Targeted inhibition in tumors with ALK dependency

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Abstract: The oncogenic function of gene translocations involving the anaplastic lymphoma kinase (ALK) was first reported in rare subtypes of non-Hodgkin’s lymphoma almost two decades ago. More recently, aberrant ALK signaling was found to be an oncogenic driver in subsets of non-small cell lung cancer (NSCLC), particularly in patients with little or no tobacco smoking history. The advent of molecularly targeted therapies that inhibit ALK has allowed the pairing of ALK inhibitors such as crizotinib as treatment for ALK-positive NSCLC, yielding dramatic responses and long-term disease control. The clinicopathologic features of ALK-driven NSCLC, the clinical development of ALK inhibitors, and the genetic determinants of acquired resistance to ALK inhibition are among the topics covered in this review.

Keywords: targeted inhibition, tumors, ALK dependency

Introduction

Lung cancer has traditionally been regarded as a collection of diseases defined by characteristic histologic features, primarily encompassed by the classifications of adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell carcinoma. However, an appreciation for the importance of genetic subtypes in non-small cell lung cancers (NSCLC) was ushered in by the discovery of activating mutations (eg, del746_A750 or L858R) in the epidermal growth factor receptor gene (EGFR) in a subset of NSCLCs.1–3 This was the first understanding that some lung cancers could be dependent on one gene or signaling pathway for the maintenance of the malignant phenotype, a phenomenon termed “oncogene addiction”.4 The dependency of these mutant NSCLCs on aberrantly active EGFR signaling confers exquisite sensitivity to inhibition by small molecule tyrosine kinase inhibitors, such as erlotinib and gefitinib. Previous to this discovery, many years of clinical investigation had been spent without an appreciation for the role of tumor genetics as a powerful predictor of patient outcome upon treatment with erlotinib and gefitinib.

Remarkably, another genetic subtype of lung cancer emerged with the discovery of oncogenic anaplastic lymphoma kinase (ALK) fusion genes in NSCLC.5 ALK is a member of the insulin receptor superfamily of receptor tyrosine kinases. In normal human physiology, ALK expression is limited to the adult brain, and the function is unclear, although it may be involved in specific aspects of neuronal development.6,7 Preclinical studies in ALK knockout mice have identified a role for ALK in the hippocampus, particularly involving neurogenesis and spatial memory.8,9 The role of ALK in oncogenesis was first established by the cloning of the fusion gene
nucleophosmin-ALK in anaplastic large cell lymphoma.\textsuperscript{10} While ALK fusions can result from translocation at multiple chromosomal sites, the NPM-ALK t(2;5) translocation occurs in approximately 80\% of cases of anaplastic large cell lymphomas that harbor ALK activation. In anaplastic large cell lymphoma, ALK is activated via proximity to strong 5′ promoters, and/or through inappropriate oligomerization leading to constitutive ALK activation.\textsuperscript{11} A role for ALK in lung cancer was discovered when NIH3T3 cells were infected with a retroviral cDNA expression library prepared from a clinical lung adenocarcinoma specimen. Recovery of cDNA from one of the transformed foci revealed a fusion gene encoding a portion of the echinoderm microtubule-associated protein-like protein (EML4) fused to the tyrosine kinase domain of ALK.\textsuperscript{3,5} EML4-ALK fusions result from rearrangement within chromosome 2 (inv (2)(p21p23)) and fusion of the 5′ portion of EML4 with the 3′ portion of ALK. The resulting chimeric protein contains an N-terminus derived from EML4 and a C-terminus that contains the entire intracellular tyrosine kinase domain of ALK. Multiple forms of the EML4-ALK fusion have since been identified, all of which encode the same cytoplasmic portion of ALK, but contain varying lengths of EML4.\textsuperscript{12,13} In NSCLC, ALK may fuse with a variety of other partners including TFG (TRK-fused gene), KIF5B, PTPN3, and KLC1, although these fusion products are comparatively rare.\textsuperscript{14-17}

Multiple centers have since screened retrospective series of NSCLCs for the presence of ALK rearrangements using multiple diagnostic approaches. Several clinicopathologic features have emerged as characteristic for ALK gene rearrangement, and are discussed as follows.

