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O R I G I N A L   R E P O R T

Detection of Circulating Tumor Cells Harboring a Unique *ALK* Rearrangement in *ALK*-Positive Non–Small-Cell Lung Cancer

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A B S T R A C T

Purpose
The diagnostic test for *ALK* rearrangement in non–small-cell lung cancer (NSCLC) for crizotinib treatment is currently done on tumor biopsies or fine-needle aspirations. We evaluated whether *ALK* rearrangement diagnosis could be performed by using circulating tumor cells (CTCs).

Patients and Methods
The presence of an *ALK* rearrangement was examined in CTCs of 18 *ALK*-positive and 14 *ALK*-negative patients by using a filtration enrichment technique and filter-adapted fluorescent in situ hybridization (FA-FISH), a FISH method optimized for filters. *ALK*-rearrangement patterns were determined in CTCs and compared with those present in tumor biopsies. *ALK*-rearranged CTCs and tumor specimens were characterized for epithelial (cytokeratins, E-cadherin) and mesenchymal (vimentin, N-cadherin) marker expression. *ALK*-rearranged CTCs were monitored in five patients treated with crizotinib.

Results
All *ALK*-positive patients had four or more *ALK*-rearranged CTCs per 1 mL of blood (median, nine CTCs per 1 mL; range, four to 34 CTCs per 1 mL). No or only one *ALK*-rearranged CTC (median, one per 1 mL; range, zero to one per 1 mL) was detected in *ALK*-negative patients. *ALK*-rearranged CTCs harbored a unique (3'5') split pattern, and heterogeneous patterns (3'5', only 3') of splits were present in tumors. *ALK*-rearranged CTCs expressed a mesenchymal phenotype contrasting with heterogeneous epithelial and mesenchymal marker expressions in tumors. Variations in *ALK*-rearranged CTC levels were detected in patients being treated with crizotinib.

Conclusion
ALK rearrangement can be detected in CTCs of patients with *ALK*-positive NSCLC by using a filtration technique and FA-FISH, enabling both diagnostic testing and monitoring of crizotinib treatment. Our results suggest that CTCs harboring a unique *ALK* rearrangement and mesenchymal phenotype may arise from clonal selection of tumor cells that have acquired the potential to drive metastatic progression of *ALK*-positive NSCLC.

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INTRODUCTION

Non–small-cell lung cancer (NSCLC) accounts for approximately 80% of lung cancers and remains a major cause of cancer death in developed countries.[1] A fusion gene between the anaplastic lymphoma kinase (*ALK*) gene and echinoderm microtubule-associated protein-like 4 (*EML4*) was recently identified in 3% to 7% of unselected patients with NSCLC.[2,3] The discovery that the EML4-ALK fusion protein kinase was a potent oncogenic driver in NSCLC[4-6] fueled the rapid development of crizo-

tinib, a drug inhibitor of ALK,[7,8] and its accelerated approval by the US Food and Drug Administration for treating patients with advanced *ALK*-positive NSCLC.[9,10] Crizotinib was approved with a companion diagnostic test, the Vysis *ALK* Break Apart FISH Probe Kit (Abbott Molecular, Des Plaines, IL). Detection of an *ALK* rearrangement is currently performed on small biopsies or fine-needle aspirates but is hindered by the limited tissue quantities available. Tumor tissue is difficult to obtain in patients with advanced/metastatic NSCLC for whom surgery is rarely a component of treatment. Finding

alternative and more effective means of diagnosing an *ALK* rearrangement is a critical issue for identifying patients with NSCLC who may benefit from treatment with crizotinib.

Circulating tumor cells (CTCs) may represent a noninvasive and easily accessible source of tumor material for assessing predictive molecular biomarkers and screening patients eligible for targeted treatments. Recent advances in technology have enabled significant progress in detecting CTCs.[11] By using the CellSearch platform (Veridex, Raritan, NJ), a method based on the detection of epithelial cells that express epithelial cell adhesion molecule (EpCAM), CTC levels were observed to be prognostic in various metastatic solid tumors, including NSCLC and SCLC.[12-16] By using an enrichment technique based on blood filtration (ISET [isolation by size of epithelial tumor cells]), the prognostic value of CTCs was also reported in patients with resected NSCLC.[17] We and others have reported that larger numbers of CTCs are identified by using this technique compared with the CellSearch method in NSCLC,[18-20] most likely because CTCs that express markers of epithelial-mesenchymal transition (EMT) and that have lost epithelial features can be missed by CellSearch. In this regard, CTCs expressing a mesenchymal phenotype were recently reported in patients with NSCLC.[19,21,22] Because the number of cells is critical for exploiting CTCs as predictive biomarkers of personalized treatments, we developed a fluorescent in situ hybridization (FISH) method on filters (filter-adapted FISH [FA-FISH]) that was optimized for high cell recovery. Our objective was to determine whether an *ALK* rearrangement could be detected in CTCs of patients with *ALK*-positive NSCLC by using this method.

PATIENTS AND METHODS

Patients

This study was approved by our institutional review board and local ethics committee. Informed written consent was obtained from all patients enrolled onto two clinical trials (IDRCB A2008-A00585-50 and IDRCB A2008-A00373-52). Peripheral blood samples were collected from all patients. An optional second sample was collected from patients undergoing crizotinib treatment (at day 30, 45, or 90, depending on patient visits).

