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Native and rearranged *ALK* copy number and rearranged cell count in NSCLC: Implications for *ALK* inhibitor therapy

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Abstract

Background—Anaplastic Lymphoma Kinase positive (*ALK*+) non-small cell lung cancer (NSCLC) responds to *ALK* inhibitors. Clinically, 15% cells showing rearrangements by break-apart FISH classify tumors as positive. Increases in native and rearranged *ALK* copy number also occur.

Methods—1426 NSCLC clinical specimens (174 *ALK*+ and 1252 *ALK* negative), and 24 *ALK* negative NSCLC cell lines were investigated. *ALK* copy number and genomic status were assessed by FISH.

Results—Clinical specimens with 0–9%, 10–15%, 16–30%, 31–50% and >50% of *ALK*+ cells were found in 79.3%, 8.5%, 1.4%, 2.7% and 8.1% of cases, respectively. Increased native *ALK* copy number (≥ 3 copies/cell in ≥ 40% cells) was detected in 19% of *ALK*+ and 62% of *ALK* negative tumors. In *ALK* negative tumors, abundant focal amplification of native *ALK* was rare (0.8%). Other atypical patterns occurred in ~6% of tumors. Mean native *ALK* copy number ranged from 2.1–6.9 in cell lines and was not correlated with crizotinib sensitivity (IC₅₀s 0.34–2.8 μM) ($r=0.279$, $p=0.1764$). Neither native, nor rearranged *ALK* copy number, nor percentage cells positive correlated with extra-central nervous system progression free survival in *ALK*+ patients on crizotinib.

Conclusions—8.5% of cases are below the established positivity threshold by 5%. Further investigation of *ALK* by other diagnostic techniques in such cases may be warranted. Native *ALK* copy number increases alone are not associated with sensitivity to *ALK* inhibition *in vitro*. However, rare complex patterns of increased native *ALK* in patients should be studied further as atypical rearrangements contained within these may otherwise be missed.

Keywords

ALK; FISH; copy number; borderline; crizotinib

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Introduction

Anaplastic Lymphoma Kinase (*ALK*) gene rearrangements represent the primary oncogenic driver in a subset of lymphomas and solid tumors.¹ In all of the rearrangements, the promoter and a region encoding a 5' dimerization motif from another gene is fused to the region encoding the 3' kinase domain of *ALK* leading to constitutively active expression of *ALK* within the resulting fusion protein. In *ALK* rearranged (*ALK*+) non-small cell lung cancer (NSCLC), the most common 5' fusion partner is echinoderm microtubule-associated protein-like 4 (*EML4*), although other, less common, 5' partners have also been described.¹

ALK+ NSCLC treated with crizotinib, a multi-targeted tyrosine kinase inhibitor with activity against *ALK* as well as *ROS1* and *MET*, is associated with a high response rate and prolonged progression free survival (PFS).²⁻⁴ Within the initial and ongoing crizotinib clinical trials in NSCLC, *ALK* status was determined by fluorescence *in situ* hybridization (FISH) using the Vysis break-apart probeset (Abbott Molecular), the only companion diagnostic for crizotinib licensed by the FDA to date.^{5,6} The break-apart FISH testing involves DNA probes binding 5' (green-labeled) and 3' (orange-labeled, usually appearing as red in most microscope settings) of the common fusion breakpoint in *ALK*. When a rearrangement occurs either a split or single red signal is noted in a cell (Figure 1). Only a single rearranged signal is required to call a cell positive, however not every cell in an *ALK* + tumor will show a detectable rearrangement by FISH, presumably, in part, due to false cellular negatives.⁷ Moreover, a proportion of cells even in normal tissues can appear to show a rearrangement by FISH due to presumed false cellular positives.⁵ Similarly, not every cell in an *ALK*+ tumor will show a detectable rearrangement by FISH, presumably, in part, due to false cellular negatives.^{5,7} The *ALK*FISH assay was originally developed for the detection of *ALK* rearrangements in lymphomas, which are associated with a range of different 5' fusion partners primarily reflecting chromosomal translocations.⁸ Conversely, *EML4*, the most common fusion partner in NSCLC, resides on the same chromosome 2p arm as *ALK* and the rearrangement is caused by a paracentric inversion. Consequently, lymphomas are associated with subtly different cytogenetic patterns of positivity than those seen in NSCLC. When the break-apart FISH assay was first modified for use in NSCLC, a natural gap in the continuum of the percentage of *ALK* positive cells in lung tumors was identified that appeared to reliably distinguish between those assumed to be true positive tumors and those whose low positive cell counts were assumed to only reflect the background noise of the assay.⁵ In the initial studies of crizotinib in NSCLC, >15% of tumor cells were required to show a rearrangement in order to classify a tumor as *ALK*+.^{2,5,7} Later, with the approval of the assay as a companion diagnostic by the US Food and Drug Administration (FDA), 15% was taken as the accepted cutpoint.⁶ In addition to the break-apart FISH assay, several other *ALK* diagnostic techniques have also been developed, including using immunohistochemistry to look for the aberrant re-expression of the *ALK* protein and reverse transcriptase PCR to look for the presence of the abnormal fusion transcripts.⁶ Case reports of tumors negative by FISH but determined to be *ALK*+ by one of these other techniques who have responded to *ALK* inhibitors have been published, raising the possibility that the established FISH assay may miss an unquantified proportion of true positive cases.⁹⁻¹¹ Due to the rarity of *ALK*+ NSCLC, the identification of the FISH cutpoint as one that could reliably distinguish true positive tumors from true negative tumors was inevitably based on a relatively small initial dataset.⁵ With far more NSCLC cases currently tested by *ALK* FISH, there is now the potential to more accurately re-explore whether the threshold value chosen still defines a true gap in the continuum of the assay and whether it should remain the sole determinant of *ALK* positivity in NSCLC.

