Optimizing the Detection of Lung Cancer Patients Harboring Anaplastic Lymphoma Kinase (*ALK*) Gene Rearrangements Potentially Suitable for ALK Inhibitor Treatment

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Abstract

Purpose: Anaplastic lymphoma kinase (*ALK*) rearrangements, associated with sensitivity to an experimental ALK/MET inhibitor, occur in 3% to 5% of non–small cell lung cancers. Intratumoral fluorescence *in situ* hybridization (FISH) heterogeneity has been reported. We explored the heterogeneity basis, the requirements for accurately determining *ALK* FISH positivity, and the effect of enriching the tested population using clinical and molecular factors.

Experimental Design: Lung cancer patients were screened by *ALK* and *MET* FISH and for *EGFR* and *KRAS* mutations.

Results: Thirteen ALK-positive cases were identified from 73 screened patients. Gene copy number increases occurred together with classic rearrangements. All positive cases were adenocarcinomas, 12 were *EGFR/KRAS* wild-type, and 1 had a coexistent *EGFR* exon 20 mutation. No association with *MET* amplification occurred. ALK positivity was associated with <10–pack-year smoking status (*P* = 0.0004). Among adenocarcinomas, without *KRAS* or *EGFR* mutations, with <10–pack-year history, 44.8% of cases were ALK positive. *ALK* FISH positivity was heterogeneous, but mean values in tumor areas from *ALK*-positive patients (54% of cells; range, 22-87%) were significantly higher than in adjacent normal tissue or tumor/ normal areas from ALK-negative patients (mean, 5-7%). Contiguous sliding field analyses showed diffuse heterogeneity without evidence of focal *ALK* rearrangements. One hundred percent sensitivity and specificity occurred when four or more fields (∼60 cells) were counted.

Conclusions: Intratumoral *ALK* FISH heterogeneity reflects technique, not biology. The clinical activity of ALK/MET inhibitors in ALK-positive patients probably reflects ALK, but not MET, activity. Prescreening by histology, *EGFR/KRAS* mutations, and smoking status dramatically increases the ALK-positive hit rate compared with unselected series. *Clin Cancer Res; 16(22); 5581–90. ©2010 AACR.*

Transforming rearrangements of the *ALK* gene have been reported in a few human hematologic and solid malignancies (1, 2). These in-frame gene rearrangements place the ALK kinase domain under the promoter control of another gene, with its intracellular localization and function potentially influenced by the specific NH$_2$-terminal protein fusion partner expressed (1). In 2007, an *ALK* gene rearrangement creating an in-frame fusion protein between ALK and echinoderm microtubule-associated protein-like 4 (EML4) was described in non–small cell lung cancer (NSCLC; ref. 3). Since then, several different transforming in-frame fusion variants of *EML4-ALK* in NSCLC have been described with different *EML4* and *ALK* breakpoints, in addition to other rarer non-*EML4* fusion partners, including *TFG* and *KIF5B* (3–8).

The incidence of *ALK* gene rearrangements detected by either real-time PCR (RT-PCR) or fluorescence *in situ* hybridization (FISH) seems to be low in largely unselected NSCLC series (∼3-5%; ref. 9). However, ALK status, at least that determined by FISH, does seem to be extremely important as a specific treatment sensitivity marker with respect to small-molecule inhibitors of ALK (9, 10).

The ability to accurately identify a patient as truly ALK positive or negative using any given screening technique is essential for appropriate therapy selection and outcome assessment. Recent reports of significant intratumoral heterogeneity of EML4-ALK fusions detected by FISH have raised questions about both whether *ALK* gene rearrangements could easily be missed and, if subclones with and

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Translational Relevance

Rare *ALK* gene rearrangements detected by a specific fluorescence *in situ* hybridization assay are associated with marked sensitivity to a small-molecule inhibitor of ALK currently in clinical development in advanced non–small cell lung cancer. Reports of intratumoral heterogeneity of *ALK* rearrangements have raised issues about the true importance of ALK in carcinogenesis and the possibility of high false-positive/false-negative screening results. Here, we show that the apparent heterogeneity reflects technique rather than biology, determine the optimal number of fields to count to ensure maximal sensitivity and specificity, and confirm that when >4 fields are counted the >15% cell positivity cut point used in the current trials falls within the range accurately distinguishing positive from negative tumors and from nontumor regions. In addition, we show how a combination of clinical and other molecular factors identifies a population particularly enriched for *ALK* gene rearrangements that could significantly increase the ALK-positive screening hit rate in the future.

without *ALK* rearrangements commonly coexist in tumors, whether they may be later rather than primary transforming events in human lung cancers *in vivo* (11). In addition, in the absence of universal screening, being able to identify, in advance, which patients are most likely to possess *ALK* gene rearrangements would enable treating physicians to prioritize which patients to refer for ALK screening (e.g., by FISH) and possible enrollment within an ALK inhibitor trial.

Here, we report on a detailed exploration of the heterogeneity of ALK-positive signals across tumor and surrounding normal tissue and the specific cytogenetic characteristics of those who tested positive using FISH break-apart probe technology. We also report on how selecting patients on the basis of histology, *EGFR* and *KRAS* mutation status, and smoking history has significantly enriched our numbers testing positive for *ALK* gene rearrangements compared with previously reported unselected series.