FISH Assay on Tumor Tissue

The dual-color FISH assay using the Vysis LSI *ALK* Break Apart Rearrangement Probe Kit (Abbott Molecular) is described in the Data Supplement. The Vysis *ALK* Break Apart FISH Probe Kit consists of two probes adjacent to the 3' (red) and 5' (green) ends of *ALK*. In cells with a native *ALK* status, the overlapping of probes results in a fused (3'5', yellow) signal. The two characteristic *ALK*-rearrangement split patterns are the split of the 3' (red) and 5' (green) probes (a distance of more than two signal diameters is considered a split) or an isolated single or amplified 3' (red) signal. Signals were enumerated in at least 100 tumor nuclei, and FISH-positive cases were defined as those with more than 15% split or isolated signals.[9,23]

Immunohistochemistry on Tumor Tissue

The immunohistochemistry and semiquantification of protein expression protocols are described in the Data Supplement.

CTC Detection by CellSearch and Enrichment by ISET

CTC enrichment by ISET (Rarecells, Paris, France) was performed on 10 mL of blood as previously reported.[16,20,24,25] CTCs were enumerated by using CellSearch (Veridex) on 7.5 mL of blood as previously described.[12-14,20] Experimental approaches used to characterize CTCs on filters are detailed in the Data Supplement.

FA-FISH Assay of Enriched CTCs on Filters

Each step of the FA-FISH method was optimized for highest cell recovery as described in the Data Supplement. Hybridization was performed by using the Vysis LSI Dual Color *ALK* Break Apart Rearrangement Probe Kit (Abbott Molecular). Filters were analyzed by trained experimenters (E.P., A.B., M.O.). The *ALK* status was validated by an experienced cytogenetician (N.A.). Immunofluorescent staining of filters was performed according to a method we previously established.[22] Monoclonal antibodies, imaging, cell lines, culture methods, and statistical analysis were described in the Data Supplement.

RESULTS

Detection of ALK *Rearrangement in CTCs*

Thirty-two patients with metastatic NSCLC, including 18 *ALK*-positive and 14 *ALK*-negative patients, were examined herein. All 18 *ALK*-positive patients were tested for an *ALK* rearrangement by using the US Food and Drug Administration–approved Vysis *ALK* Break Apart FISH Probe Kit in tumor biopsies of primary tumors (eight patients) or metastases (10 patients). The *ALK* rearrangement was identified by FISH in 17 patients, four of whom needed a second biopsy because initial FISH results were negative ($<$ 15% rearranged cells) or not interpretable. For one patient (P8) with a negative FISH test, the *EML4-ALK* fusion transcript was detected by reverse transcriptase polymerase chain reaction (RT-PCR) in the tumor specimen. The percentage of *ALK*-rearranged cells in tumor biopsies ranged from 25% to 97% in 17 of the 18 patients (median, 30%; Table 1). Among the 14 *ALK*-negative patients included, six patients had a *KRAS*-mutated adenocarcinoma, and nine patients had a *KRAS*-nonmutated adenocarcinoma or squamous cell carcinoma and a negative FISH test (Data Supplement). In blood samples, median values of zero CTCs per 7.5 mL (range, zero to 16 CTCs per 7.5 mL) were detected by CellSearch in *ALK*-positive and *ALK*-negative patients, respectively (Table 1; Data Supplement). By using blood filtration and FA-FISH, CTCs bearing an *ALK* rearrangement were detected in all 18 *ALK*-positive patients with a mean value of 11 CTCs per 1 mL and a median value of nine CTCs per 1 mL (range, four to 34 CTCs; Table 1; Fig 1; Data Supplement). All *ALK*-positive patients had four or more *ALK*-rearranged CTCs per 1 mL of blood (Table 1; Fig 1). No or only one *ALK*-rearranged CTC was detected in blood samples obtained from the 14 patients with *ALK*-negative NSCLC (mean, 0.64 CTCs per 1 mL; median, one CTC per 1 mL; range, zero to one CTC per 1 mL; Fig 1; Data Supplement). Receiver operating characteristic curve analysis indicated that a cutoff value of four *ALK*-rearranged CTCs per 1 mL blood had a sensitivity and a specificity of 100% (Fig 1). The concordance between CTCs and tumors quantified by the κ coefficient was 99.99%. Total CTCs and CTC subpopulations were identified in independent experiments by combining four-color immunofluorescent staining (cytokeratins/vimentin/CD45/4,6-diamidino-2-phenylindole [DAPI]) and cytomorphologic analysis (Data Supplement).[22] Mean and median percentages of *ALK*-rearranged CTCs were 63% and 57% (range, 28% to 100%), respectively, in *ALK*-positive patients and were 4% and 4% (range, 0% to 100%) in *ALK*-negative patients (Table 1; Data Supplement).

ALK-rearrangement patterns present within tumor specimens and also in CTCs of *ALK*-positive patients are described in Table 2. As is commonly observed, two types of split patterns were detected in tumors. Seven patients had a split of the red and green (3'and 5') signals. Five patients had isolated red signals, consisting of either a unique (3') or several (3' $\geq$ 2) red signals. Five other patients had both a split of the red and green signals and isolated red signals (Fig 2A). Each of these rearrangement patterns was detected in tumor cells