Age
The presence of ALK fusion proteins is associated with a relatively young population of NSCLC patients. A set of 266 primary NSCLCs from Chinese patients was analyzed by reverse transcriptase-polymerase chain reaction and sequencing, as well as immunohistochemistry. The median age of 209 patients with adenocarcinoma histology was 64 years, which was also the median age of patients whose tumors were negative for ALK, while the median age of ALK-positive cases was 59 years.\textsuperscript{18} Similarly, in another series, EML4-ALK tumors were identified in patients with an average age of 56 years versus 64 years for EML4-ALK-negative patients.\textsuperscript{19} Shaw et al reported a median age of 52 years for patients with ALK-rearranged tumors as compared with a median age of 64 years in patients with wild-type tumors.\textsuperscript{20} Similar results were reported by Rodrig et al.\textsuperscript{21}

Smoking
ALK fusions in NSCLC have been detected in tumors from both smokers and nonsmokers.\textsuperscript{18} However, multiple studies have observed that EML4-ALK is strongly associated with a history of never or light smoking.\textsuperscript{18-24} In one of these series, a >10 pack-year smoking history was seen in only four of 47 (9\%) ALK-positive patients.\textsuperscript{20}

Tumor histology
EML4-ALK gene fusions have been identified predominantly in NSCLCs with adenocarcinoma histology. In the series from Wong et al, 11 of 13 EML4-ALK-positive cases were found in 209 adenocarcinomas, while the remaining two cases were classified as “other” histology.\textsuperscript{19} Kim et al identified ALK rearrangement by fluorescence in situ hybridization (FISH) in 19/465 cases of lung cancer, with 18 positive cases occurring in adenocarcinomas and one case classified as “other”.\textsuperscript{25} Interestingly, EML4-ALK-positive cases are significantly more likely than EGFR mutant or wild-type tumors to have a solid pattern with abundant signet ring cells,\textsuperscript{20,21,26-27} although the biological or clinical significance of signet ring cells in EML4-ALK lung cancers is unclear. Another characteristic of ALK-positive NSCLCs is correlation with thyroid transcription factor-1 positivity, suggesting that these cancers may have a terminal respiratory unit etiology.\textsuperscript{19}

Prevalence
In the initial report by Soda et al, 75 NSCLCs in Japanese patients were screened for EML4-ALK by reverse transcriptase polymerase chain reaction, with identification of the fusion gene in five of 75 (6.7\%) cases.\textsuperscript{5} Institutional series have reported ALK fusions in similar percentages of unselected NSCLC patient populations. Several studies in Asian lung cancer populations have identified EML4-ALK in 4\%\textsuperscript{25} and 5.3\%\textsuperscript{18} of NSCLCs, and 4.3\% of lung adenocarcinomas.\textsuperscript{19} Similarly, ALK rearrangements were identified in 5.6\% (20/358) of lung adenocarcinomas from a Western patient population.\textsuperscript{20} Despite its rarity, the identification of ALK-rearranged tumors can be facilitated through enrichment using the clinicopathologic characteristics noted above. For instance, in a cohort of 141 NSCLC patients enriched to identify tumors with ALK rearrangement or EGFR mutation, 13\% harbored EML4-ALK, and 22\% harbored an activating EGFR mutation, and these mutations were mutually exclusive.\textsuperscript{20} However, subsequent studies have reported concurrent EGFR mutations and ALK rearrangements in rare NSCLC tumors.\textsuperscript{28} In the cohort of 141 NSCLC patients, the percentage of EML4-ALK positivity increased to 33\% in
patients who were never/light smokers and whose tumors did not contain EGFR mutations.\textsuperscript{20}

**Cellular pathways involved in ALK signaling**

Wild-type ALK is a receptor tyrosine kinase belonging to the insulin receptor superfamily, and ligand binding leads to dimerization and kinase activation. ALK fusion genes encode proteins that generally reside in the cytoplasm rather than in the plasma membrane, and these fusion proteins are constitutively active independent of ligand. ALK activation results in phosphorylation on Y1604, and in turn triggers multiple downstream signaling pathways, including those for RAS/MEK/ERK, JAK/STAT, and PI3K. Roles for each of these pathways have been demonstrated for NPM-ALK in anaplastic large cell lymphoma.\textsuperscript{29} In the case of EML4-ALK, in vitro studies have demonstrated activation of MEK/ERK signaling, with some studies also indicating roles for STAT3 or PI3K/AKT pathways.\textsuperscript{23,30–32}