Materials and Methods

Patients

All lung cancer patients tested received treatment or consultation from the University of Colorado Thoracic Oncology Program. Since mid-2008, all new NSCLC patients seen at the University of Colorado have had routine testing of their tumors for epidermal growth factor receptor (EGFR) expression by immunohistochemistry, *EGFR* gene copy number by FISH, and *EGFR*, *KRAS*, and *TP53* mutational status done through the on-site Clinical Laboratory Improvement Amendments–certified Colorado Molecular Correlates (CMOCO) Laboratory (12). In addition, to identify patients for entry into defined molecular cohorts treated within the phase I study of crizotinib (PF-02341066), we began to screen tumor biopsies from selected NSCLC patients for *MET* gene copy number alterations and for *ALK* gene rearrangements by FISH. An Institutional Review Board–approved protocol approved in early 2009 permits both retrospective and prospective clinical correlates to be made on all in-house patients in whom molecular analyses have been conducted within CMOCO.

CMOCO mutation tests

Mutational analysis was done as previously described (13). Briefly, tumor-rich regions were identified in each block of formalin-fixed, paraffin-embedded (FFPE) tissue. Tissue cores were then obtained from these regions using a microarrayer device and 1-mm arrayer needles. Tissue cores were deparaffinized by soaking in xylene overnight and subsequently digested with protease K. DNA was isolated from the incubation mixture using a QIAcube robotic workstation (Qiagen, Inc.) extraction protocol. *EGFR* exons 19, 20, and 21 and *KRAS* exon 2 were then amplified and sequenced using a ABI model 3730 capillary gel sequencer. Mutations were identified by visual inspection of the resulting chromatograms and automated scanning using Mutation Surveyor v3.24.

MET and *ALK* FISH testing

Unstained 4-μm sections of FFPE tumor tissue were submitted to dual-color FISH assays using two probe sets. The *MET/CEP7* probe set was developed in house with a DNA insert encompassing *MET* (RP 11-95I20 bacterial artificial chromosome clone) labeled with SpectrumRed and the SpectrumGreen CEP7 probe (Abbott Molecular). The LSI *ALK* (anaplastic lymphoma kinase) Dual Color, Break-Apart Rearrangement Probe (Abbott Molecular) was designed to detect rearrangements in 2p23 encompassing the *ALK* gene. This probe set includes a 250-kb DNA fragment telomeric to *ALK* (3′ end) labeled in Spectrum-Orange and a 300-kb fragment centromeric to *ALK* (5′ end) labeled in SpectrumGreen.

For both probe sets, assays were done according to a protocol previously described (14). Signals were enumerated in at least 50 tumor nuclei per core using an epifluorescence microscope with single interference filters sets for green (FITC), red (Texas red), and blue (4′,6-diamidino-2-phenylindole) as well as dual (red/green) and triple (blue, red, green) band-pass filters.

In the *MET* FISH assay, specimens were classified as carrying gene amplification when the *MET/CEP7* ratio was >2 or when >10% tumor cells carried clusters or >15 copies per cell of *MET* signals. For documentation, images were captured using a charge-coupled device (Photometrics) and merged using dedicated software (CytoVision, Genetix). In the *ALK* FISH assay, the native state appears as fused

or adjacent red/green (yellow) signals, whereas a rearrangement with a breakpoint at the 2p23 *ALK* region produces single red and single green signals. Red and green signals separated by a gap larger than 2 signal diameters were considered split. The occurrence of an *ALK* rearrangement (*ALK* FISH positive) was concluded if >15% of tumor cells showed split red and green signals (15) and/or single red signals; otherwise, the specimen was classified as *ALK* FISH negative.

In the detailed FISH analyses to investigate intratumoral heterogeneity and the presence of the rearrangement in nontumor cells, the whole section was scanned using a contiguous "sliding strategy." In addition to the 13 positive cases detected among the Colorado CMOCO patients, an additional 4 positive Caucasian cases from Italy identified in other studies were also included in these analyses. Beginning at a selected "starting corner" and using the high-power objective, a tumor area was selected (T1) and ~15 representative tumor cells were analyzed. Then, the microscope stage was moved 2 mm along the Y axis and another tumor area (T2) was chosen for analysis of 15 tumor cells. A similar sequence of steps was then used to reach the third location (T3), and so on. In the event that a selected location included nontumor cells such as lymphocytes, macrophages, or smooth muscle cells, this area was identified as N1, and 15 of these cells were analyzed. When reaching an area without tissue, the microscope stage was moved 2 mm along the X axis and the stage was again moved 2 mm along the Y axis, looking for tumor (T) and normal (N) areas. This strategy guaranteed that specimens were scanned in entirety, that no overlap occurred among different areas, and that no cell was counted in more than one area. Following this schema, from 7 to 20 tumor areas and from 5 to 15 nontumor areas were analyzed in each specimen.

Statistical analyses

Unless otherwise specified, for the analyses of clinical and CMOCO markers on the patient samples, the Fisher's exact test was used to assess correlation between categorical variables and Student's *t* test was used to assess association between the distributions of treatment outcome. All reported *P* values are two-sided unless otherwise specified, and we considered a test as statistically significant if $P < 0.05$.