				Tumor		CTCs			
						Characterized by ISET			
Patient	Sex	Age (years)	Smoking Status (No. of pack-years)[a]	Biopsy Origin[b]	% of Rearranged Cells[c]	Rearranged CTCs (/1 mL)	% of Rearranged CTCs[d]	Total CTCs[e] (/mL)	CTC Counts by CellSearch (/7.5 mL)
P1	M	32	3	Node (MS)	97	9	56	16	0
P2	M	35	0	Pleura (MS)	47	9	53	17	0
P3	F	40	0	Pleura (MS)	30	5	50	10	2
P4	M	54	0	Pleura (MS)	30	4	44	9	N/A
P5	F	79	0	Lung (PT)	60	10	40	25	0
P6	F	69	0	Node (MS)	43	34	100	28[f]	0
P7	M	69	20	Lung (PT)	27	7	28	25	16
P8	M	48	3.5	Pleura (MS)	RT-PCR+[g]	24	96	25	12
P9	F	70	40	Lung (PT)	61	6	40	15	0
P10	M	53	0	Lung (PT)	30[h]	7	41	17	6
P11	F	25	0	Lung (PT)	68	7	44	16	0
P12	F	44	12.5	Lung (PT)	29	9	90	10	0
P13	F	36	0	Node (PT)	77	4	57	7	1
P14	M	48	5	Pleura (MS)	62[i]	11	65	17	0
P15	F	42	0	Lung (PT)	25[h]	7	70	10	5
P16	F	52	0	Node (MS)	26[h]	9	64	14	0
P17	F	42	10	Lung (PT)	25	11	100	10[f]	0
P18	F	57	0	Node (MS)	28	25	100	18[f]	0

Table 1. Numbers and Percentages of *ALK*-Rearranged Cells in Tumors and in CTCs of *ALK*-Positive Patients

Abbreviations: *ALK*, anaplastic lymphoma kinase; CTC, circulating tumor cell; F, female; ISET, isolation by size of epithelial tumor cells; M, male; MS, metastatic site; N/A, not available; PT, primary tumor; RT-PCR, reverse transcriptase polymerase chain reaction.
[a]All patients were former smokers or never-smokers (0).
[b]Tumor biopsy was obtained from either PT or MS.
[c]Percentage of rearranged tumor cells determined by FISH in tumor samples.
[d]Proportion of *ALK*-rearranged CTCs determined by FA-FISH (filter-adapted FISH) among total numbers of CTCs determined in independent experiments by combining four-color immunofluorescent staining with cytomorphologic examination.
[e]Total numbers of CTCs per milliliter were calculated as the mean of CTCs identified by combining four-color immunofluorescent staining with cytomorphologic examination in 3×1 mL of blood.
[f]In these three patients, numbers of *ALK*-rearranged CTCs were slightly greater compared with the total number of CTCs identified by phenotypic analysis. This difference is due to the fact that numbers of CTCs may differ between each spot of the filter.
[g]The biopsy was negative by FISH but positive by RT-PCR.
[h]Two tumor biopsies were analyzed: the first biopsy was negative ($< 15\%$ of rearranged cells) but the second biopsy was positive.
[i]FISH failed but ALK protein was detected by immunohistochemistry in the first biopsy. The second biopsy was positive by FISH.

presenting either a single ($3'/5'$ or yellow signal) or several ($3'/5' \geq 2$ or yellow signals) native copies of *ALK*, the latter corresponding to a gain of *ALK* copies. Tumor cells presenting only a gain of *ALK* copies were observed in seven patients. In contrast to that observed within tumors, the *ALK*-rearrangement pattern identified in CTCs consisted exclusively of the split of the red and green ($3'$ and $5'$) signals (Table 2), which was associated with either a single copy or a gain of native *ALK* copies (Fig 2A). An isolated red signal pattern was never detected in CTCs. CTCs harboring this split pattern and a single native copy of *ALK* were observed as isolated cells or within clusters (Table 2; Fig 2A). CTC clusters had variable sizes and carried one or two *ALK*-rearranged CTCs, with other cells harboring two native *ALK* gene copies (normal status; Fig 2A). The nature of cells bearing a normal *ALK* status could not be formally identified by using only FA-FISH, although the large size of cells within these clusters is compatible with CTCs harboring a normal *ALK* status (Fig 2A). Furthermore, clusters carrying both CD45-positive hematopoietic cells and CTC(s) were also detected in experiments combining immunofluorescent staining and cytomorphologic analysis (Data Supplement). A few patients presented an important contingent of isolated CTCs harboring a gain of *ALK* copies ($3'/5' \geq 2$; Table 2; Fig 2B). On the basis of total CTCs numbers, the number of *ALK*-rearranged CTCs, and the number of CTCs with gains of *ALK* copies, we estimated that CTCs harboring a

normal *ALK* status represented a non-negligible subset of CTCs ranging from 0% to 42% of total CTCs in half the patients examined (Data Supplement).