In addition to the percentage of cells manifesting rearrangements, the break-apart FISH assay also provides information on the copy number of both the native and rearranged *ALK*

genes per cell. The later development of rearranged *ALK* copy number gain (CNG) compared to baseline pre-crizotinib levels is one of several different identified mechanisms of acquired resistance to crizotinib.^{12,13} Yet, increases in copy number of both rearranged and native *ALK* relative to the diploid state in inhibitor-naïve specimens also occur.^{5,14} *In vitro*, increases in the copy number of native *ALK* in NSCLC cell lines have been associated with crizotinib sensitivity in the 1–3 μ M range.¹⁵ However, the clinical significance of baseline native/rearranged *ALK* copy number to crizotinib sensitivity at physiological exposures remains unclear.¹⁶

We have previously shown that neither the positive cell count, the baseline native copy number, nor the baseline rearranged copy number showed any significant association with the maximal percentage shrinkage per RECIST version 1.0 in *ALK*+ tumors treated with crizotinib.⁷ However, not all of the clinical benefit from a drug may manifest as tumor shrinkage and correlations between the different cytogenetic features of *ALK* positivity (cell count or copy number of native or rearranged signals) and progression free survival (PFS) endpoints in *ALK* FISH+ tumors treated with crizotinib have not been previously reported.

Here we explore whether 15% reflects a clear biological distinction in the frequency of *ALK*+ cells in over a 1400 tested cases of NSCLC, as well as the details of variation in native *ALK* copy number in tumors with and without *ALK* rearrangements; the significance of baseline native and rearranged copy number on PFS outcomes on crizotinib in *ALK*+ patients and of native copy number on crizotinib outcomes in *ALK* negative cell lines at IC50s compatible with clinically achievable exposures.

Methods

Clinical specimens

NSCLC clinical specimens tested for *ALK* between October 2008 and December 2010 within the Colorado Molecular Correlates (CMOCO) laboratory were identified and investigated. Copy number and genomic status (rearranged/native) of *ALK* was assessed using Vysis break-apart FISH as previously described.^{5,7} An IRB-approved protocol at the University of Colorado permitted clinical correlates to be made on patients in whom molecular analyses have been conducted within the CMOCO laboratory.

