Published in final edited form as:

Cancer. 2012 September 15; 118(18): 4486–4494. doi:10.1002/cncr.27411.

Correlations between the percentage of tumor cells showing an *ALK* gene rearrangement, *ALK* signal copy number and response to crizotinib therapy in *ALK* FISH positive non-small cell lung cancer

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Abstract

Background—FISH, using break-apart red (3') and green (5') *ALK* probes, consistently shows rearrangements in < 100% of tumor cells in *ALK*+ NSCLC. Increased copy numbers of fused and rearranged signals also occur. Here we explore correlations between the percentage of *ALK* positive cells and signal copy number and their association with response to *ALK* inhibition.

Methods—Ninety *ALK*+ NSCLC cases were evaluated. The percentage of positive cells, pattern of positivity (split, single red, or both) and copy number of fused, isolated red and green signals were recorded. Thirty patients had received crizotinib.

Results—Increased isolated red signal copy number (contributing to both single red and split patterns of positivity) correlated with a higher percentage of *ALK*+ cells ($r = 0.743$, $p = <0.0001$). Mean fused copy number was negatively associated with isolated red signal copy number ($r = -0.409$, $p = <0.0001$). Neither percentage cells positive ($r = 0.192$, $p = 0.3$), nor copy number of isolated red ($r = 0.274$, $p = 0.195$) correlated with maximal tumor shrinkage with crizotinib.

Conclusions—The strong association between increased copy number of key *ALK* signals and percentage positive cells suggests that the <100% rate of cellular positivity in *ALK*+ tumors is due to technical factors not biology. In *ALK*+ tumors, neither percentage cells positive, nor signal copy number appear to be informative variables for predicting benefit from *ALK* inhibition. The inverse relationship between fused and isolated red copy number suggests *ALK*+ may be a distinct 'near diploid' subtype of NSCLC developing before significant chromosomal aneusomy occurs.

Keywords

ALK; FISH; copy number; crizotinib

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Financial Disclosures: None declared for any authors

Background

Activation of Anaplastic Lymphoma Kinase (ALK) has been described as a primary oncogenic event in a number of different human cancers.¹ In most cases, activation is through a chromosomal rearrangement placing one of several different 5' fusion partners and their associated promoter, upstream of the 3' kinase domain of *ALK*.² Rearrangements of the *ALK* gene occur in 3–5% of non-small cell lung cancers (NSCLC), most notably in adenocarcinomas.¹ The most common 5' fusion partner in NSCLC is *EML4*, but other, rarer 5' fusion partners, notably *KIF5B* and *TFG*, have also been described.^{3–5} There are various potential methods for detecting ALK positivity in solid tumors, including using immunohistochemistry to look for the aberrant expression of the kinase domain, using RT-PCR with primers directed against specific fusion pairings and using fluorescence in-situ hybridization (FISH) with break apart probes that bind 5' and 3' of the common breakpoint in *ALK* to look directly for a gene rearrangement.^{1,6}

NSCLC patients proven to have an *ALK* gene rearrangement using FISH as the screening technique commonly manifest rapid, dramatic and prolonged responses to crizotinib, a small molecule inhibitor of *ALK* and *MET*.^{7,8} Within all of the crizotinib studies, *ALK* positivity by FISH has been defined as >15% of tumor nuclei demonstrating either split and/or single 3' (red in the most commonly used probe-color pairing) fluorescence patterns.^{7,8} To count as positive for a rearrangement with a split pattern, splitting of the 5' (green) and 3' (red) signals must occur by more than two signal diameters. Single 5' (green) patterns and increased copy numbers of native (fused) and rearranged signals have also been reported, but their biological significance, beyond simply reflecting the chromosomal instability of many cancers, remains uncertain.^{9,10}

We have previously shown that the proportion of positive cells within an overall *ALK* FISH positive (*ALK*+) tumor covers a broad range above 15% but below 100% (e.g. 22–87%).⁹ While some have interpreted such findings as evidence against *ALK* gene rearrangements being both a primary and/or necessary oncogenic event in NSCLC, other explanations exist.¹¹ In conjunction with the presence of positive signals in up to 11% of non-tumor and negative tumor cells, we hypothesized that most of the variation above zero in the negative and non-tumor areas and the variation below 100% in the positive tumor areas reflected technique rather than biology.⁹ Specifically, individual cells may be counted as positive but not possess a true rearrangement (false positives) or may be counted as negative when they do in fact possess a rearrangement (false negatives).