In the setting of anaplastic large cell lymphoma, preclinical work had explored the ability of selective ALK inhibition to affect tumor growth. Small hairpin RNAs against the catalytic domain of ALK led to apoptosis and tumor regression in ALK-positive anaplastic large cell lymphoma cell lines, demonstrating the promise of ALK inhibition as a potential therapeutic strategy in patients with this disease.\textsuperscript{33} Subsequently, small molecule compounds of the diamino-pyrimidine and aminopyrimidine classes (TAE684 and PF02341066) were generated\textsuperscript{34,35} and shown to target ALK selectively in vitro.\textsuperscript{36} PF-02341066, (R)-3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine (subsequently termed crizotinib) was identified as an inhibitor of the ALK and mesenchymal-epithelial transition factor (c-MET) tyrosine kinases through biochemical screens. In cell-based assays, crizotinib inhibited the tyrosine phosphorylation of NPM-ALK with an IC\textsubscript{50} of 24 nmol/L in two anaplastic large cell lymphoma cell lines, Karpas 299 and SU-DHL-1. Crizotinib inhibited wild-type and activated MET with an IC\textsubscript{50} of 11 nmol/L across a panel of multiple cell lines, and was found to be selective for ALK and MET over a panel of more than 100 other kinases.\textsuperscript{36} In addition, crizotinib has subsequently been found to have significant activity against ROS1 kinase.\textsuperscript{37}

**Clinical experience with ALK inhibition in lung cancer**

A first-in-human Phase I clinical trial of crizotinib in patients with advanced solid tumors was initiated in 2006 at three US institutions. Using a standard 3 + 3 dose-escalation design, the trial established continuous twice-daily administration of 250 mg crizotinib as the recommended Phase II dose. The drug was well tolerated, with mild gastrointestinal toxicities being the most common drug-related adverse events.\textsuperscript{38} The dose-escalation portion of the trial was in progress at the time when EML4-ALK was first discovered in NSCLC.\textsuperscript{5} At that time, under an institutional research protocol, early efforts were being made to screen clinical NSCLC samples for the ALK fusion gene by FISH. Early indications of the activity of crizotinib were observed when two NSCLC patients who had progressed on multiple prior therapies for advanced disease, and whose tumors harbored ALK gene rearrangement, experienced marked symptomatic improvement while being treated on the dose-escalation trial.\textsuperscript{39}

This prompted expansion of the Phase I trial to include five additional investigational sites (representing the US, Australia, and Korea), and large-scale screening of NSCLCs for ALK gene rearrangements by FISH was undertaken. By a data cutoff of April 2010, approximately 1500 NSCLC tumors had been screened for ALK gene rearrangement, and 82 patients with ALK-rearranged tumors were enrolled into an expansion cohort of the Phase I clinical trial receiving crizotinib 250 mg twice daily.\textsuperscript{39} The data from this ongoing trial were subsequently updated to include 119 ALK-positive NSCLC patients enrolled with a data cutoff of October 29, 2010.\textsuperscript{40} The baseline clinicopathologic features that emerged from this trial reflected the unique characteristics previously reported in NSCLC tumors with ALK gene rearrangement. Of the 119 patients enrolled on trial, the median age was 51 (range 21–79) years. A history of light or never smoking characterized the majority of patients enrolled on the crizotinib study, with 72% of enrolled patients having a history of never smoking. Ninety-seven percent of the 119 enrolled NSCLC patients with ALK gene rearrangements had tumors with adenocarcinoma histology, frequently with the presence of signet ring cells.\textsuperscript{40}

In the initial report of 82 patients with ALK-rearranged NSCLCs treated with crizotinib, the response rate was 57%, with multiple patients experiencing clinical improvement and disease shrinkage within several weeks of starting drug.\textsuperscript{39} In the updated series of 119 patients, 86% of whom had received at least one prior line of therapy for advanced disease, 116 patients evaluable for response demonstrated an overall response rate of 61% (95% confidence interval [CI] 52–70) with 69 patients achieving a partial response. A further 31 patients experienced stable disease as their best response. By the data cutoff, median progression-free
survival in this heavily pretreated cohort of 119 patients was 10.0 months (95% CI 8.2–14.7) with 50 events (42%; 40 progressive disease events), 69 patients (58%) censored, and 59/69 (86%) in follow-up for progression-free survival.40,41