Clinical response and PFS data

A subset of the tested patients received treatment at the University of Colorado and had clinical data on crizotinib outcomes available (n=33). Patients were treated with crizotinib both within reported clinical studies and as standard of care following FDA licensing.^{3,4,17} Best objective responses of target lesions per RECIST version 1.0 were assessed on patients with measurable disease following crizotinib treatment. Radiographic disease assessments were performed at baseline prior to commencing the inhibitor and continued after every other 21 or 28 day cycle (depending on the clinical trial involved or physician preference when treated off-study). PFS was calculated from the first dose of the crizotinib to documented radiographic or clinical progression per RECIST, or change in systemic therapy (other than discontinuation). Extra-central nervous system (eCNS) PFS was then calculated excluding CNS only events. We used eCNS PFS as the primary PFS variable in the analyses due to the high CNS failure rate with crizotinib in *ALK*+ NSCLC that may represent low drug exposure more than biological evolution.^{4,18,19}

In vitro cell line sensitivity data

Twenty-four *ALK* and *ROS1* rearrangement negative cell lines (A459, Calu-3, H1975, H2009, H2122, H322C, H3255, H358, HCC2279, HCC4006, HCC4011, HCC44, PC9,

H125, H1703, H1299, H1334, H460, H661, H157, H226, H520, HCC95, NE18) were analyzed by ALK FISH and the mean copy number of the native *ALK* gene per cell was determined. Two *ALK* rearrangement positive (H2228 and H3122) cell lines were used as positive controls. The IC₅₀ of crizotinib was determined for each cell line using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) growth assay. Briefly, 1,000 to 2,000 viable cells were plated in 100 μ l of growth medium in 96-well plates (Corning, Ithaca, NY) and incubated overnight at 37°C. Crizotinib, 0 to 10 μ M, was added and the plates were incubated for 5 days, after which the tetrazolium salt was added to a final concentration of 0.4 mg/mL to each well. The medium was aspirated after 4 hours, and the reduced MTT product was solubilized by adding 100 μ l of 0.2 N HCl in 75% isopropanol and 23% Milli-Qwater to each well. Thorough mixing was done using a Titertek multichannel pipetman. The absorbency of each well was measured at 490 nm using an automated plate reader (Molecular Devices, Sunnyvale, CA).

Statistical analyses

The difference between ALK- and ALK+ cases on mean native *ALK* copy number was analyzed using a two-sample t-test. Frequency of native *ALK* copy number gain between ALK- and ALK+ was analyzed using Fisher's exact test. Correlation between mean native *ALK* copy number and crizotinib sensitivity was analyzed using Spearman's correlation. In the survival analysis with eCNS PFS as the outcome, native and rearranged *ALK* copy number, and percentage positive cell count were categorized with their respective quartiles; Cox Proportional Hazards regression was then used to analyze these associations.

All statistical analyses were performed using SAS/STAT software version 9.3 of the SAS System for Windows. Copyright © 2002–2010 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

Results

A total of 1426 NSCLC clinical specimens tested for *ALK* rearrangements within the CMOCO laboratory (174 ALK+ and 1252 ALK negative by standard criteria) were identified. In ALK+ crizotinib-treated patients eCNSPFS data was available on 33 patients.

Clinical specimens with 0–9%, 10–15%, 16–30%, 31–50% and >50% of ALK+ cells were found in 79.3%, 8.5%, 1.4%, 2.7% and 8.1% of NSCLC cases, respectively (Figure 2). The proportions, in detail, among the ALK negative cases are shown in Table 1.

Mean native *ALK* copy number was significantly higher in ALK negative than in ALK+ cases (2.8, SD 0.93, range 1.2–11.4 vs. 1.8, SD 0.79; range 0.6 to 5.3; $p < 0.0001$) (Figure 3). Frequency of native *ALK* copy number gain (defined as ≥ 3 copies/cell in $\geq 40\%$ cells) was significantly higher in ALK negative tumors (62%) than in ALK+ tumors (19%) ($p < 0.0001$).

In ALK negative tumors, although native *ALK* copy number was elevated the distribution of signals within the nucleus was largely diffuse, suggestive of the occurrence of polysomy of chromosome 2 or partial polysomy of 2p23 (Figure 4A). Abundant focal amplification of native *ALK* was rare (0.8%) (Figure 4B), whereas scanty amplification ($< 10\%$ of tumor cells) occurred in 1.1%, and duplication of the entire native *ALK* or of the 3' *ALK* end occurred in 3.5% (2.9% and 0.6%, respectively) (Figures 4C and D). Single green signals (extra copies of the 5' *ALK* with loss of 3' *ALK*) were observed in 1.2% of these specimens.