If cellular heterogeneity is related to technique and not to biology, we hypothesized that the following would be true. Firstly, as a single red pattern may be easier to detect than a split pattern, the overall proportion of *ALK*+ cells should be higher in tumors expressing the single red pattern of positivity than the split pattern. Secondly, it should be harder to miss a true positive pattern when the number of signal copies is increased (given that only one split red/green or single red pattern per cell is required to call a cell positive), therefore there should be a positive correlation between the number of 3' (red) signals (contributing to both single red and split patterns of positivity) and the percentage of *ALK*+ cells in an *ALK*+ tumor. In contrast, fused signal copy number should not be associated with the proportion of *ALK*+ cells. As the 5' (green) signal contributes to both positive (split) and negative (single green) patterns, correlation with *ALK*+ may not be apparent for the overall 5' signal copy number, but should be obvious when only those cases with a split pattern of positivity are analyzed. Finally, there should be no correlation between the percentage of *ALK*+ cells present in any *ALK*+ tumor and the maximal percentage tumor shrinkage per RECIST seen in that tumor after therapy with crizotinib. To test these hypotheses, as well as the potential biological significance of *ALK* copy number alterations in *ALK*+ tumors, we explored

correlations between the percentage of *ALK*+ cells, patterns of *ALK* positivity and signal copy number and whether these variables are associated with response to *ALK* inhibition in *ALK*+ NSCLC.

Methods

The University of Colorado Thoracic Oncology Program performs routine molecular screening for multiple molecular abnormalities including *EGFR* and *KRAS* mutations and *ALK* gene rearrangements on all NSCLC cases with available tumor tissue. Testing is performed in-house within the CLIA-certified Colorado Molecular Correlates (CMOCO) laboratory for the purposes of directing patients towards the most appropriate targeted therapy. Screening for *ALK* gene rearrangements by FISH began in late 2008 with the goal of identifying patients for entry into defined molecular cohorts within the Phase I study of crizotinib (PF-02341066).⁷ Initially only patients with metastatic disease were screened, but later, beyond the crizotinib study, all NSCLC patients regardless of stage from the first point of contact with the program were screened. *ALK* FISH testing using the LSI *ALK* Dual Color, Break-Apart Rearrangement Probe (Abbott Molecular), designed to detect rearrangements in 2p23 encompassing the *ALK* gene, was conducted as previously described.^{7,9} This probe set included a 250 kilobase DNA fragment telomeric to *ALK* (3' end) labeled in SpectrumOrange and a 300 kb fragment centromeric to *ALK* (5' end) labeled in SpectrumGreen. Signals were enumerated in at least 50 tumor nuclei in standard 4–5µm thick sections, using an epifluorescence microscope with single interference filters sets for green (FITC), red (Texas red) and blue (DAPI) as well as dual (red/green) and triple (blue, red, green) band pass filters. Tumor areas were marked by a lung pathologist in a hematoxylin and eosin-stained slide prior to FISH analysis to facilitate the identification of tumor rich regions by the FISH technologist. Because the 3' *ALK* orange probe is detected by an interference filter with emission/excitation within the red wavelength, its fluorescence signal is seen as red and identified as a 'red' signal in this study.

On FISH testing, the occurrence of an *ALK* gene rearrangement was concluded if >15% of tumor cells showed a split red and green and/or single red (residual 3') pattern, otherwise the specimen was classified as *ALK* FISH negative. Tumor specimens from the first 90 *ALK*+ NSCLC patients were examined. The percentage of tumor cells positive for a rearrangement, the pattern of positivity (split, single red or both), and the copy number of the fused, isolated red and isolated green signals contributing to these patterns were recorded (Figure 1A and B). Of note, throughout this manuscript, the term 'signal' is used to refer to the individual spot representing homology to a specific probe sequence, whereas 'pattern' is used to refer to how the different signals present in a given tumor cell. In addition, the term 'single' is reserved for the description of a specific pattern, whereas 'isolated' is used in reference to a specific probe signal. For example, an isolated red signal may be part of a split pattern or a single red pattern of positivity, depending on whether an isolated green signal either does or does not co-exist in the same cell.