The presence of CTCs bearing this unique *ALK* rearrangement—the split of the red and green ($3'$ and $5'$) signal—was particularly intriguing among patients who did not have this pattern within tumor specimens and only had red ($3'$ or $3' \geq 2$) signals. For this reason, tumor specimens from four of these five patients were re-examined to determine whether a minor subset of tumor cells harboring a split of the red and green ($3'$ and $5'$) rearrangement had been missed. In one patient (P2), a second area of rearranged cells bearing a red and green split signal or bearing isolated red signals was identified. This second area had initially not been sought since the threshold of 15% of *ALK*-rearranged cells had been obtained on initial examination. In patient P16, two rearranged cells with a split of the red and green signals were detected with a frequency less than 1/1,000 tumor cells after reanalysis. For the last two patients (P1, P9), no cells bearing the red and green split rearrangement were detected on second analysis. Overall, this unique *ALK*-rearrangement pattern was consistently detected in all patient CTCs, regardless of the frequency of cells harboring this rearrangement within the tumor specimen. These findings suggested that CTCs bearing this unique *ALK* rearrangement
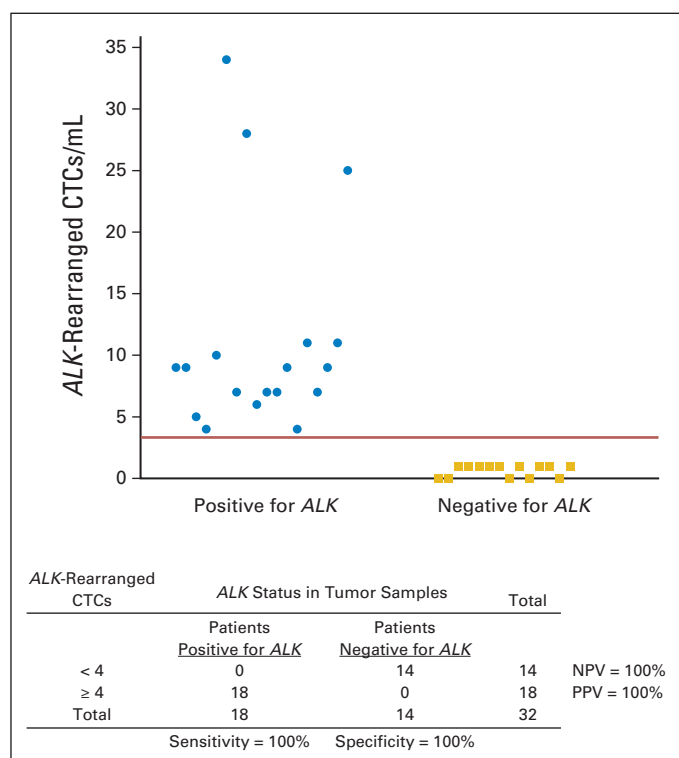
Fig 1. Determination of the anaplastic lymphoma kinase (*ALK*) –rearranged circulating tumor cells (CTCs) optimal cutoff value in *ALK*-positive and *ALK*-negative patients. Top, prevalence of *ALK*-rearranged CTCs in *ALK*-positive and *ALK*-negative patients. Bottom, determination of the cutoff value for four *ALK*-rearranged CTCs per 1 mL blood by using receiver operating characteristic curve analysis. NPV, negative predictive value; PPV, positive predictive value.

may have acquired invasive and migratory properties that are lacking in tumor cells with other *ALK*-rearrangement patterns.

Characterization of EMT Markers in ALK-*Rearranged CTCs*

This hypothesis prompted us to analyze the expression of EMT markers in *ALK*-rearranged CTCs. We established a two-step method that combined four-color immunofluorescent staining and FA-FISH. CTCs were stained on filters according to two distinct combinations of markers (vimentin/cytokeratins/CD45/DAPI or N-cadherin/E-cadherin/CD45/DAPI). Next, DAPI$^+$CD45$^-$ cells were selected, analyzed, and precisely located on the filters. After performing FA-FISH, the FISH signals of DAPI$^+$CD45$^-$ cells were analyzed, and both the immunofluorescent and FISH signals were precisely relocated. Cells from six patients (P7, P9, P13, P14, P16, P18) were assayed by combining immunofluorescent staining and FA-FISH. Positive controls resulted from spiking normal peripheral blood with the A549 lung cancer cell line (positive for both epithelial and mesenchymal markers; Fig 3A). Similar results were obtained in all six patients. As shown in Figures 3B and 3C, *ALK*-rearranged CTCs were positive for mesenchymal markers, vimentin and N-cadherin, with a moderate expression level that was significant but generally lower than that of hematopoietic cells. Cytokeratin markers were not detected in *ALK*-rearranged CTCs. Expressions of cytokeratins or of both cytokeratins and vimentin were detected in CTCs bearing a native *ALK* status in the one *ALK*-negative and six *ALK*-positive patients (Data Supplement). Although a strong E-cadherin expression was detected in the A549 cell

line (Fig 3A), this marker was undetectable in CTCs, including those positive for cytokeratins (data not shown).

Characterization of ALK-*Positive Tumors for Epithelial and Mesenchymal Marker Expression*

The observation that *ALK*-rearranged CTCs expressed a mesenchymal phenotype led us to analyze tumor samples for epithelial and mesenchymal marker expression (Appendix Table A1, online only). All tumors (except those from P4 with a sarcomatoid carcinoma) had a prominently epithelial phenotype with moderate to strong expression of cytokeratins and E-cadherin. However, the expressions of epithelial and, more importantly, mesenchymal markers were not homogeneous between tumors. Tumors (from P7, P13, P14, P15, P18) with moderate expression of cytokeratins and E-cadherin harbored significant vimentin and N-cadherin expression. This finding could reflect different levels of EMT among tumors, as previously described in lung adenocarcinoma.[27] Thus, although *ALK*-rearranged CTCs appeared to have a homogeneous mesenchymal phenotype, tumor tissues presented greater heterogeneity for epithelial and mesenchymal marker expression.

In addition to the intertumor heterogeneity of epithelial and mesenchymal marker expression, we observed significant intratumor heterogeneity within biopsies. Examples are shown in Figure 4 for two representative patients bearing a prominent mesenchymal phenotype (P4) or a prominent epithelial phenotype (P17). Interestingly, a high expression of ALK protein correlated with areas that were negative for cytokeratins (P17) or in areas with lower cytokeratin and higher vimentin expression (P4). These data show that *ALK*-rearranged CTCs consistently harbored a mesenchymal phenotype, regardless of the level of mesenchymal marker expression within individual tumor specimens.