To assess whether *ALK* rearrangement was a focal event, we used a window of four consecutively scored high-power objective fields generated through the sliding strategy described above (e.g., areas T1-T4, T2-T5, etc.) through all the tumor areas tested for *ALK* rearrangement in each individual ALK-positive patient. We hypothesized that variability resulting from technical aspects of the FISH assay would produce a diffuse effect, and positive signals (>15% cells positive for rearrangement) would be seen in most fields tested. In contrast, if independent ALK-positive and ALK-negative subclones existed, we would expect to see differences between fields in the same tumor. *ALK* rearrangement was considered focal if only one of the four areas analyzed was positive, whereas all other combina-

tions were taken as proof that *ALK* rearrangement was a diffuse event.

To determine the optimal number of high-power microscope fields and tumor nuclei required to accurately define a patient's tumor as positive or negative for *ALK* rearrangement, we randomly selected two to seven tumor areas from ALK-positive and ALK-negative patients in 100 permutations. If the average percent of positive cells in the permutation was >15%, we called that permutation ALK positive, and vice versa for ALK negative. Using large numbers of tumor areas to generate what we considered definitive ALK-positive and ALK-negative status, we could compute the number of true positives (TP) and true negatives (TN), false positives (FP), and false negatives (FN) for permutations with lower numbers of counted fields. We defined TP and TN as the tumor areas that were obtained and correctly determined as ALK positive and negative in patients who, overall, were concluded as ALK positive and negative, respectively. FP was defined as tumor areas sampled from ALK-negative patients but determined as ALK positive because these areas showed >15% of cells positive for rearrangement patterns. FN was defined as tumor areas obtained from ALK-positive patients but wrongly classified as ALK negative. From this permutation test, we could calculate sensitivity [TP/(TP + FN)] and specificity [TN/(TN + FP)] for the number of high-power microscope fields and number of tumor nuclei required to determine ALK positivity.

Results

Patients tested for *ALK* gene rearrangements

Between June 2008 and October 2009, a total of 73 lung cancer patients were considered for testing with the *ALK* FISH break-apart assay within the University of Colorado Thoracic Oncology Program. Of these, four cases could not be analyzed due to insufficient tumor material remaining in the biopsy specimen and three cases could not be analyzed due to assay failure, in one case due to DNA degradation following decalcification of a bone biopsy specimen. From the remaining 66 specimens, 13 cases were positive for *ALK* gene rearrangements (20%).

Cytogenetic patterns of *ALK* positivity

Table 1 shows the descriptive statistics for each of the 13 patients classified as positive for the *ALK* rearrangement, including the mean copy number per cell (and SD) for the normal signal (fused 3′ red–5′ green *ALK*), for the split/single 3′ *ALK* (red), and the split/single 5′ *ALK* (green) signals, and the distribution of tumor cells according to the possible patterns combining these different signals. Normal signals are frequently seen in cells that also display *ALK* gene rearrangements. The classic *EML4-ALK* fusion, as well as translocations between *ALK* and other genes, should typically show distinct red and green signals separated within the same cell. However, either due to nuclear truncation or true physical deletion of chromosomal

Table 1. Descriptive statistics for each of the 13 CMOCO patients classified as positive for the *ALK* rearrangement, including the mean copy number per cell (and SD) for the normal signal (3'/5' *ALK*, fused red/green), for the single 3' *ALK* (red) and the single 5' *ALK* (green) signals, and the distribution of tumor cells according to the possible patterns combining these three types of signals

ALK-positive patient	Fused (3'/5')		Single 3' ALK		Single 5' ALK		Percentage of cells with given patterns							% Cells positive for ALK rearrangement
	Mean	SD	Mean	SD	Mean	SD	Only 3'/5'	Only 3'	Only 5'	3'/5' and 3'	3'/5' and 5'	3' and 5'	3'/5', 3' and 5'	
1	3.63	2.68	1.18	1.41	0.06	0.24	40%	0%	0%	54%	2%	0%	4%	58%
2	2.45	1.25	0.61	0.63	0.74	0.76	40%	0%	0%	3%	6%	4%	47%	54%
3	2.7	1.07	0.54	0.68	0.78	0.93	42%	0%	0%	4%	12%	0%	42%	46%
4	1.69	1.07	0.42	0.59	0.38	0.6	57%	0%	0%	10%	6%	9%	18%	37%
5	1.61	0.75	0.31	0.53	0.31	0.56	69%	0%	0%	5%	3%	6%	17%	28%
6	1.75	0.67	1.18	1.03	0.14	0.35	24%	0%	0%	62%	7%	1%	6%	69%
7	2.4	1.21	0.79	0.86	0.79	0.88	33%	0%	0%	16%	14%	3%	34%	53%
8	0.56	0.92	2.46	2.44	1.05	1.45	17%	20%	1%	6%	0%	42%	14%	82%
9	1.72	0.7	1.16	0.91	0.1	0.36	30%	2%	0%	60%	0%	2%	6%	70%
10	1.6	0.86	0.88	0.82	0.91	0.87	33%	0%	0%	4%	3%	8%	52%	64%
11	1.72	0.88	0.94	0.74	0.16	0.42	24%	0%	0%	62%	6%	2%	6%	70%
12	0.98	0.8	1.86	0.95	1.92	1.03	6%	0%	0%	4%	0%	22%	68%	94%
13	3.1	1.25	0.5	0.61	0.64	0.72	46%	0%	0%	2%	10%	0%	42%	44%

segments, atypical patterns including only single red (3' end) and only single green (5' end) signals were also observed. To compile the frequency of positive cells for *ALK* rearrangements, the sum of cells carrying split red and green and single red (*ALK* 5' region) signals was considered, in the presence or absence of normal fused signals. The predominant *ALK*-positive FISH pattern was fusion and split red and green signals in eight patients (62%), whereas in four (31%) it was fusion and single red, and in one (7%) it was single red and single green signals. Multiple copies per cell of single reds, clusters of red and green signals, and clusters of fusion signals were also seen, suggesting that increases in gene copy number occur in addition to rearrangements. Representative variations in the cytogenetic patterns of *ALK* rearrangements are shown in Fig. 1.