Of the 90 *ALK*+ patients, 30 received crizotinib (PF-02341066) within the Phase I study and had available response outcomes on therapy.^{7,8} 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 and continued after every other 28 day cycle, or if clinically indicated, until disease progression as determined by the clinic physician. The lowest sum of the diameter of target lesions at any assessment while on treatment was calculated against baseline measurements to determine maximum tumor response (percent tumor shrinkage). RECIST response data were then correlated with the individual's *ALK* profile (percent *ALK* positive cells, signal pattern and signal copy number).

An IRB-approved protocol at the University of Colorado permitted clinical correlates to be made on all in-house patients in whom molecular analyses have been conducted within the Colorado Molecular Correlates laboratory (CMOCO). In addition, patients within the crizotinib Phase I study signed informed consent for treatment and data capture. The protocol for the crizotinib Phase I study was approved by the institutional review board at each institution.

Linear associations between the percentage of *ALK*+ cells and signal copy number or response to therapy were summarized by Spearman correlations using two-sided p-values. A two-sided Student's t-test was performed to compare the average percentage of *ALK*+ cells between different patterns of positivity (split vs. single red).

Results

Percentage of *ALK*+ cells according to patterns of positivity and correlation with signal copy number

The mean percentage of *ALK*+ cells was 56% (range: 18–100%) in the 90 *ALK*+ patient dataset and 59% (24–94%) in the 30 crizotinib-treated patient subset. In the overall dataset, 49 patients (54%) demonstrated a split signal pattern of positivity, 33 patients (37%) demonstrated a single red pattern of positivity and 8 patients (9%) demonstrated aspects of both patterns (cf. Figure 1). In the crizotinib-treated dataset, 18 patients (60%) demonstrated a split signal pattern of positivity, 10 patients (33%) demonstrated a single red pattern of positivity and 2 patients (7%) demonstrated aspects of both patterns.

The mean and range of cells positive by pattern of positivity is shown in Table 1. Tumors with a single red pattern of positivity had a significantly higher mean percentage of *ALK*+ cells than tumors with a split pattern of positivity (74% (26–100%) vs. 48% (18–82%), respectively; $p < 0.0001$). However, both patterns demonstrated a similarly wide range of percentage positive cells.

The correlations between the percentage of *ALK*+ cells, overall and by different patterns of positivity (split, single red and mixed), and the mean fused, isolated red and isolated green signal copy number are shown in Table 1. Increased copy number of isolated red signal (contributing to both single red and split patterns of positivity) strongly correlated with a higher percentage of cells positive for a rearrangement ($r = 0.743$, $p = < 0.0001$) (Figure 2). The correlation with isolated red signal copy number gain was stronger for the single red pattern than for the split pattern of positivity ($r = 0.884$, $p = < 0.0001$ vs. $r = 0.558$, $p = < 0.0001$, respectively).

Isolated green signal copy number (contributing to the split pattern of positivity and the single green negative pattern) was loosely associated with a lower percentage of cells positive for a rearrangement (negative association with higher percentage) ($r = -0.264$, $p = 0.012$). However, when only those tumors with a split pattern of positivity were assessed, the correlation between percentage cells positive and isolated green signal copy number strongly reversed ($r = 0.91$, $p = < 0.0001$).

Fused signal copy number was negatively associated with the percentage of cells positive for a rearrangement ($r = -0.466$, $p = < 0.0001$), particularly if the tumor was positive with a split as opposed to a single red pattern of positivity ($r = -0.44$, $p = 0.002$ vs. $r = -0.042$, $p = 0.016$).

Correlations between copy number of different signals (fused, isolated red and isolated green)

The correlations between the mean copy number per cell of fused signals and isolated red and isolated green signals are shown in Table 2. While there was no correlation between mean fused and isolated green signal copy number (all patterns), mean fused copy number was negatively associated with isolated red signal copy number ($r = -0.409$, $p = <0.0001$). As the isolated green signal is not specifically associated with *ALK* positivity per se, contributing as it does to both positive (split) and negative (single green) patterns, we re-analyzed the correlation between mean fused and isolated green signal copy number looking only in tumors with split as opposed to single green patterns of positivity. A similar inverse relationship to that seen with isolated red signal copy number was noted between the isolated green (split pattern) copy number and the fused signal copy number, however it did not reach statistical significance ($r = -0.425$, $p = 0.13$).

