NSCLC Patients with an Isolated 5′ Green Signal Pattern in ALK FISH Testing May Be Positive for Complex EML4-ALK Rearrangement with 3′ Deletion and May Benefit from Crizotinib Targeted Therapy

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Introduction

Non-small cell lung cancer (NSCLC) is a molecularly heterogeneous disease. Anaplastic lymphoma kinase (ALK) gene rearrangement is seen in ~5% of patients with NSCLC, most commonly resulting from an intrachromosomal inversion, inv(2)(p21p23) leading to oncogenic fusion that joins the EMAP-like protein 4 (EML4) and ALK with 15 different variants reported to date.[1-5] Accurately identifying patients who are positive for ALK gene rearrangement is essential for selecting effective crizotinib targeted therapy. Currently the result reporting guide in the FDA approved companion diagnostic FISH test using breakapart (ba) probes considers only the separation and isolated 3′ red signal patterns as positive. However, we have periodically observed the isolated 5′ green pattern in 3 – 4% of cases with structural ALK abnormalities. We applied an EML4-ALK fusion translocation probe on 10 cases with an isolated 5′ green signal pattern to confirm the EML4-ALK rearrangement. Based on case study and genomic position mapping of the ALK ba probe in relation to the ALK gene and its tyrosine kinase domain (Figure 1), we conclude that the 3′ flanking red probe of the Abbott® ALK probe set does not cover the ALK gene per se, whereas 5′ probe covers the entire intracellular tyrosine kinase domain. The 3′ red probe deletion with a remaining 5′ green signal only may not necessarily affect the transcription and expression of rearranged EML4-ALK and may represent a regional complex chromothripsis event in NSCLC. Patients showing this FISH pattern should be considered positive for the EML4-ALK rearrangement and hopefully eligible for crizotinib treatment in the foreseeable future.

Materials and Methods

FISH was tested on FFPE tumor tissues following ALK ba probe kit instructions (Abbott® Molecular). A confirmative EML4-ALK inversion/translocation FISH testing was performed using a probe set by Kreatech (Figure 2) with a 360kb 3′ALK red probe and a 350kb 5′EML4 green probe. Ten cases with an isolated 5′ green signal were included in this study. In addition, 3 cases with diminished signal patterns were presented to back our discussion/conclusion. Briefly, tumor areas were selected by reviewing pathologists, serial unstained 5-µm sections of FFPE tumor tissue were prepared for FISH with deparaffinization and pretreatment performed using the VP 2000 Processor (Abbott® Molecular) and corresponding programs. The StatSpin® ThermoBrite® was utilized for the denaturation (73°C for 6 minutes) and hybridization process (37°C overnight). Attempts were made to obtain available information on clinical, pathologic and other lab findings such as EGFR and KRAS mutations. The ALK gene location including its tyrosine kinase domain and the ALK ba probe coverage and their corresponding genomic positions were derived from UCSC-hg19, 2009 (Figure 1).

Results

Isolated 5′ green signals along with fusion signals were observed in 48 – 100% of interphase cells (median 70.5%) by the ALK ba probe, and all 10 cases were positive (20-92%; median 36%) by the EML4-ALK probe with one to multiple copies of fusion signals. Six patients had EGFR and 4 had KRAS mutation tests done; two of them were positive for the L858R in exon 21 and the L747_P753del7insS in exon 19 respectively for the EGFR gene. This is significant compared to 2 positives out of 83 ALK ba probe positive cases during the same testing period. One of the 4 patients with KRAS mutation detection done showed a p.G12C (c.34G>T) mutation in codon 12 **(Table 1)**. **Figure 4** shows two representative cases with isolated 5′ green signal patterns by the ALK ba probe and the EML4-ALK inversion positive fusion signals by the translocation probe. Figure 5 shows diminished signal patterns in three cases with partial probe deletions (5′ deletion in 2 and 3′ deletion in 1), all of these three cases were positive for EML4-ALK rearrangement by the translocation probe. One of two cases with diminished (partially deleted) 5′ probe signal also showed a coexistence of the L861Q mutation in exon 21.

Mapping the ALK ba probe shows that the green probe (chr2:29307035-29749070) covers 3′ C-terminal of the ALK gene including the entire tyrosine kinase domain (chr2:29419636-29445481), but the red probe (chr2:28972311-29272436) does not cover any portion of the ALK gene (chr2:29416093-30143505) **(Figure 1)**.

Results

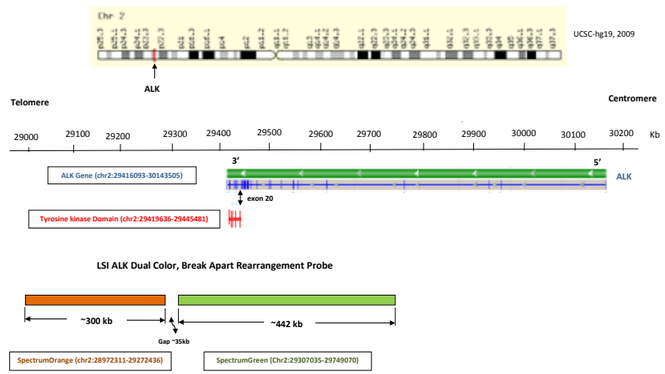


Figure 1
Genomic Positions of ALK Gene and LSI ALK Break Apart Rearrangement Probe