Among the 119 ALK-positive NSCLC patients, crizotinib was generally well tolerated. Adverse events were usually grade 1 or grade 2, and consisted of visual effects [mostly trails of light at times of light accommodation (62%), nausea (49%), diarrhea (43%), vomiting (35%), and edema (28%)]. Increases in liver function tests were seen, particularly affecting alanine transaminase. These occasionally reached grade 4 in severity but were reversible upon cessation of the study drug. Most patients were able to restart crizotinib at a lower dose without recurrence of dose-limiting transaminitis.40

Given the role of ALK rearrangement as a predictive marker of crizotinib response in NSCLC, the effect of crizotinib on overall survival was evaluated in a 30-patient subgroup of the originally reported Phase I cohort of 82 ALK-positive NSCLC patients receiving crizotinib.41 The overall survival of these patients was compared with that of 23 patients with ALK-positive advanced NSCLC from the US and Australia who had received any second-line treatment, but had not received crizotinib, as well as to a group of ALK-negative/EGFR mutation-negative control patients. Because patients in the comparison groups were identified from non-Korean sites, the 30 patients with crizotinib-treated ALK-positive tumors did not include those patients that had been treated at the Korean Phase I institution, and furthermore represented only those patients treated in the second-line or third-line treatment setting. At the time of reporting, the median overall survival of crizotinib-naive patients was 6 months, while the median overall survival of the crizotinib-treated cohort had not yet been reached (95% CI of 14 months to not reached). As compared with patients having tumors lacking ALK rearrangement or EGFR mutation, there was no statistically significant difference in survival for the crizotinib-naive ALK-positive disease group, suggesting that crizotinib treatment improves outcomes in a subset of lung cancers that would otherwise have a poor prognosis.41 The caveat to this analysis is its retrospective nature; however, the widespread availability of crizotinib soon after identification of the role of ALK in NSCLC (see below) has rendered a retrospective analysis the only manner in which to evaluate a crizotinib-naive ALK-positive group of NSCLC patients. Interestingly, a retrospective analysis of PFS in crizotinib-naive patients treated with pemetrexed found that patients with ALK-positive NSCLCs experienced significantly longer progression-free survival than patients with ALK-negative tumors, indicating that, while not prognostic, ALK positivity may be a predictor of benefit from pemetrexed as well as crizotinib.42 A larger retrospective study has since demonstrated that ALK-positive and ALK-negative NSCLC patients have similar outcomes after pemetrexed-based therapy, although nonsmoking status may be predictive of benefit from pemetrexed.43

The success of the expansion cohort of ALK-positive NSCLCs treated with crizotinib led to the implementation of Phase III trials in the second-line and first-line setting, randomizing ALK-positive NSCLC patients to crizotinib treatment versus standard chemotherapy. The second-line trial has reached its goal accrual, and the results have been presented at the European Society of Medical Oncology congress in 2012. In 2011, on the basis of the response rate seen in 255 patients from the Phase I trial and a single-arm Phase II trial (NCT00932451), crizotinib received accelerated US Food and Drug Administration FDA approval in patients with advanced NSCLC harboring ALK rearrangement, along with a companion diagnostic test for ALK rearrangement.