Correlations between percentage cells positive, signal copy number and maximal percentage tumor shrinkage on crizotinib

Among the 30 crizotinib treated patients the mean maximal tumor shrinkage per RECIST was 58% (range: 0–100%). Given the small numbers in the subgroups, there appeared to be little significant difference in the extent of tumor shrinkage seen by pattern of positivity: split ($n = 18$; mean = 58%; 0–100%), single red ($n = 10$; mean = 65%; 31–100%) and mixed ($n = 2$; mean = 24%; 16–32%).

Neither percentage cells positive ($r = 0.192$, $p = 0.3$) (Figure 3), nor isolated red ($r = 0.274$, $p = 0.195$) (Figure 4), nor isolated green (split pattern) ($r = 0.438$, $p = 0.117$) nor fused signal ($r = -0.247$, $p = 0.187$) copy number correlated with the maximal percentage tumor shrinkage per RECIST with crizotinib.

Conclusions

ALK gene rearrangements in NSCLC are highly predictive of clinical benefit from crizotinib, a small molecule inhibitor of *ALK* and *MET*.⁷ In most cases, treatment with crizotinib results in tumor shrinkage. The objective response rate in the first-in-man Phase I study of crizotinib was 61%, with a median duration of response of 48 weeks.⁸ In addition, when the waterfall plot of RECIST-defined changes in tumor size on treatment is examined, although not everyone reached the criteria for a RECIST-defined objective response, nearly 90% of patients manifested some degree of tumor shrinkage from the drug.⁸ Within the Phase I and other studies of crizotinib in *ALK* positive NSCLC, *ALK* positivity is determined using FISH with the Abbott Molecular break-apart probe set as the screening technique. We have previously shown that there is a broad range of percentage cells positive within tumors meeting the >15% criterion for positivity.⁹ Using separate FISH probes for both *EML4* and *ALK*, Martelli et al similarly noted *EML4-ALK* rearrangements in 50–100% of cells of their *ALK* positive tumors.¹¹ As we know that the presence of *EML4-ALK* alone is sufficient to act as a transforming event, the fact that not all cells in the tumor appear to demonstrate a gene rearrangement initially seems confusing.³ Based on their findings, Martelli et al concluded that *ALK* rearrangements may represent a late, rather than the primary, oncogenic event, resulting in the co-proliferation of different *ALK* positive and *ALK* negative clones within established tumors.¹¹ The alternative explanation for *ALK*+ tumors to not show positivity in all cells is that there is a consistent cell-by-cell false negative rate. Consistent with this is the observation that FISH analysis of cell pellets derived from *ALK* positive cell lines, such as NCI-H3122 and NCI-H2228, also do not show rearrangements in every cell (data not shown). In theory, such false negatives could occur due to compression or folding of the DNA randomly impacting the distance between the

sequences homologous to the red and green probes, nuclear sectioning causing loss of the 3' (red) probe binding site, aberrant probe hybridization or observer error. Observer error may be particularly problematic in lung cancer, where the predominant 5' fusion partner is *EML4* that resides only 12 MB centromeric from *ALK* on chromosome 2p. The paracentric inversion that generates the *EML4-ALK* fusion causes only minimal separation of the 3' and 5' *ALK* probes and therefore may be harder to spot than rearrangements involving genes mapped on different chromosomes seen in other *ALK* positive cancers.^{9,12} We have similarly shown that there is also the potential for a cell-by-cell false positive rate with up to 11% of non-tumor cells or tumor cells within protocol-defined *ALK* negative tumors appearing to demonstrate rearrangement patterns.⁹ Just as with false negatives, there are also numerous technical factors that could lead to false positive patterns. Among them are the stretching of the DNA leading to artificial separation of the red and green probe binding sites from each other, nuclear sectioning causing loss of the 5' (green) probe binding site, aberrant probe hybridization or observer error. Whether tumors with any percentage of positive cells will respond to crizotinib, or whether these are indeed false positive cells (as the detection of positive patterns in non-tumor tissue suggests can sometimes be the case) is unknown. Further exploration of the cell-by-cell false positivity rate would require second screening techniques such as immunohistochemistry and potentially wider access to specific inhibitors in the clinic to see if borderline tumors respond. Exploration of the potential for false cellular negatives in *ALK* FISH testing is, however, already possible via retrospective analyses set up to address a series of specific hypotheses (Table 3). We have previously shown that the heterogeneity in cellular positivity appears to be a diffuse and not a focal phenomenon within positive tumors, suggesting that clonal gain or loss of rearrangements is not the dominant basis of the heterogeneity.⁹ To further explore whether technique rather than biology underlies this heterogeneity, we analyzed in detail the association between patterns of positivity (split vs. single red patterns) and percentage cells positive and between signal copy number (of fused, isolated red and isolated green signals) and percentage cells positive (Figure 1). The higher percentage of positive cells seen in tumors with a single red pattern of positivity (74%), compared to the percentage seen in tumors manifesting a split pattern (48%) ($p < 0.0001$), suggest that difficulties in detecting the narrow probe separation in the dominant *EML4-ALK* paracentric inversion may indeed contribute towards 'missed' true positive cells (Table 1 and Table 3).