Monitoring of ALK-*Rearranged CTCs During Treatment With Crizotinib*

ALK-rearranged CTCs were monitored in five patients at baseline and during crizotinib treatment. Three spots (3 × 1 mL) were analyzed per blood sample for quantitative analysis of *ALK*-rearranged CTCs. The levels of CTC subsets bearing different *ALK* pattern abnormalities are presented in the Data Supplement. These data mainly highlighted the heterogeneity of responses to crizotinib in CTC subsets. The levels of CTCs harboring an *ALK* rearrangement and a single *ALK* native copy (3′/5′, 3′ and 5′) decreased to different degrees in four patients (P6, P8, P14, P18). Despite the small size of our cohort, these data show that monitoring quantitative and qualitative changes of CTCs bearing *ALK* abnormalities is possible in patients undergoing crizotinib therapy.

DISCUSSION

By combining blood filtration and FA-FISH (a FISH assay optimized for CTC characterization), levels of four or more *ALK*-rearranged CTCs per 1 mL of blood were detected in all 18 *ALK*-positive patients tested, but no or only one *ALK*-rearranged CTC was detected in 14 *ALK*-negative patients. The cutoff value of four or more *ALK*-rearranged CTCs per 1 mL provided a sensitivity and a specificity of 100% for predicting the *ALK*-rearrangement status present within the tumors in our cohort. A threshold of 15% of *ALK*-rearranged cells,

Table 2. Description of *ALK* Rearrangement Patterns in Tumors and in CTCs

	Tumor								CTCs						
		Rearranged Cells*						Cells With Gain of ALK Copies (3'/5' > 2)	Total Rearranged CTCs (/1 mL)	Rearranged CTCs (/1 mL)				Only Gain of ALK Copies (/1 mL)	
										3'/5', 3' and 5'		3'/5' ≥ 2, 3' and 5'		3'/5' > 2	
Patients	% of Rearranged Cells	3'/5', 3' and 5'	3'/5' ≥ 2, 3' and 5'	3'/5', and 3'	3'/5', and 3' ≥ 2	3'/5' ≥ 2, and 3'	3'/5' ≥ 2, and 3' ≥ 2			Isolated	In Clusters	Isolated	In Clusters	Isolated	In Clusters
P1	97	0	0	13	50	0	34	0	9	7	0	2	0	8	0
P2	47	0	0	47	0	0	0	0	9	5	3	1	0	1	0
P3	30	0	2	0	0	10	18	0	5	5	0	0	0	2	0
P4	30	8	22	0	0	0	0	54	4	3	0	1	0	3	0
P5	60	30	30	0	0	0	0	0	10	5	0	5	0	11	0
P6	43	43	0	0	0	0	0	32	34	22	10	2	0	1	0
P7	27	7	0	0	0	20	0	28	7	6	0	1	0	25	0
P8	RT-PCR+	—	—	—	—	—	—	—	24	21	3	0	0	4	0
P9	61	0	0	48	8	5	0	0	6	4	1	1	0	5	0
P10	30	31	0	0	0	0	0	0	7	7	0	0	0	3	0
P11	68	0	0	41	0	19	8	0	7	5	1	1	0	4	0
P12	29	12	0	0	0	17	0	0	9	6	3	0	0	5	0
P13	77	77	0	0	0	0	0	0	4	3	0	1	0	0	0
P14	62	9	0	13	2	28	10	15	11	11	0	0	0	1	0
P15	25	25	0	0	0	0	0	13	7	5	2	0	0	0	0
P16	26	0	0	26	0	0	0	0	9	5	3	1	0	4	0
P17	25	25	0	20	0	0	0	1	11	7	4	4	0	1	0
P18	28	8	0	20	0	0	0	2	25	17	4	4	0	1	0