Molecular diagnosis of ALK rearrangement

FISH has been used as the diagnostic method in all of the crizotinib studies. However, a variety of diagnostic approaches have been developed to identify activated ALK, including reverse transcriptase polymerase chain reaction and immunohistochemistry, in addition to in situ hybridization (FISH or chromosomal in situ hybridization). The approach of reverse transcriptase polymerase chain reaction and direct sequencing allows resolution of the genomic structure of EML4-ALK and has identified multiple isoforms of the fusion gene. The predominant isoforms, sometimes referred to as variant 1 and variant 2, arise through fusion of intron 13 of EML4 to intron 19 of ALK, and from fusion of EML4 intron 20 to ALK intron 19, respectively. Other isoforms arise through fusion of intron 6 of EML4,12 as well as exon 2 and exon 14 of EML4.15,18 Although reverse transcriptase polymerase chain reaction is sensitive, provides more detail about EML4-ALK structure, and has even been used in sputum samples to detect rearrangement, the widespread use of this approach is limited by its dependence on sample quality, which is a consideration when using archival paraffin-embedded specimens. FISH uses two fluorescently labeled probes that flank the break point of the EML4 and ALK fusion gene (Vysis LSI ALK dual-color,
break-apart probe, Abbott Laboratories, Abbott Park, IL), giving rise to signals that can be identified as split or isolated (indicating EML4-ALK rearrangement) or superimposed (indicating wild-type status). This analysis can be performed on archival paraffin-embedded tumor specimens, making it amenable to routine clinical use. However, interpretation of FISH results can be challenging, requiring experience in the interpretation of fluorescence microscope images in samples in which only a subset of cells may be evaluable for split signals. Because EML4-ALK is not expressed to a significant extent in normal tissues, immunohistochemistry for expression of the fusion protein is an attractive approach that enables analysis of paraffin-embedded tumor specimens in a fashion already routine in clinical practice. In one study, Kim et al compared chromosomal in situ hybridization with immunohistochemistry using an antibody to ALK (Novocastra Laboratories Ltd, Newcastle-upon-Tyne, UK) and found the two methods to be strongly correlated. Furthermore, intercalated antibody-enhanced polymer methods have been used to improve the sensitivity of immunohistochemistry. Because US Food and Drug Administration approval for crizotinib was granted using the Vysis probe for FISH as the approved companion diagnostic, FISH is the diagnostic method of choice at this time. Recently, improved antibodies have become more available and may favor the use of immunohistochemistry in the future.

### Resistance

While FISH or immunohistochemistry is adequate to diagnose ALK-positive disease, the utility of reverse transcriptase polymerase chain reaction and direct sequencing will become more important as we seek to learn more about molecular determinants of response and resistance to targeted ALK inhibitors. The majority of patients treated with crizotinib experience disease shrinkage or stabilization, and progression-free survival exceeds what would be expected with standard chemotherapy. However, acquired resistance to crizotinib challenges the further management of these patients. In addition, primary refractoriness to crizotinib limits the treatment in a small percentage of patients with ALK-positive tumors. Mechanisms of resistance are the subject of intensive research, and multiple mechanisms have been described. Resistance mutations within EML4-ALK were first reported by Choi et al, who identified two subpopulations of cancer cells from the pleural effusion of a patient who initially experienced disease shrinkage on crizotinib, but then developed resistance to treatment. One subclone of resistant cells harbored a 4374G→A substitution, resulting in a C1156Y amino acid change adjacent to the N-terminal of the predicted helix alpha C as well as close to the upper edge of the ATP-binding pocket in the fusion protein. The other mutation was found at L1196M, located at the bottom of the ATP-binding pocket, in a position analogous to the “gatekeeper” position previously found in chronic myelogenous leukemia with BCR-ABL (T315I) resistant to imatinib and in EGFR (T790M) resistant to erlotinib. Doebele et al reported multiple mechanisms of resistance identified in 11 patients who had developed disease progression after initial response to crizotinib treatment, including: a new mutation, G1269A, found within the kinase region of ALK in two patients; a copy number gain seen in two patients (5-fold and >4-fold); the presence of an EGFR L858R mutation, despite ALK positivity in the original diagnostic transbronchial biopsy specimen; and several instances of K-ras mutations in codon 12. Sasaki et al identified an L1152R mutation in the pleural fluid of a patient with NSCLC who progressed after initial disease shrinkage on crizotinib biopsied post-progression. This L1152R mutation had not been detected in the original tumor specimen prior to crizotinib treatment. In a cell line generated from the pleural fluid tumor cells containing L1152R, roles for activation of MET and EGFR signaling as a resistance mechanism were also identified. While crizotinib was able to inhibit phosphorylation of MET, EGFR activation appeared to be stimulated by the ability of this cell line to secrete amphiregulin, and required concurrent treatment with a pan-ERBB inhibitor to inhibit EGFR phosphorylation. A role for epidermal growth factor–mediated signaling in ALK-positive NSCLC tumor cell lines resistant to crizotinib was also demonstrated by Tanizaki et al. Activation of these pathways through endothelial and fibroblast secretion of EGFR and MET ligands has also been demonstrated, suggesting that paracrine signals from tumor microenvironments may also contribute to resistance.