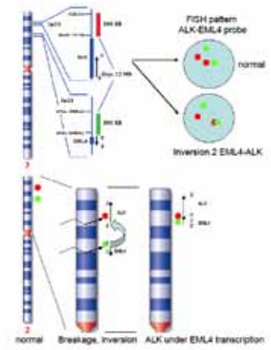


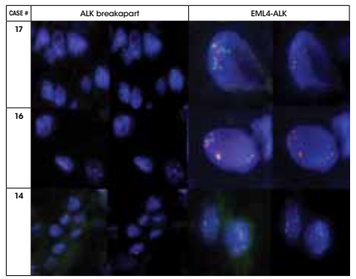
Figure 2
The EML4-ALK inversion/translocation probe signal patterns and the underlying chromosomal rearrangement (courtesy of Kreatech, Inc., Durham, NC)

Case #	Results	% POS Abbott® FDA	% POS Kreatech	EGFR	KRAS
1	NEG w 3′ del	68	36	NEG	NEG
2	NEG w 3′ del	72	34	NEG	POS
3	NEG w 3′ del	59	30	ND	ND
4	NEG w 3′ del	63	32	POS	NEG
5	NEG w 3′ del	81	92	ND	ND
6	NEG w 3′ del	62	20	NEG	ND
7	POS 3′ del	90	64	ND	ND
8	NEG w 3′ del	69	56	ND	ND
9	NEG w 3′ del	100	38	POS	NEG
10	NEG w 3′ del	90	88	NEG	NEG
11	POS w dim 5′	74	34	POS	FAILED
12	POS w dim 5′	92	58	ND	ND
13	POS w dim 3′	82	82	NEG	NEG
14	POS Control w 5′ del	88	66	NEG	NEG
15	POS Control	88	40	NEG	ND
16	POS Control	70	80	NEG	ND
17	NEG Control	0	4	ND	ND

- ND = not done
- dim = diminished signal

Table 1
Result Comparison Using ALK ba (ABBOTT®) and EML4-ALK (KREATECH) Probes

Figure 3

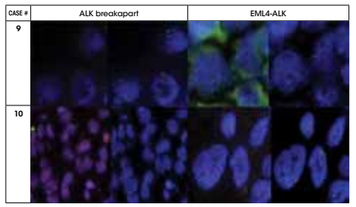


Negative control: Case 17 shows multiple copy of ALK with no rearrangement and a normal EML4-ALK pattern.

Positive control: Case 16 shows a separation of red and green signals and an abnormal fusion positive pattern for the EML4-ALK probe.

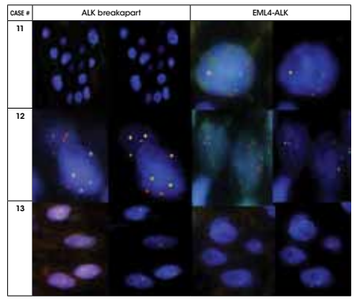
Positive control: Case 14 shows an atypical pattern with fusions and red only signals and an abnormal fusion positive pattern for the EML4-ALK probe.

Figure 4



Two cases show an isolated green signal pattern which is considered negative by the FDA guidelines. Concurrent EML4-ALK probes on the same patients are fusion positive.

Figure 5



Cases 11 and 12 show abnormal diminished signal patterns due to a partial deletion of the 5′ green signal probe, and the concurrent EML4-ALK probe tests are fusion positive. Case 13 shows a 3′ red diminished (partially deleted) probe signal, and the concurrent EML4-ALK probe test is fusion positive.

Discussion and Conclusion

1. NSCLC is biologically and genomically heterogeneous. The less commonly observed FISH pattern of isolated 5′ green signal may well represent a molecular subset of complex EML4-ALK gene rearrangements with a concomitant 3′ red probe deletion confirmed by an inversion/translocation FISH test in our study. The complex chromosomal alterations and the higher dual mutation rate with EGFR/KRAS suggest clonal selection or regional chromothripsis. We suggest that further verification by RT-PCR should be performed as a reflex test. Based on our findings and experience, this pattern accounts for 3 – 4% of structurally altered ALK abnormal cases or ~0.2% of all tested cases in ALK ba FISH testing (14/6945): 4 of the 1,155 cases prior to crizotinib approval and 10 of the 5,790 post approval. These patients are currently considered negative for ALK rearrangement[4-9] and are missing the crizotinib targeted therapy.
2. It might be misconceived with the probe mapping information that the tyrosine kinase domain is covered by the 3′ red probe of the ALK ba probe set and thus the red signal needs to be present to be considered ALK rearrangement positive. The red probe is actually located outside the ALK gene per se, whereas the green signal covers the intracellular portion of the gene including the tyrosine kinase domain as shown in **Figure 1**. Even though the recombination of ALK with EML4 or other genes in all variants up to date are involved in exon 20, regional or whole genome rearrangements in NSCLC are very complex and some reported cases showed characteristics of chromothripsis.[10-12] Studies on NSCLC genomes with this isolated 5′ green signal FISH pattern and the cases with partial deletions of either the 3′ or the 5′ probe by next-generation sequencing including deep sequencing will reveal genomic alterations of various FISH patterns that are currently considered negative.
3. It is intriguing to note that 2 of the 10 cases with an insolated 5′ green signal pattern and 1 of the 3 cases with a diminished signal pattern were positive for a coexisting EGFR mutation. Although EGFR mutations are anticorrelated with KRAS mutations, coexistence of EGFR mutations and ALK rearrangements has been occasionally reported; this is significant compared with ALK ba positive cases. NSCLC with an isolated 5′ green signal pattern or other variant patterns may be indicative of genomic complexity, chromothripsis and/or intra-tumor heterogeneity with the presence of multiclones or clonal evolution in tumor progression.[11-13] Receptor tyrosine kinase coactivation networks in cancer is also a recent subject of intense investigation as they play critical roles in influencing tumor response to targeted therapeutics and manifestation of cancer phenotypes.[14,15]

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