Abbreviations: *ALK*, anaplastic lymphoma kinase; CTC, circulating tumor cell; RT-PCR, reverse transcriptase polymerase chain reaction.
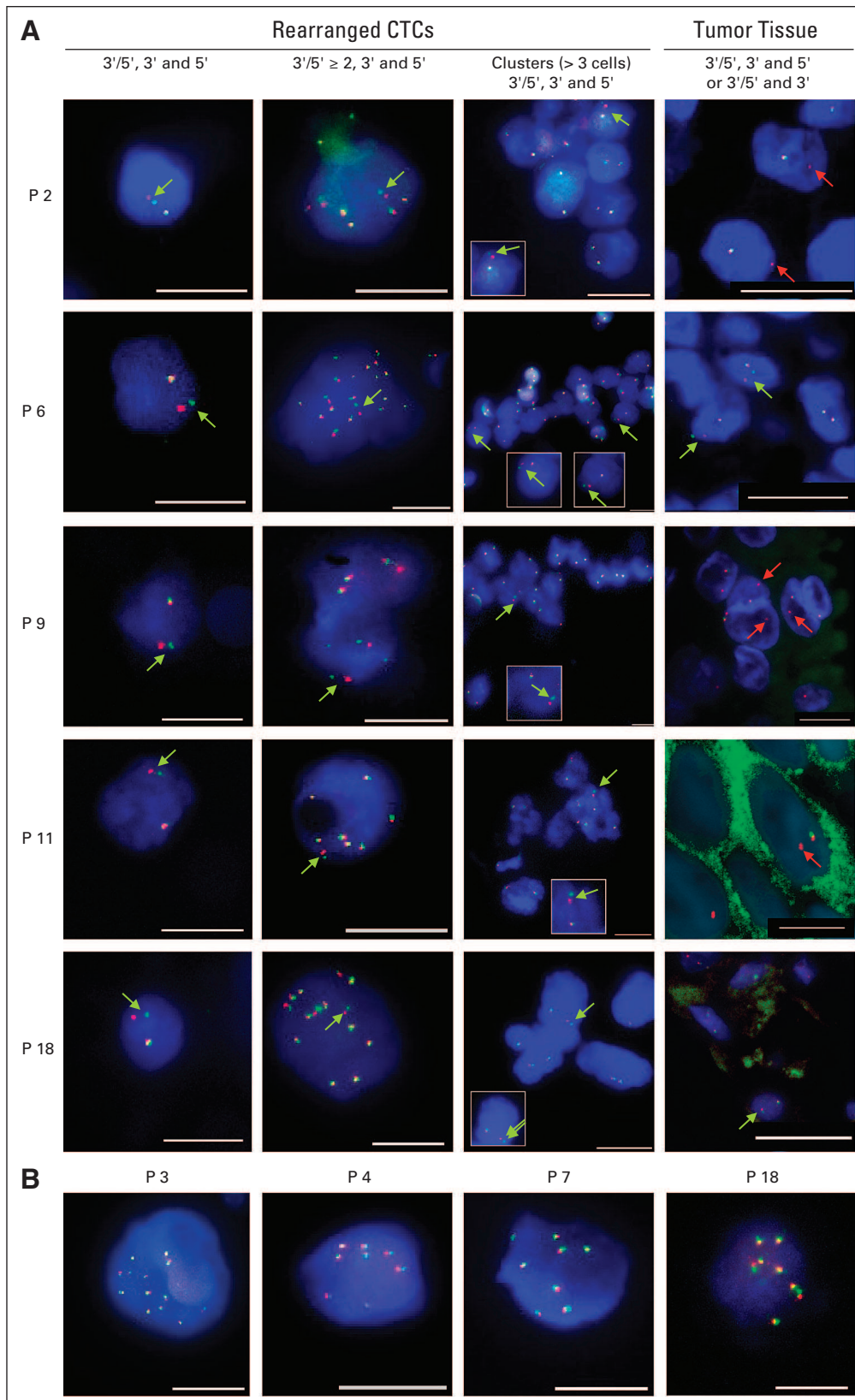*Number of rearranged cells for 100 counted tumor cells.

Fig 2. Detection of anaplastic lymphoma kinase (*ALK*) gene abnormalities in circulating tumor cells (CTCs) and tumor specimens of *ALK*-positive patients. (A) Examples of isolated or clusters of *ALK*-rearranged CTCs detected by filter-adapted fluorescent in situ hybridization (FISH) and of *ALK*-rearranged tumor cells in tumor specimens detected by FISH. Green arrows show an *ALK* rearrangement with a split 3′ and 5′ (red/green) signal. Red arrows show an *ALK* rearrangement with only the 3′ signal. (B) Examples of isolated CTCs with a gain of native *ALK* copies. Scale: white bars correspond to 10 μm.