Details and consequences of heterogeneity in *ALK* positivity

As depicted in Fig. 2 and Table 2, tumor areas tested in ALK-positive patients had an average of 53.8% of cells with a positive signal, which was significantly higher than in the adjacent normal areas in ALK-positive patients (6.81%; $P = 2.2 \times 10^{-8}$, t test), as well as in both
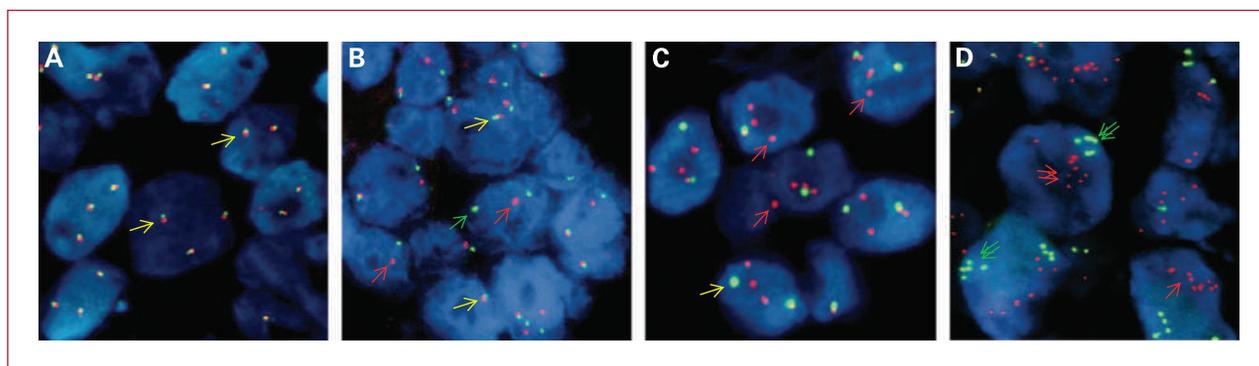
Fig. 1. NSCLCs hybridized with the *ALK* break-apart FISH probe (Abbott Molecular). Native ALK status (indicated by yellow arrows) shows fusion of the probes adjacent to the 3' and 5' ends of the gene, labeled, respectively, with red and green fluorophores. Rearranged *ALK* is indicated by the presence of split 3' (red arrows) and 5' (green arrows) signals, including single red signals. A, *ALK* FISH-negative specimen. B, "classic" *ALK* FISH-positive specimen showing split red and green signals. C, *ALK* FISH-positive specimen showing two or three copies of single red signals per cell. D, *ALK* FISH-positive specimen showing evidence of gene copy number increase with clusters of multiple single red and single green signals (double arrows).
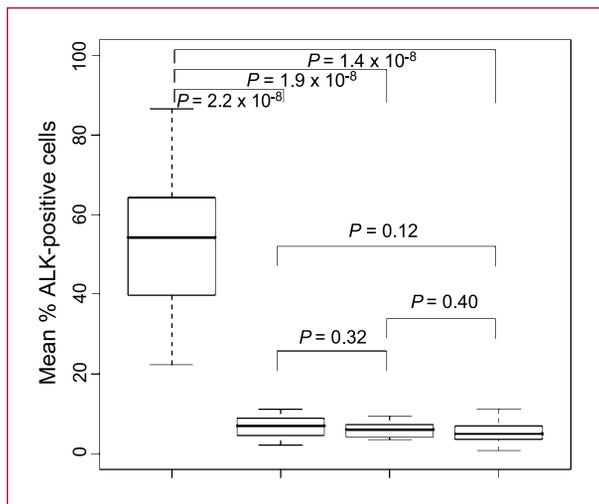
Fig. 2. Box-and-whisker plot of ALK-positive and ALK-negative samples in tumor and adjacent normal areas. Seventeen ALK-positive and 15 ALK-negative patients were analyzed to determine the % of ALK-positive cells from various tumor and adjacent normal areas from each individual patient. *P* values were computed by Welch two-sample *t* test. The box portion of the box-and-whisker plot includes 50% of the data. The bold line in the box represents the median of the data, and the borders of the box represent the lower quartile (25% percentile) and the upper quartile (75% percentile) of the data. The upper and lower whiskers represent the maximum and minimum % of ALK-positive cells.

tumor (5.98%; $P = 1.9 \times 10^{-8}$, t test) and adjacent normal areas (5.26%; $P = 1.4 \times 10^{-8}$, t test) in ALK-negative patients. To explore whether intratumoral ALK heterogeneity was a diffuse or focal event, the sliding window technique was used. From 16 to 26 four-area "sliding windows" were available for analysis in each of the 17 ALK-positive specimens and not a single focal event (1 of 4 windows positive) was detected.