Given that only one split or single red pattern per cell is required to call a cell positive, if cells are falsely called negative because a positive pattern is missed in individual cells, we hypothesized that increases in the signal copy number associated with positive patterns should be correlated with a higher percentage of cells being called positive within any tumor. The strong positive association between increases in isolated red signal copy number and percentage of *ALK* FISH+ cells ($r = 0.743$, $p = < 0.0001$) and between isolated green signal copy number (when part of a split pattern of positivity) and percentage of *ALK*+ cells ($r = 0.91$, $p = < 0.0001$) appears to support this hypothesis (Tables 1 and 3 and Figure 2). The fact that the correlation is even stronger for the isolated red signals when only tumors with a single red pattern of positivity were considered, compared to those with a split pattern, also supports the idea that single red patterns may be easier to spot than split patterns ($r = 0.884$, $p = < 0.0001$ vs. $r = 0.558$, $p = < 0.0001$, respectively). As a corollary, the hypothesis is also supported by the observation that overall isolated green signal copy number (contributing to both the split pattern of positivity and the single green negative pattern) did not show a strong association with the percentage of cells positive for a rearrangement ($r = -0.264$, $p = 0.012$). Similarly, fused signal copy number was negatively associated with the percentage of cells positive for a rearrangement ($r = -0.466$, $p = < 0.0001$), particularly if the tumor was positive with a split as opposed to a single red pattern of positivity ($r = -0.44$, $p = 0.002$ vs. $r = -0.042$, $p = 0.016$), consistent with fused and split patterns being the hardest to distinguish.

If all the variation in percentage cells positive within a positive tumor only reflects technical aspects of *ALK* FISH testing rather than true biological heterogeneity, the proportion of positive cells should not influence therapeutic outcomes. Specifically, we hypothesized that there should be no correlation between the percentage of positive cells present in any positive tumor and the maximal percentage tumor shrinkage per RECIST seen in that tumor with crizotinib, which was indeed the case ($r = 0.192$, $p = 0.3$) (Table 3, Figure 3).

Copy number alterations in *ALK* related signals have recently been described.^{9,10} While we have exploited these alterations to address questions about false cellular negatives in *ALK*+ tumors, whether copy number alteration reflected biological variation that was directly clinically relevant was unknown. Here we have shown that neither isolated red ($r = 0.274$, $p = 0.195$) (Figure 4), nor isolated green (split pattern) ($r = 0.438$, $p = 0.117$), nor fused signal ($r = -0.247$, $p = 0.187$) copy number correlated with the maximal percentage tumor shrinkage per RECIST with crizotinib.

Potential flaws in the logic underlying these response-related data include the fact that the percentage of positive cells and the signal copy number mostly have been assessed on archival material, whereas the tumor itself may have changed biologically prior to commencing crizotinib. In addition, the estimate of tumor shrinkage per RECIST, although expressed as a percentage is based on proportional change in a very specific measurement calculation (the sum of the longest diameter of a number of pre-specified target lesions) (RECIST version 1.0). Therefore the proportional shrinkage may not reflect the exact magnitude of the biological response. For example, it may not be as direct a measure as assessing changes in tumor volume, and may underestimate the clinical activity of crizotinib in the correlations. However, based on our data, the lack of correlation between either percentage cells positive, or fused/rearranged signal copy number and crizotinib response does suggest that these are not relevant biological variables to follow with respect to benefit from *ALK* inhibition.