which represents two standard deviations above the mean cell count in negative tumor samples,[23] is currently used to diagnose *ALK* rearrangement by FISH on paraffin-embedded tumor samples. Because of small CTC numbers in blood, we propose to use the number of *ALK*-rearranged CTCs per volume of blood rather than the percentage of *ALK*-rearranged CTCs as a cutoff value for establishing the diagnosis of *ALK* rearrangement. Although further studies are needed to confirm our data in larger cohorts of *ALK*-positive and
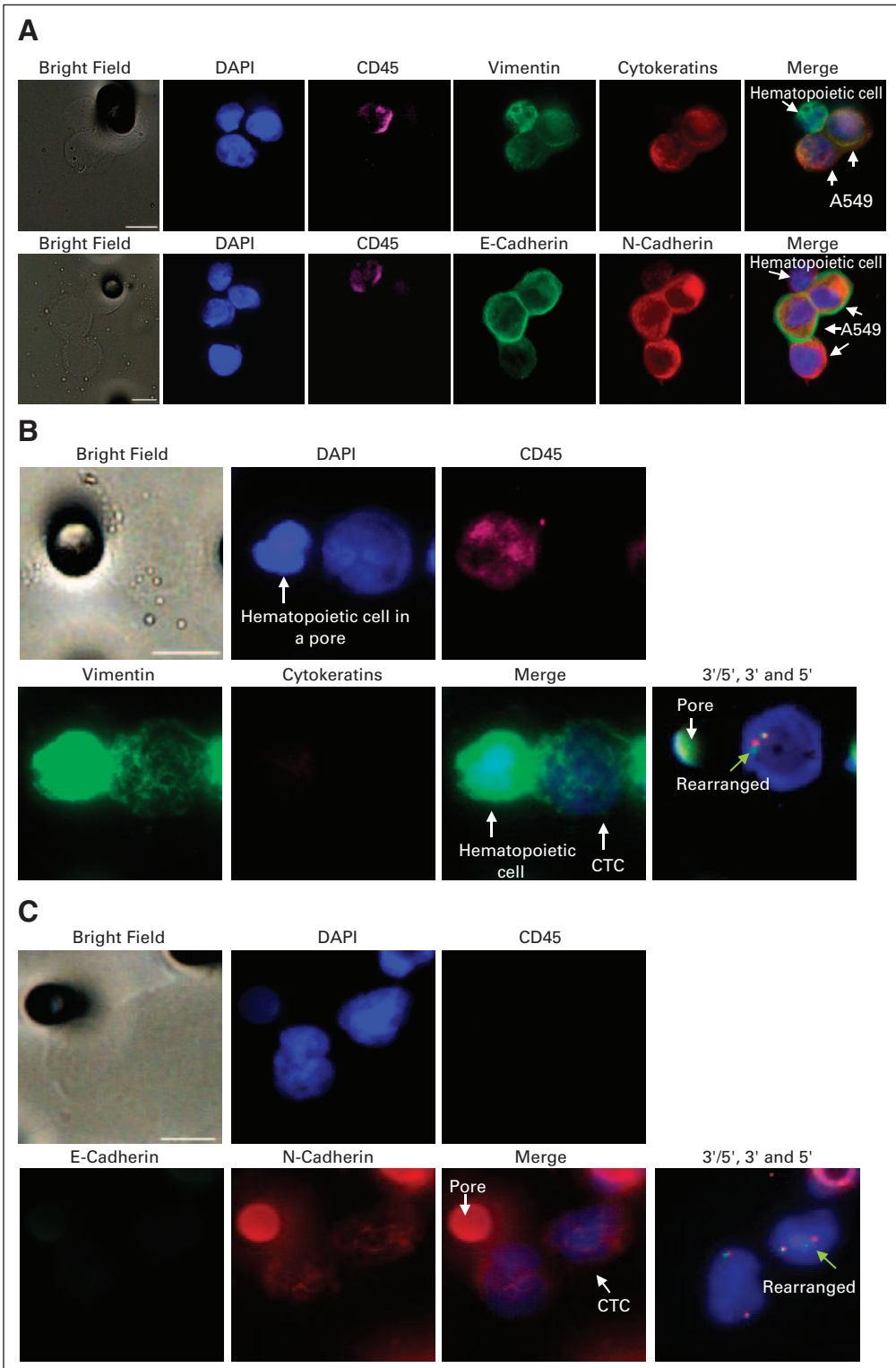
Fig 3. Epithelial mesenchymal transition marker detection in anaplastic lymphoma kinase (*ALK*) –rearranged circulating tumor cells (CTCs). (A) Positive control examples of four-color immunofluorescent staining (vimentin/cytokeratins/CD45/4,6-diamidino-2-phenylindole [DAPI] and N-cadherin/E-cadherin/CD45/DAPI) experiments. Positive controls resulted from having the A549 lung cancer cell line spiked into peripheral blood of a healthy patient. (B) Representative example of vimentin/cytokeratins/CD45/DAPI immunofluorescent staining of *ALK*-rearranged CTCs in an *ALK*-positive patient. (C) Representative example of N-cadherin/E-cadherin/CD45/DAPI immunofluorescent staining of *ALK*-rearranged CTCs in an *ALK*-positive patient. Pores are indicated. Green arrows show *ALK* rearrangement with a split 3′ and 5′ (red/green) signal. Scale: white bars correspond to 10 μm.

ALK-negative patients and to establish definite cutoff values of *ALK*-rearranged CTCs, our study provides the first proof-of-concept that CTCs can be used for highly sensitive and highly specific diagnostic testing of *ALK* rearrangement in patients with NSCLC.