Table 3 depicts results of statistical simulations on two to seven randomly selected tumor areas (100 permutations) from individual ALK-positive and ALK-negative patients. Large numbers of tumor areas were used to generate "true" ALK-positive and ALK-negative status. The sensitivity and specificity of using different lower numbers of high-power microscope fields and number of tumor nuclei required to determine true ALK positivity are shown. Maximum sensitivity and specificity occurred when four tumor areas (~60 cell nuclei) were analyzed.

Clinical and molecular factors associated with ALK positivity

Of the 66 analyzed specimens in our series, 61 were adenocarcinoma, 1 was a small cell lung cancer, 1 was carcinoid, 2 were squamous cancers, and 1 was a large cell neuroendocrine cancer. The primary histology was reviewed in house, and in all 13 ALK-positive cases, the underlying histology was described as adenocarcinoma of the lung. All other analyses described below were only conducted among the adenocarcinoma population.

In the first 55 adenocarcinomas screened for *ALK* gene rearrangements in whom both *EGFR* and *KRAS* status was known, 7 of the 42 ALK-negative cases showed *EGFR* mutations (4 exon 19 deletions, 1 L858R point mutation, and 2 S768I point mutations), whereas among the 13 ALK-positive cases only 1 showed an *EGFR* mutation (exon 20 point mutation S768I). None of the 55 patients had a *KRAS* mutation. The smoking status in pack-years of each of these 55 patients and their mutational status are shown graphically in Fig. 3.

Among our 13 *ALK* FISH-positive cases, *MET* copy number was also available in 9 (6 with *MET* high polysomy but no gene amplification and 3 with no or low *MET* copy number gain). Among the closest comparable group {i.e., ALK-negative never/light smoking adenocarcinomas without either *EGFR* [tyrosine kinase inhibitor (TKI) sensitivity mutations] or *KRAS* mutations}, *MET* information was available in all 16 cases (5 with *MET* high polysomy but no gene amplification, and 11 negative for any *MET*-related FISH changes). Taken together, although *ALK* FISH-positive cases seemed to be significantly correlated with *MET* high polysomy ($P < 0.0001$, Fisher's exact test), they were not correlated with *MET* gene amplification. No correlation between *ALK* FISH-positive cases with *TP53* mutational status could be established: none of the 11 ALK FISH-positive cases tested for *TP53* showed *TP53* mutations, whereas 5 of the 41 ALK-negative cases tested were *TP53* mutant ($P = 0.288$, Fisher's exact test).

Among our 13 ALK-positive patients, the mean and median ages at diagnosis were 55.4 and 53 years, respectively (range, 34-75), and the gender split was 9F:4M. With regard to race, 12 of our ALK-positive patients were Caucasian and 1 was Hispanic. Twelve of the ALK-positive patients were resident in Colorado at the time of diagnosis and 1 was resident in South Carolina. Our reference

Table 2. ALK-positve and ALK-negative samples in tumor and adjacent normal areas

| Histology | Patient category | | | |
| | ALK Positive | | ALK negative | |
	Tumor	Nontumor	Tumor	Nontumor
No. areas tested (range)	16 (7-20)	8 (5-15)	8 (18-20)	8 (5-10)
Mean no. cells scored (range)	15 (12-16)	14 (11-19)	15 (14-16)	13 (11-15)
% Cells positive for rearrangement (range)	53.80 (22.25-86.62)	6.81 (2.14-11.14)	5.98 (3.51-9.45)	5.26 (0.71-11.21)

Table 3. Sensitivity and specificity of the ALK FISH assay based on different number of tumor areas/nuclei scored compared with definitive status set by analysis of entire specimen following a contiguous sliding field strategy

	Number of tumor areas					
	2	3	4	5	6	7
% Sensitivity	98.6	99.3	100	100	100	100
% Specificity	96.6	100	100	100	100	100
No. nuclei scored	~30	~45	~60	~75	~90	~105

population was not sufficiently diverse to make any meaningful conclusions about either race or geographic factors on the ALK positivity rate.

Retrospective treatment efficacy results on advanced ALK-positive cases

Progression-free survival (PFS) for patients with advanced disease was calculated from the start of therapy until documented radiographic or clinical progression. Of our 13 ALK-positive cases, advanced disease treatment outcome for platinum-based chemotherapy and for EGFR TKIs were available in 7 (2 ongoing and censored at data gathering) and 3 (none ongoing) patients, respectively. For platinum-based chemotherapy, mean PFS was 3.3 months (range, 2-5 months). For EGFR TKI therapy, mean PFS was 5.3 months (range, 3-9 months). For the ALK-negative cases known to lack EGFR TKI sensitivity mutations and KRAS mutations, treatment outcome for platinum-based chemotherapy and EGFR TKI were available in 22 (4 ongoing and censored at data gathering) and 6 (1 ongoing and censored at data gathering) patients, respectively. For platinum-based chemotherapy, mean PFS was 7.0 months (range, 1-19 months). For EGFR TKI therapy, mean PFS was 6.5 months (range, 1-22 months). Of note, for the ALK-negative patients with EGFR TKI sensitivity mutations, treatment outcome for EGFR TKI therapy was available in 2 (1 ongoing and censored at data gathering) patients, with a mean PFS of 18 months (12 and 24 months). Although numerically ALK-positive patients had a shorter PFS with platinum-based therapy and a comparable PFS on EGFR TKI therapy to ALK-negative EGFR/KRAS wild-type patients (both of these groups appearing to have a much shorter PFS on EGFR TKI therapy than known EGFR mutant patients), given the small sample size of the different subgroups, and the high proportion of patients still on therapy, we did not pursue formal statistical comparisons on this treatment data set.