Finally, while there was no correlation between mean copy number of fused and isolated green signal copy number, mean fused copy number was negatively associated with isolated red signal copy number ($r = -0.409$, $p = <0.0001$) (Table 2). The inverse relationship between fused and isolated red signal copy number suggests that *ALK* gene rearrangements may not be a manifestation of simple chromosomal instability in these cancer cells. Specifically, this raises the possibility that while there may be some selection pressure for increases in *ALK* signal copy number, or that the region around the *ALK* gene may be particularly fragile, overall *ALK* gene rearrangements tend to originate in a distinct 'near-diploid' state. Similar near-diploid states have been described previously, notably in the microsatellite instability subtype of colorectal cancer.¹³ This suggests that *ALK* rearrangements may occur early in the cancer development process, before instability changes leading to significant chromosomal aneusomy occur.

In conclusion, our detailed analyses based on correlations between the percentage of *ALK*+ cells, patterns of *ALK* positivity and mean signal copy number per tumor cell and association of these variables with response to *ALK* inhibition in *ALK* FISH+ NSCLC, support the hypothesis that heterogeneity in the percentage cells positive within *ALK*+ tumors reflects technical rather than biological variation (Table 3). The lack of correlation between either percentage of cells positive or signal copy number and response to crizotinib suggests that these are not relevant biological variables to capture with respect to predicting benefit from *ALK* inhibition in *ALK*+ tumors. In addition, the negative association revealed between copy numbers of fused signals and isolated red signals suggests the novel hypothesis that *ALK* gene rearrangements occur early in cancer development, preceding

significant chromosomal aneusomy. Additional studies are warranted to investigate further whether *ALK*⁺ NSCLC is indeed a ‘near-diploid’ subtype of NSCLC.

Acknowledgments

Funding Source: Colorado Lung Specialized Program of Research Excellence P50CA58187

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Figure 1A

Fused (native) pattern:

Single green (5') pattern:

Split pattern:

Single red (3') pattern:

Figure 1B

Fused (native) pattern:

Single green (5') pattern:

Split pattern:

Single red (3') pattern:

Figure 1.

(A) Schematic diagram of the different cellular positive (split or single red) and negative (fused or single green) patterns for *ALK* FISH testing. (B) Schematic diagram exploring how individual fused, isolated red and isolated green signals are counted and contribute to the different positive and negative patterns seen.

Figure 2.

Correlation between the percentage of *ALK*⁺ cells and the isolated red signal copy number in *ALK*⁺ tumors. The ellipse represents the 95% bivariate normal prediction area for a new observation.

Figure 3.

Correlation between the percentage of *ALK*+ cells and the maximal percentage tumor shrinkage per RECIST with crizotinib in *ALK*+ tumors. The ellipse represents the 95% bivariate normal prediction area for a new observation.

Figure 4.

Correlation between the isolated red signal copy number and the maximal percentage tumor shrinkage per RECIST with crizotinib in *ALK*⁺ tumors. The ellipse represents the 95% bivariate normal prediction area for a new observation.

Table 1

Correlation between % of *ALK*⁺ cells and mean copy number per cell of fused (FCN), isolated red (IRCEN) and isolated green (IGCN) signals

Pattern of <i>ALK</i> rearrangement positivity	N	Variable	% <i>ALK</i> ⁺ vs. Mean fused signal copy number (FCN)				
			Mean	Minimum	Maximum	r	p
All <i>ALK</i> ⁺	90	% <i>ALK</i> ⁺	0.56	0.18	1	-0.466	<0.0001
		FCN	1.77	0.56	5.25		
<i>ALK</i> ⁺ with split pattern	49	% <i>ALK</i> ⁺	0.48	0.18	0.82	-0.440	0.002
		FCN	1.77	0.56	5.24		
<i>ALK</i> ⁺ with single red pattern	33	% <i>ALK</i> ⁺	0.74	0.26	1	-0.042	0.016
		FCN	1.57	0.68	3.63		
<i>ALK</i> ⁺ with mixed pattern	8	% <i>ALK</i> ⁺	0.37	0.25	0.6	-0.619	0.102
		FCN	2.54	1	5.25		