Among ALK negative lung cancer cell lines, mean native *ALK* copy number per cell ranged from 2.1 to 6.9 and was not correlated with in vitro crizotinib sensitivity (IC₅₀s 0.34–2.8

uM) ($r=0.279$, $p=0.1764$) (Figure 5A). Using a two-side unpaired t-test, the IC₅₀ for crizotinib was no different for those cells with native *ALK* copy number gain (≥ 3 copies of native *ALK* in $\geq 40\%$ or more of cells) compared to all other lines, defined as those with balanced or loss of native *ALK* copy number ($p=0.21$) (Figure 5B). In ALK+ patients treated with crizotinib, neither native ($p=0.2859$), nor rearranged ($p=0.4115$) *ALK* copy number, nor percentage cell positive ($p=0.0748$) correlated with eCNS PFS with crizotinib.

Discussion

Screening patients with advanced NSCLC for an *ALK* rearrangement is now considered part of standard care based on the dramatic and prolonged objective responses seen when these patients are directed toward an ALK inhibitor such as crizotinib.^{2,4,17} Consequently, accurately identifying the patients who will derive most benefit from these inhibitors is imperative. Here, we describe our exploration of several different aspects of the *ALK* FISH break-apart assay, the only ALK diagnostic assay that is currently FDA approved.

Initial data suggested that a cutpoint of $\geq 15\%$ or $>15\%$ of cells showing an *ALK* rearrangement by break-apart FISH for classifying a tumor as ALK+ would generate few borderline cases. For example, our own series had previously suggested that cell counts in positive tumors began at 22% and in negative tumors/normal tissue stopped at 11%.⁵ Specifically, the $\geq 15\%$ cutpoint used in the initial crizotinib studies and the $>15\%$ cutpoint adopted by the FDA appeared to occur in a naturally occurring gap in the continuum of the assay. However, when analyzing a much larger series we can now see that a considerable proportion of ‘negative’ cases closely approach the established cutpoints (Figure 2, Table 1). Overall 8.5% of cases occur in the 10–15% range (9.7% of ALK negative cases). Although $\geq 15\%$ would be considered positive in some studies and negative in others, the number of cases with exactly 15% was very low, and therefore the overall conclusion is not changed dependent on whether $\geq 15\%$ or $>15\%$ is chosen as the criterion for positivity. Consequently, a second ALK diagnostic technique (e.g. immunohistochemistry) is recommended for such borderline cases to more accurately identify the true genomic status of *ALK*.⁶

As FISH analyses can also offer information about the copy number of both the native and rearranged *ALK* gene, several studies have already explored the importance of *ALK* copy number to outcomes with crizotinib. Baseline increases in copy number of both rearranged and native *ALK* relative to the diploid state are both known to occur and associations between native copy number and sensitivity to high levels (1–3uM) of crizotinib have previously been reported *in vitro*.^{5,14,15} In our current analyses, we did confirm the frequent increase in native *ALK* copy number reported by others. Intriguingly, the native *ALK* mean copy number was significantly higher in ALK negative than in ALK+ cases (2.8, SD 0.93, range 1.2–11.4 vs. 1.8, SD 0.79; range 0.6 to 5.2; $p<0.01$) (Figure 3). Increased native *ALK* copy number (≥ 3 copies/cell in $\geq 40\%$ cells) was detected in 19% of ALK+ and 62% of ALK negative tumors ($p<0.001$). The lower native *ALK* copy number in ALK+ NSCLC suggests *ALK* rearrangement occurs early in tumorigenesis, preceding chromosomal instability, consistent with our previous studies.⁷ As ALK positivity primarily occurs in those with little or no smoking history, it is perhaps not surprising that these data suggest that ALK positive lung cancer may reflect a ‘near-diploid’ or genetically ‘simple’ cancer.^{5,7}

We have previously demonstrated no relationship between either native/rearranged copy number or the percentage of cells positive for a rearrangement with the maximal tumor shrinkage per RECIST seen in ALK+ patients on crizotinib.⁷ As not all clinical benefit may be manifest through the methods for determining tumor shrinkage used by RECIST, we therefore attempted to correlate these same parameters with lack of tumor progression as assessed through the eCNS PFS of ALK+ patients on crizotinib. However, again we were

unable to demonstrate any significant correlation between eCNS PFS and either the native or rearranged copy number or the percentage of cells positive for a rearrangement in the tumor by FISH. With regard to rearranged signals, we have previously shown a strong correlation between the copy number of the rearranged gene and the percentage of cells counted as positive in the tumor, consistent with differences in cell counts in positive tumors largely reflecting missed positive cells and not true biological negatives.⁷ Consequently, beyond the importance of the cell count in determining whether a tumor is positive or not in the first place, it is perhaps not surprising that PFS shows no more correlation with these variables than tumor response does. With regard to native copy number, additional discussion is required. Unlike with the prior in vitro data conducted at supra-physiological exposures, our own data suggest no association between native copy number and sensitivity to crizotinib in ALK negative cell lines treated at lower exposures that are more comparable to those used when studying ALK+ cell lines (Figure 5).^{12,15,16}

