At our institution, we routinely perform EGFR, KRAS molecular testing and ALK ICC on the same cellular smear. For these patients, using the CT technique reduces the need for repeat FNAs, thereby reducing potential patient morbidity and health care costs. Technically, CT is simple to perform and can be easily learned by a cytotechnologist or histotechnologist. There is no requirement for special equipment to perform this technique and the cost is relatively low. The only disadvantage of CT technique is that at least 5 hours of manual preparation is required to perform this technique, and this usually results in delaying the case for another day.

Although there have been evidences that ALK IHC can be used as a screening test for FISH test, we don’t have enough evidence for using ALK ICC on cell-transferred smear as a screening test. In our current practice, ALK FISH test performed on tissue or CB is still the first choice for detecting ALK rearrangements. ALK ICC is performed only when the CB lacks adequate cellularity. Clinicians are informed of the possibility of a false negative ALK ICC test. Decisions of whether or not to repeat biopsy are made incorporating the ALK ICC result with other factors such as age, gender, smoking history, and EGFR/KRAS mutation status.

The major limitation of current study is the relatively small sample size, especially the small number of FISH positive cases (n=7). Common limitations of a retrospective study, such as selection bias and information errors, also exist. A prospective study with a large number of cases is needed for further characterization of the test.

In conclusion, our study showed that ICC performed on the FNA smears using the CT
technique is an alternative method for assessment of $ALK$ rearrangement especially when the direct smears are highly cellular and the CB lacks adequate cellularity.
Table 1. Scoring system for ALK by ICC.

<table>
<thead>
<tr>
<th>Score</th>
<th>Staining pattern</th>
<th>ALK status</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No staining</td>
<td>Negative</td>
</tr>
<tr>
<td>1+</td>
<td>Faint, focal cytoplasmic staining in less than 50% of tumor cells</td>
<td>Negative</td>
</tr>
<tr>
<td>2+</td>
<td>Moderate, granular cytoplasmic staining (also can partly present strong staining) in more than 50% of tumor cells</td>
<td>Positive</td>
</tr>
<tr>
<td>3+</td>
<td>Strong, granular cytoplasmic staining in more than 75% tumor cells, diffusely homogeneity in distribution.</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Table 2. Comparison between the results of ALK ICC and ALK FISH.

<table>
<thead>
<tr>
<th>ALK ICC</th>
<th>ALK FISH positive</th>
<th>ALK FISH negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (2+/3+)</td>
<td>5 (10.6%)</td>
<td>1 (2.1%)</td>
<td>6 (12.8%)</td>
</tr>
<tr>
<td>Negative (0/1+)</td>
<td>2 (4.3%)</td>
<td>39 (83.0%)</td>
<td>41 (87.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>7 (14.9%)</td>
<td>40 (85.1%)</td>
<td>47 (100%)</td>
</tr>
</tbody>
</table>

Abbreviations: ALK, anaplastic lymphoma kinase; FISH, fluorescence in situ hybridization; ICC, immunocytochemistry.
**Figure Legends**

Figure 1. Representative images of cases that were positive on ALK ICC and FISH. A) Papanicolaou-stained direct smears before CT. B) ALK ICC staining using D5F3 antibody. Original magnification: 400X.

Figure 2. Representative images of cases that were ALK ICC negative but FISH positive. A) Papanicolaou-stained direct smears before CT. B) ALK ICC staining using D5F3 antibody. Original magnification: 400X.

Figure 3. Representative images of the case that was ALK ICC positive but FISH negative. A) Papanicolaou-stained direct smears before CT. B) ALK ICC staining using D5F3 antibody. Original magnification: 400X.
References