Pattern of <i>ALK</i> rearrangement positivity	N	Variable	% <i>ALK</i> ⁺ vs. Mean isolated red signal copy number (IRCEN)				
			Mean	Minimum	Maximum	r	p
All <i>ALK</i> ⁺	90	% <i>ALK</i> ⁺	0.56	0.18	1	0.743	<0.0001
		IRCEN	0.66	0.01	2.32		
<i>ALK</i> ⁺ with split pattern	49	% <i>ALK</i> ⁺	0.48	0.18	0.82	0.558	<0.0001
		IRCEN	0.51	0.01	1.86		
<i>ALK</i> ⁺ with single red pattern	33	% <i>ALK</i> ⁺	0.74	0.26	1	0.884	<0.0001
		IRCEN	0.95	0.02	2.32		
<i>ALK</i> ⁺ with mixed pattern	8	% <i>ALK</i> ⁺	0.37	0.25	0.6	0.898	0.002
		IRCEN	0.41	0.28	0.66		

Pattern of <i>ALK</i> rearrangement positivity	N	Variable	% <i>ALK</i> ⁺ vs. Mean isolated green signal copy number (IGCN)				
			Mean	Minimum	Maximum	r	p
All <i>ALK</i> ⁺	90	% <i>ALK</i> ⁺	0.56	0.18	1	-0.264	0.012
		IGCN	0.36	0	1.92		
<i>ALK</i> ⁺ with split pattern	49	% <i>ALK</i> ⁺	0.48	0.18	0.82	0.910	<0.0001
		IGCN	0.58	0.16	1.92		
<i>ALK</i> ⁺ with single red pattern	33	% <i>ALK</i> ⁺	0.74	0.26	1	-0.492	0.004

Pattern of <i>ALK</i> rearrangement positivity	N	Variable	% <i>ALK</i> + vs. Mean isolated green signal copy number (IGCN)				r	p
			Mean	Minimum	Maximum			
		IGCN	0.06	0	0.26			
<i>ALK</i> + with mixed pattern	8	% <i>ALK</i> +	0.37	0.25	0.6		0.762	0.028
		IGCN	0.26	0.08	0.4			

Table 2

Correlation between mean copy number per cell of fused signals versus isolated red and isolated green signals.

Variable (copy number per cell)	N	Mean	Minimum	Maximum	Correlation to mean fused signal copy number	
					r	p
Fused signal	90	1.77	0.56	5.25		
Isolated red signal		0.66	0.01	2.32	-0.409	<0.0001
Isolated green signal		0.36	0	1.92	-0.007	0.951

Table 3Hypotheses relating to *ALK* FISH heterogeneity

	Technique related	Biology related	Resolution
Distribution of positive cells within a positive tumor	Expected to be diffuse	Expected to be focal	Sliding windows analysis looking for areas where only 1 out of 4 high power field 'windows' met positivity criteria failed to demonstrate a single focal event out of 17 analyzed tumors (Figure 2) (Camidge et al, 2010).
Effect of pattern of positivity (single red vs. split) on percentage cells positive	Single red pattern should be easier to spot and be associated with a higher percentage of cells being positive	No differences expected	Tumors with a single red pattern of positivity had a higher mean percentage of positive cells compared to those with a split pattern of positivity (74% vs. 48%, respectively) ($p < 0.0001$)(Table 1).
Effect of signal copy number (fused, isolated red and isolated green signals) on percentage cells positive	Increased isolated red signal copy number (contributing to both split and single red patterns of positivity) and increased isolated green signal copy number (in split pattern positive tumors) should increase the chances of a true positive pattern being seen in any given cell and be associated with a higher percentage of cells being called positive	No correlation expected	Isolated red signal copy number strongly correlated with a higher percentage of cells positive ($r = 0.743$, $p = < 0.0001$) (Table 1, Figure 2). Isolated green signal copy number (split pattern of positivity) was strongly correlated with a higher percentage of cells positive ($r = 0.909$, $p = < 0.0001$) (Table 1). Fused signal copy number was negatively associated with the percentage of cells positive if the tumor had a split pattern of positivity ($r = -0.44$, $p = 0.0016$ vs. $r = -0.042$, $p = 0.016$), consistent with fused and split patterns being the hardest to distinguish.
Correlation between percentage cells positive and maximal percentage tumor shrinkage with crizotinib	No correlation expected	Correlation expected	Percentage cells positive did not correlate with the maximal percentage tumor shrinkage per RECIST with crizotinib ($r = 0.192$, $p = 0.3$) (Figure 3).