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[4] H. Mano, personal communication.

Discussion

The original identification of EML4-ALK as a potential early driver of lung carcinogenesis came through a screen for transforming cDNAs isolated from a lung adenocarcinoma known to be both EGFR and KRAS wild-type, occurring in a 62-year-old male Japanese with a low smoking history (4.5 pack-years)[4] (3). The importance of detecting ALK gene rearranged NSCLCs relates to the apparent high predictive value of the gene rearrangement with regard to sensitivity of the tumor to small-molecule inhibitors of ALK (16). In the first-in-man phase I study of crizotinib (a potent dual inhibitor of ALK and MET), an objective response rate of 53% and a disease control rate at 8 weeks of 79% were reported following its use in advanced NSCLC patients known to harbor ALK gene rearrangements, detected with the break-apart ALK FISH probe (10). Crizotinib is now being explored in ALK FISH-positive advanced NSCLC patients study compared with standard second-line chemotherapy in an international randomized phase III study.

Recently, the apparent intratumoral heterogeneity of ALK gene rearrangements and the presence of rearrangements in nontumor cells have both been raised as areas of concern. Using separate FISH probes for both EML4 and ALK, 2.7% of NSCLCs cases were noted to be EML4-ALK positive in 603 tissue microarray–screened tumors (11). However, rearrangements were only detected in 50% to 100% of cells in positive tumors. Based on this finding, the authors concluded that ALK rearrangements may represent a late, rather than an early, tumorigenic event, resulting in the coproliferation of different clones (both ALK positive and ALK negative) within established tumors. Using RT-PCR, Martelli et al. detected EML4-ALK fusion transcripts in 7.5% of 120 frozen NSCLC samples (17). However, EML4-ALK transcripts were also detected in frozen nontumor lung samples taken at the time of the initial tumor resection in 10 of 67 patients, all of whom were negative for EML4-ALK in their tumors. Among eight paraffin-embedded tumor specimens positive for the RT-PCR transcript in the same study, the frequency of FISH-positive cells ranged from only 1.2% to 9% of cells.

Different ALK assays will undoubtedly have different FP and FN rates. ALK gene rearrangement screening in NSCLC using the FISH break-apart probe described here is technically sophisticated, significantly more so than, for example, FISH that is designed to simple estimate increases in gene copy number. Even among break-apart probe tests, ALK gene rearrangements generally require significant expertise for accurate assessment. When molecular fusions occur between genes mapped in different chromosomes or far distant within the same chromosome, the sequences at the 3′ and 5′ end of the gene targeted by the break-apart probe will be physically separated and the red and green signals representing these sequences will be easily verified as split. However, the EML4-ALK fusion, which is considered to be the most

Fig. 3. Scatter plot of patient number [adenocarcinomas of known EGFR and KRAS status (marked)] versus smoking status in pack-years. The vertical line separates ALK-positive from ALK-negative cases. ALK FISH positivity was statistically significantly enriched among never/light smokers (<10 pack-years; P = 0.0004, Fisher's exact test; proportion positive among never/light smoking adenocarcinomas = 13 of 33, 39%; proportion positive among those with >10–pack-year smoking history = 0 of 22).
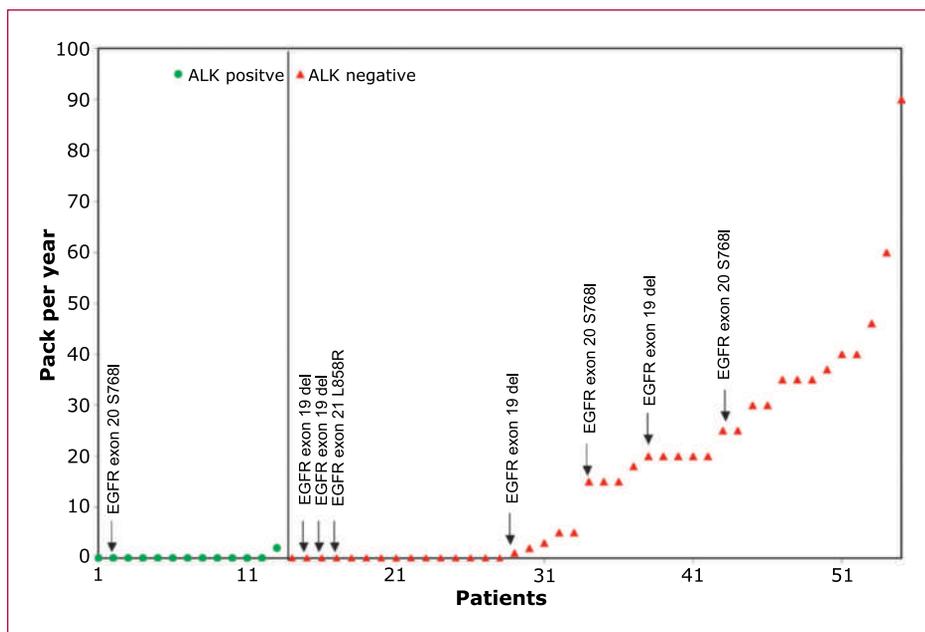
common *ALK* gene rearrangement in NSCLC, is a consequence of a paracentric inversion on 2p such that the 3′ and 5′ *ALK* sequences are only separated by ~12 Mb. In interphase chromatin, the signals generated by these two probes, although split, will therefore usually show as closely "paired" not independently distributed in the nucleus as with other break-apart probes. However, the FISH break-apart probe is the ALK-related marker that has been most extensively explored in relation to clinical activity following ALK inhibitor intervention. We were able to successfully conduct *ALK* FISH testing in 90% (66 of 73) of our initial screening cohort. The detailed cytogenetic rearrangement patterns seen in ALK-positive tumors (Table 1 and Fig. 1) reveal the potential for activating chromosomal deletions (single red 3′ *ALK*) and fusion/truncation gene copy number increases, in addition to the classic split signal occurring with the *EML4-ALK* inversion, to contribute to the transforming potential of ALK in NSCLC. Single green signals (5′ *ALK*) also occur, but as these do not represent the kinase domain of *ALK*, they do not contribute to the definition of ALK positivity.