Additional kinase domain mutations have been identified by Katayama et al, including G1202R, S1206Y, and a threonine insertion mutation, 1151Tins. In vitro, cell lines engineered to express the kinase domain mutations C1156Y, L1196M, L1152R, G1202R, S1206Y, and 1151Tins were highly resistant to crizotinib at concentrations that inhibited intact EML4-ALK. Interestingly, distinct mutations possessed differential degrees of in vitro resistance or sensitivity to newer second-generation ALK inhibitors, suggesting that there may be clinical implications to the choice of ALK inhibitor used to treat patients. Nonetheless, preliminary results from the Phase I study of the potent, selective ALK inhibitor LDK378 demonstrated a response rate of 81%.
(21/26 cases) in ALK-positive NSCLC patients who had previously progressed on crizotinib. While the mechanisms of acquired resistance to crizotinib were not presented with these data, the impressive activity seen in this trial suggests the potential utility of LDK378 across a variety of resistance mutations in ALK.51

Other resistance mechanisms that have been identified include ALK gene amplification and KIT gene amplification,50 pointing to the diversity of acquired resistance mechanisms seen in ALK-positive NSCLC and to the future challenges in designing effective single-agent and combination treatment regimens. Importantly, ALK is a HSP90 client protein; HSP90 inhibition has activity against ALK-rearranged NSCLC52 and was found by Katayama et al to be active against the various kinase domain resistance mutants tested in vitro.50 Therefore, HSP90 inhibition may be a promising alternative strategy to treat or even prevent the emergence of resistance.

**ALK in other diseases**

While the preponderance of recent data comes from NSCLC, the therapeutic potential of ALK inhibition is relevant to a variety of other tumor types as well. In anaplastic large-cell lymphoma, the disease in which ALK molecular abnormalities were first identified, crizotinib has led to marked responses in ALK-positive relapsed chemotherapy-refractory disease.53 Crizotinib has also demonstrated activity in a case of an inflammatory myofibroblastic tumor harboring a RANBP-ALK fusion.54 In neuroblastoma, activating ALK mutations have been identified in both sporadic and familial cases. In vitro data suggest that these tumors may also be sensitive to ALK inhibitors, and crizotinib is being evaluated in a clinical trial enrolling neuroblastoma patients.55-57 Whether aberrant ALK has therapeutic implications for more common malignancies other than NSCLC has yet to be determined. ALK fusion has been rarely identified in renal cell cancers,58 and ALK fusions in breast and colon cancers have been described.59 Screening of clinical colorectal specimens by FISH has not supported this finding, although increases in gene copy number were seen.60 Next-generation sequencing may provide additional insights into the role of ALK in colorectal cancer.61 ALK gene amplification was reported in approximately 75% of patients in inflammatory breast cancer, and enrollment of such patients into clinical trials of ALK inhibitors is ongoing.51,62

**Conclusion**

Although the role of activated ALK as a driver of oncogenesis has been known since the discovery of NPM-ALK in anaplastic large cell lymphoma, its role in NSCLC and the clinical availability of selective inhibitors has brought ALK to the forefront of clinical investigation. The activity of crizotinib in NSCLCs that have been screened for ALK gene rearrangement has demonstrated that prospective molecular analyses in the early phases of clinical trials can identify the tumors most sensitive to targeted therapies and facilitate the development of new agents for those patients most likely to benefit. As the role of ALK is explored in additional tumor types, there will likely be other settings in which the success of crizotinib in ALK-rearranged NSCLC is duplicated.

As with all cancer therapies, the acquisition of resistance is a challenge to the ongoing successful treatment of patients with ALK-rearranged NSCLC. Recent data point to a variety of mechanisms of resistance with potentially multiple implications for therapy. As crizotinib resistance emerges, subsequent options for treatment may include second-generation ALK inhibitors such as LDK378, combinations of targeted agents, or HSP90 inhibitors. The optimal role for these approaches has yet to be determined, but further study will rely upon rebioy of resistant tumors and molecular analyses of these specimens. In NSCLC, as in other diseases, there is still much to be learned about ALK.

**Disclosure**

The authors report no conflicts of interest in this work.

**References**