When looking at the pattern of native copy number gain in ALK negative patients, there is little suggestion of specific selection of this region of the genome by the cancers. Abundant focal amplification of native *ALK* was rare in ALK negative tumors (0.8%); scanty amplification (<10% tumor cells) occurred in only 1.1%, and most CNG occurred without significant amplification. However, duplication of the entire native *ALK* or of the 3' *ALK* and 5' *ALK*, occurred in 3.5% of the ALK negatives. Instead of simply reflecting the overall aneusomy of the cancer, such patterns may represent direct or inverse duplications or more complex rearrangements specifically involving the region of interest. In all such cases, it may be possible that the rearrangement still leads to fusion of ALK with an activating partner. For example, Lipson et al described the in frame fusion *C2orf44:ALK* in colorectal cancer resulting from a 5Mb tandem duplication at 2p23 and Peled et al reported an *EML4:ALK* fusion detected by next-generation sequencing and IHC in a specimen displaying a FISH pattern that was negative by standard criteria but with complex atypical features.^{9,20} Consequently, while native ALK copy number alone does not appear to be a useful predictor of ALK inhibitor sensitivity, the possibility that complex or atypical rearrangements that exist within the rare cases of focal native *ALK* duplication or amplification has to be considered.

Extra copies of 5' *ALK* with loss of 3' *ALK* is not expected to represent activation of the ALK gene by fusion since the 5' *ALK* signal represents exons 1 to 19 and does not include the tyrosine kinase domain. However, there are reports of NSCLC with extra copies of 5' *ALK* and loss of 3' *ALK* (appearing as single green signals with the break-apart FISH probes) whose tumors were determined to carry the *EML4-ALK* fusion.¹¹ A possible explanation is that those specimens also have complex rearrangements in the 2p23–21 region and the genomic region left in the area encompassed by the 3' *ALK* probe only covers the *ALK* tyrosine kinase domain and is too small to be visually detectable in formalin-fixed paraffin-embedded sections. Therefore, just as with the borderline negative cases by percentage cell count, 'atypical' negatives – i.e. cases that do not meet the current break-apart positivity criteria but have evidence of potential complex rearrangements within the *ALK* locus, such as 3' *ALK* doublets or single 5' *ALK*, should also be considered for additional interrogation with a second ALK diagnostic technique. By capturing clinical outcomes from atypical negative and borderline negative FISH cases in which the second diagnostic suggests the true presence of an activating rearrangement, it should be possible to minimize missed therapeutic opportunities among the true inhibitor sensitive ALK positive population.

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Figure 1.

Break-apart FISH Schematic: EML4-ALK example. Modified from Camidge et al, Cancer 2012 (with permission)

Figure 2.
Distribution of NSCLC patients showing different percentage of rearrangement positive cells.

Figure 3.

Mean copy number of native ALK is significantly higher in ALK negative than in ALK+ NSCLC.

Figure 4. Patterns of native *ALK* CNG in *ALK* negative NSCLC. Where specific patterns are localized, rather than diffuse, within the cell, white arrows are used to identify these. Schematic inserts in which the 5' probe is shown as a green dot, the 3' probe as a red dot and overlap as yellow are shown to illustrate the key features of each photomicrograph.

Figure 5A

Figure 5B

$P = 0.21$ unpaired t-test, two-sided

Figure 5.

(A) No detectable correlation between native ALK CNG in ALK negative NSCLC cell lines and crizotinib IC₅₀. No association between CNG and IC₅₀ (B).

Table 1

Proportion of ALK negative cases with different percentage positive cells below >15% cutpoint for tumoral positivity

% Cells with FISH Positive Patterns	Number of ALK Negative Patients	% of ALK Negative Patients
0–1	232	18.5%
2–3	291	23.2%
4–5	277	22.1%
6–7	200	16.0%
8–9	131	10.5%
10–11	72	5.8%
12–13	26	2.1%
14–15	23	1.8%
Total	1252	100.0%