Validation of a simplified approach to detect ALK translocations in lung cancer samples by FISH

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Background: ALK rearrangements occur in 2-7% of lung adenocarcinomas (LAC) and represent targets for a class of different therapeutics that are already approved or in advanced clinical trials (i.e., Crizotinib, Ceritinib, Lorlatinib, Brigatinib). ALK alterations can be detected by sequencing, immunohistochemistry or FISH.

FISH has several advantages:
- that of an immediate morphologic control (provides data from tumor cells without background from non-neoplastic tissue),
- does a direct genomic information,
- has the capability to detect even focal aberrations in heterogeneous tumors
- rapid technology (turn around time only one to two working days)
- works on cytologic smears, cell blocks and histologic material, even with low tumor cell content.

The reason why FISH is thought to be so laborious is the large number of cases to be evaluated. This is due to the fact that aberrant signal patterns can occur (or be missed) incidentally by standard protocols, an effect which needs to be eliminated by large numbers of tumor cells counted. Therefore, we aimed to implement and validate an approach of a simplified ALK FISH analysis.

Design: We investigated a total of 374 LAC with two different ALK FISH probes, the Vyysis LSI ALK Dual Color Break-Apart Rearrangement Probe (Abbott: conventional protocol) and ZytoLight® SPEC ALK/EM4 TriCheck™ Probe (ZytoVision: simplified approach). The most important difference to notice is that the ZytoLight probe is a three colored (green, orange, aqua) combination of a break-apart and fusion probe. Therefore any signal separation between green and orange probes flanking the ALK gene, even <1 signal distance, counts as positive, but exclusively if a colocalization of 3’-ALK (orange) and EML4 (aqua) is present. This pattern indicates an ALK-EM4 inversion involving the chromosomal regions 2p23 and 2p21. The small distance between orange and green signals in these cases is due to the fact that both genes are separated by only 13 Mbase pairs.

We screened 366 LAC prospectively for ALK rearrangements and enriched the series with eight tumor known to be ALK positive. All tumors with both probes were evaluated by one pathologist (HUS) but at different times, therefore blinded for the results of the other probe. A second pathologist (KS) randomly evaluated 122/374 LAC with both probes, blinded for the results of the first analyst and the other probe.

Results: The evaluation of 20 tumor cells with the ZytoLight ALK probe (simplified approach; see Table 1 for reading strategy) resulted in a sensitivity and specificity of 100% compared to the Vyysis probe (Figures 2 & 3). Only 1% of the samples (five tumors) fell into the equivocal range requiring additional counting. 99% of all LAC could be evaluated with only 20 cells. There was an interobserver concordance of 100% based on positive/negative of tumors but percentages of aberrant cells varied between both readers (data not shown). The frequency of aberrant cells was mostly higher with the simplified approach. This led to an increased safety by reduction of those cases close the cut-off level of 15% with the Vyysis probe.

Conclusion: We could demonstrate a novel sensitive and specific evaluation approach for ALK fluorescence in situ hybridization that 99% of all LAC need an analysis as little as 20 tumor cells. The introduction of an equivocal range avoids false positive results. The advantage of the combined break-apart/fusion probe is clearly that smaller distances between orange/green can be judged as positive if there is an ALK-EM4 fusion with orange/aqua colocalization. The results in those cases become more obvious with a higher percentage of aberrant cells. The number of cases close to the cut-off is reduced. The expenditure of time per case was approximately halved. Furthermore material with low tumor cell content might still be evaluable. This novel evaluation approach is time-saving and safe, and even in cases with a low tumor cell count reliable result can be obtained.

Table 1: Comparison of the two different ALK FISH probes

<table>
<thead>
<tr>
<th>Probe composition</th>
<th>Conventional protocol</th>
<th>Simplified approach</th>
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<tbody>
<tr>
<td>Probe design</td>
<td>dual colour probe</td>
<td>triple colour probe</td>
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<tr>
<td></td>
<td>- orange ALK-3'</td>
<td>- orange ALK</td>
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<tr>
<td></td>
<td>- green ALK</td>
<td>- green ALK (LTZ)</td>
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<td></td>
<td></td>
<td>- green EML</td>
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<td></td>
<td></td>
<td>- aqua EML (KS)</td>
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<tr>
<td>Vendor</td>
<td>Abbott/Vyysis</td>
<td>ZytoVision</td>
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<td>Characteristics of aberrant tumor cells</td>
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<tr>
<td>A) Isolated orange</td>
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<td>B) Break apart (2 signal diameters apart)</td>
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<td>C) Co-occurrence of both</td>
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Scoring guidelines

1. score 50 cells
   - negative < 10% aberrant cells
   - positive > 10% aberrant cells
   - equivocal: 10-30% aberrant cells
2. if result is equivocal second reader should score 50 cells and results should be added (total 100 cells)
   - negative < 10% aberrant cells
   - positive > 10% aberrant cells

Positive cases with 15-20% aberrant cells

4 cases of 20 ALK positive tumors

Advantages of the simplified approach

- 100% specificity, 100% sensitivity compared with conventional protocol
- reduction of reading time (> 90%)
- increased safety (reduction of positive cases with only 15-20% aberrant cells)
- allows evaluation of samples with only few tumor cells (i.e., 20 cells)
- provides genomic information on ALK translocation partner (EML4 or non-EML4)

Figure 1: ALK FISH probe design of the ZytoLight® SPEC ALK/EM4 TriCheck™ Probe

Figure 2: FISH pattern of ALK-EM4 fusion with small signal separation showing orange/aqua colocalization. Different fluorescence colors (A) fusion of B-D, B) ZyGreen™/ZyOrange™ Dual Bandpass Filter, C) aqua, D) DAPI.

Figure 3: Correlation of the amount of aberrant cells between the conventional protocol and the simplified approach (cut-off level of 15%).