When the signal patterns generated by the break-apart FISH probe in nontumor tissue and in tumor samples from ALK-positive and ALK-negative tumors are compared (Fig. 2), several conclusions can be drawn. Firstly, as previously noted, not all cells within a positive tumor display the positive signal patterns for an *ALK* rearrangement. In our series, positivity occurred in 22.25% to 86.62% of the cells in ALK-positive tumors. Second, there is a clear "background noise" associated with the assay with up to 11% of cells in both nontumor sections and ALK-negative tumors scoring positive. This "noise" seems to be entirely assay related and not a true biolog-

ical phenomenon, as the signals in nontumor sections and ALK-negative tumor specimens did not differ significantly from each other, but each did differ significantly from the ALK-positive tumor areas. Importantly, the >15% cut point used in the crizotinib trials falls within the nonoverlapping area of 12% to 21% positivity that seems to accurately differentiate biology from assay variability. FP and FN signals within individual cells are likely to have multiple different etiologies, including nuclear truncation on sectioning (signals are physically lost), aberrant probe hybridization (intended target sequences do not hybridize with the labeled probe and/or the probe does hybridize to nonspecific sequences), background noise (weak specific signals mimic background nonspecific noise), and observer error (nontumor cells included in scoring, adjacent red/green incorrectly classified as fused or split). In further support of assay, rather than biological, variability driving the apparent intratumoral heterogeneity in cell positivity, we found no evidence of discrete foci of *ALK* gene rearrangements, as might occur if distinct subclones of positive and negative cells truly existed, when a series of four sliding fields were tracked across positive tumor specimens, allowing us to conclude that *ALK* gene rearrangements are truly diffuse events within ALK-positive tumors. Taken together, these data suggest that variations in the percentage of positive cells within positive tumors are unlikely to represent a true biological phenomenon, and although retrospective clinical data will be required to confirm this, we would predict that the exact percent of positive cells in a positive tumor will probably have little bearing on outcome following treatment with an ALK inhibitor.

Given the propensity for individual cells to falsely read as positive or negative with the break-apart FISH assay,

determination of the optimal number of high-power fields and cells to count is imperative for accurate reporting. In Table 2, it is clear that both sensitivity and specificity increase as the number of fields and cells increase. As expected, the worst performance was based on two tumor areas with an average of 30 cells scored, where the decision would be made with 96.6% specificity and 98.6% sensitivity. In contrast, examination of four or more tumor areas including ~60 tumor cells provided 100% sensitivity and specificity in determining ALK positivity, establishing these conditions as the minimum requirements to accurately determine the presence or absence of *ALK* rearrangements in NSCLC specimens.

Among our initial 66 specimens tested, 13 were positive for *ALK* gene rearrangements (20%). The reported incidence in unselected series is ~3% to 5%, and our high initial incidence reflects both our routine molecular screening for *EGFR* and *KRAS* mutations and an evolving awareness of the clinical and molecular characteristics associated with ALK positivity (specifically, adenocarcinomas known to be *EGFR* and *KRAS* wild-type; ref. 9). Together, these factors lead us to adopt a highly focused approach designed to enrich the tested cohort for *ALK* gene rearranged cancers. Although we can quantify the frequency of ALK positivity within subsets of the group that we have defined, it is important to note that our ALK-negative group for comparisons does not represent an unselected NSCLC population; instead, it represents the negatives seen within the same increasingly preselected group. By taking all *EGFR* mutations into consideration, our ALK positivity rate in adenocarcinomas known to be *EGFR* and *KRAS* wild-type was 12 of 47 (25.5%). The one *EGFR* mutation noted among our ALK-positive patients was an exon 20 point mutation, which has not been associated with EGFR TKI sensitivity (18).Given that the primary clinical significance of *EGFR* mutations relates to their association with sensitivity to EGFR TKIs, if we chose to only consider *EGFR* mutations associated with EGFR TKI sensitivity, our ALK positivity rate became 13 of 50 (26%). Among adenocarcinomas in whom both EGFR and KRAS status was known, there was a trend for ALK-positive cases to be enriched among those known to lack both *EGFR* (sensitivity mutations) and *KRAS* mutations (26%; 13 of 50) compared with those with known mutations (0%, 0 of 5; P = 0.245, Fisher's exact test). Whether this general lack of co-occurrence of *EGFR* or *KRAS* mutations with *ALK* gene rearrangements reflects biology (i.e., lack of a baseline selection pressure for a second oncogenic driver in the presence of an existing one), or simply the low chances of two rare events occurring together, is uncertain. With regard to the first point, although not a TKI sensitivity mutation, the coexisting exon 20 mutation noted in our series may still be a driving mutation. Similarly, within a tissue microarray,coexistence of an *ALK* gene rearrangement and a classic activating *EGFR* exon 19 deletion (E746_A750) has also been described (19). How frequently potential molecular codrivers exist when larger data sets are available, how cells with more than one driver will react when treated with specific inhibitors *de novo*, and whether upregulation or

selection of subclones with others drivers could provide a means for acquired resistance following specific inhibitor use, as has, for example, been documented with *EGFR* mutations and *MET* gene amplification, remains to be seen (20, 21). Of note, we found no association between *ALK* gene rearrangements and *MET* gene amplification, a known preclinical sensitivity factor for MET inhibition, suggesting that the clinical activity seen with crizotinib in ALK-positive cases is likely to reflect the anti-ALK activity of this dual-targeted drug (22). We did see an association with *MET* high polysomy (P < 0.0001, Fisher's exact test), although, in the absence of knowing the copy number of other chromosomes, a specific selection for chromosome 7 cannot be distinguished from a more general trend toward increased genetic instability in these cells.

A general trend toward ALK positivity being more common among those with little or no smoking history has been reported (9). In our own series, <10–pack-year smoking status, based on precedents from the *EGFR* mutation literature, was a highly statistically significant cut point for enriching for ALK-positive patients (Fig. 3; ref. 23). Moreover, when only those known to be wild-type for both *EGFR* (TKI sensitivity mutations) and *KRAS* mutations were considered, the effect was even stronger [P = 0.0002, Fisher's exact test; proportion positive among never/light smoking adenocarcinomas wild-type for both *EGFR* (TKI sensitivity mutations) and *KRAS* mutations = 13 of 29, 44.8%; proportion positive among those with >10–pack-year smoking history wild-type for both *EGFR* (TKI sensitivity mutations) and *KRAS* mutations = 0 of 21; Fig. 3]. We interpret these data as showing that smokers are more prone to getting other, non–ALK-driven, types of lung cancer, rather than smoking being somehow protective against *ALK* gene rearrangements.

Taken together, our data suggest that using aspects of histology, *EGFR, KRAS*, and smoking status to prescreen patients can enrich the population testing positive for an *ALK* gene rearrangement by ~10-fold (~45% positivity rate) compared with unselected series (9). Because *EGFR* and *KRAS* mutational testing is becoming routinely available, in the absence of universal ALK testing, this prescreening approach could significantly increase the chances of finding these rare patients, enabling treating physicians to determine who it would be most appropriate to refer for ALK screening and possible enrollment within an ALK inhibitor trial. However, although the proportions testing positive may increase with this approach, it does not guarantee that ALK-positive patients will not be missed should they exist at lower frequencies outside this group, and until very large data sets are analyzed, we would caution against being too proscriptive in terms of who not to test.

Both age and gender factors have also been reported in association with ALK positivity (9). In series where gender has been documented among adenocarcinomas with *ALK* gene rearrangements using RT-PCR, the ratios have been 6F:5M (24), 7F:4M (25), and, using the break-apart

FISH probe, F8:11M (26). Whereas the first two series were conducted in Japan and China, the third was conducted in the United States. Shaw et al. (26) reported that ALK-positive patients were significantly more likely to be males compared with patients who were either *EGFR* mutant (*P* = 0.036) or *EGFR* wild-type (*P* = 0.039). However, in the larger data set, taking our own series together with the other U.S. series or all four series together, no dominant gender difference is apparent (United States: 17F:15M; United States + Japan + China: 30F:24M). In series where age has been documented, trends toward ALK-positive patients being younger than the general lung cancer population have been reported (9, 26). Here, our median age at diagnosis was similarly low at 53 years, but because the range is so broad (34-75 in our series), we do not believe that age per se should be used as a preselection criterion for ALK screening for fear of inappropriately excluding patients.

Lung cancer is now being divided into several well-defined molecular subtypes. In addition to helping to identify a significant majority of the patients potentially appropriate for treatment with an ALK inhibitor, in the absence of widespread screening of all lung cancer cases for *ALK* gene rearrangements, the categorical factors (adenocarcinoma, *EGFR/KRAS* wild-type, and <10–pack-year smoking history) we have associated with ALK positivity may also prove a useful first step toward reexploring lung cancer epidemiology at the molecular level. Several nonsmoking-related risk factors, including radon gas exposure and various familial syndromes, have been described

for lung cancer in general (27). The specific etiology of ALK-driven lung cancers remains unknown. By looking in different series of patients preselected by the characteristics we describe here to maximally enrich for patients testing positive, any apparent regional and/or national variation in the frequency of ALK positivity may generate hypotheses relating to high-risk genetic and/or environmental factors differing between the series to then be explored in more detail in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Optimizing the Detection of Lung Cancer Patients Harboring Anaplastic Lymphoma Kinase (*ALK*) Gene Rearrangements Potentially Suitable for ALK Inhibitor Treatment

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