During EMT, epithelial carcinoma cells acquire a migratory phenotype and express mesenchymal genes, a prerequisite to tumor infiltration and metastasis.[28-30] The remarkably homogeneous mesenchymal phenotype observed in *ALK*-rearranged CTCs contrasted with the heterogeneity observed between and within tumor specimens in terms of epithelial and mesenchymal marker expression. We also observed that heterogeneity in EMT marker expression correlated with ALK protein levels within the tumor specimens of two patients.
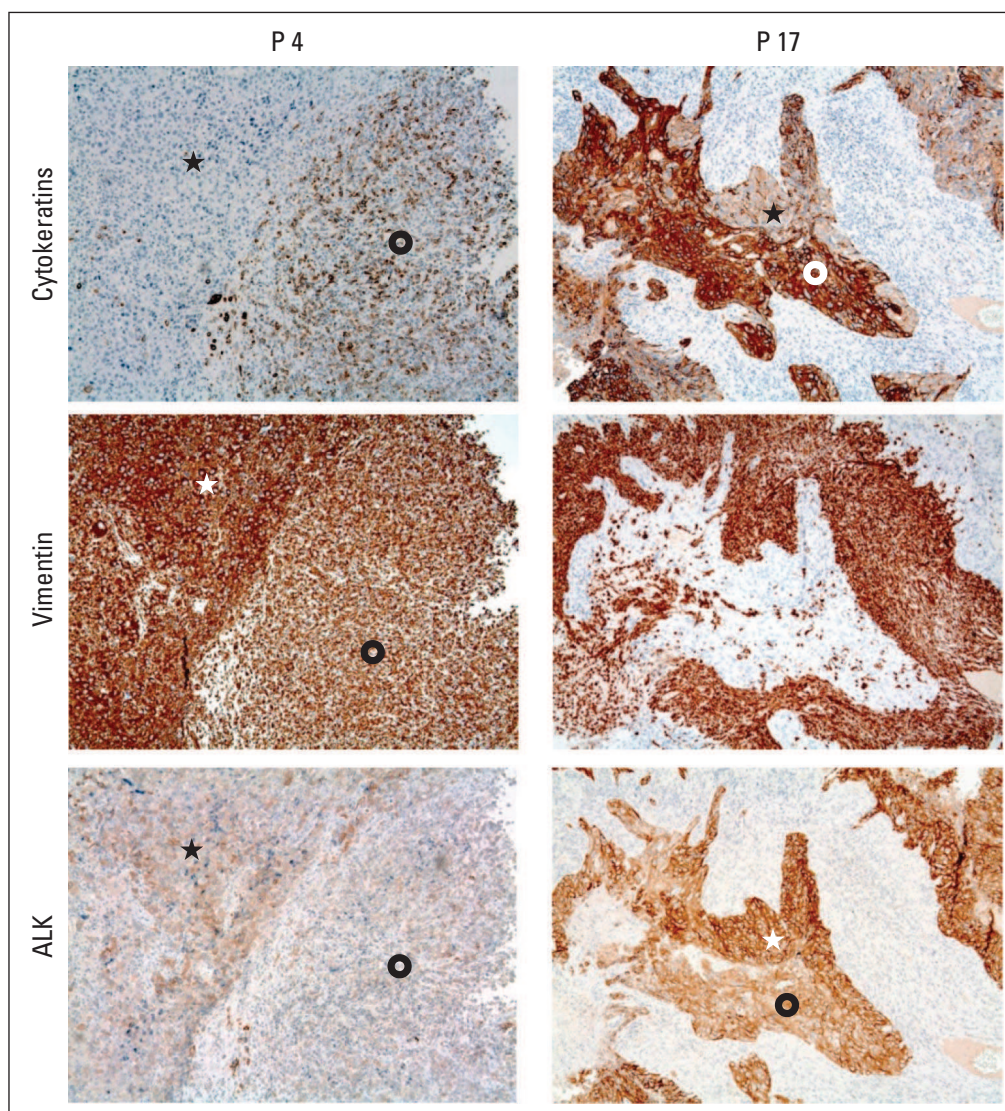
Fig 4. Intratumoral heterogeneity of cytokeratin and vimentin expression for two representative anaplastic lymphoma kinase (*ALK*) –positive patients bearing either a prominent mesenchymal phenotype (P4) or a prominent epithelial phenotype (P17) and for whom ALK expression protein could be assessed by immunohistochemistry. P4 (sarcomatoid carcinoma): area (★) shows no expression of cytokeratin, strong expression of vimentin, and strong expression of ALK; area (O) shows moderate expression of cytokeratins, moderate expression of vimentin, and low expression of ALK. P17 (solid adenocarcinoma): area (★) shows low expression of cytokeratins, no vimentin, and stronger expression of ALK intermixed with area (O), which shows strong expression of cytokeratins, no vimentin, and low expression of ALK.

Our results support a role for ALK expression in EMT induction in NSCLC and suggest that *ALK*-rearranged CTCs could originate from particular foci of EMT related to ALK protein expression. Previously, NPM-ALK fusion protein was reported to promote the expression of TWIST1 and tumor cell invasiveness through activation of the NPM-ALK Stat3 signaling pathway.[31] In lung adenocarcinoma, inhibition of E-cadherin through the expression of TWIST1 is known to be key in regulating EMT and promoting invasive properties in tumor cells.[28,29,32] Taken together, our results support the hypothesis that downstream signaling pathways triggered by ALK tyrosine kinase activation could have a role in promoting invasiveness through EMT regulation in *ALK*-positive NSCLC tumor cells, thereby generating CTCs expressing a mesenchymal phenotype and migratory properties.

The most striking finding was the unique split pattern of *ALK* rearrangement consistently identified in CTCs despite the intertumoral heterogeneity of *ALK* rearrangements and the frequency of tumor cells harboring this rearrangement within tumors. Moreover, this unique *ALK*-rearrangement split pattern was detected in CTCs of patients for whom it was not identified within the tumor. Although a single tumor biopsy sample might not be representative of the entire tumor, these results suggested that CTCs might originate from various metastatic sites. By reflecting the metastatic disease process, CTCs may be more informative of biomarker status than a single biopsy taken at a given time. This hypothesis could have important implications for developing personalized strategies. For patients with *ALK*-positive NSCLC, our results suggested that CTCs that harbor this unique *ALK* rearrangement and express a mesenchymal phenotype may result from the clonal selection of tumor cells that display migratory properties and higher invasive potential and may possibly contain highly metastatic cells, such as cancer stem cells or tumor-initiating cells. Finally, noninvasive molecular analyses performed on CTCs could be easily repeated at different time points during treatment to guide therapeutic decision making in a patient's treatment course. Our results suggest that serial evaluation of CTC subsets could be used to monitor response to crizotinib. Furthermore, CTC assessment could enable detection of resistance markers in *ALK*-positive NSCLC.

By demonstrating that *ALK* rearrangement can be reliably detected in CTCs of all patients with *ALK*-positive NSCLC, our study provides new and important perspectives for diagnosing *ALK*-positive

patients eligible for treatment with crizotinib or other ALK inhibitors and for monitoring the impact of these treatments. Our findings may offer new insights to the overall role of *ALK*-rearranged CTCs in metastatic progression resulting from the clonal selection of tumor cells undergoing EMT.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Appendix

Patients	Tumor Origin	Adenocarcinoma Subtype*	Cytokeratins	E-Cadherin	Vimentin	N-Cadherin
P4	Pleura (MS)	Sarcomatoid	− to ++	−	+ to +++	+ (FS)
P7	Lung (PT)	Solid	++	N/A	±	−
P9	Lung (PT)	Acinar and papillary	+++	++	−	−
P13	Node (MS)	Mucinous	++	++	± (FS)	±
P14	Pleura (MS)	Solid	++	++	+++	±
P15	Lung (PT)	Solid	++	+	++	±
P16	Node (MS)	Mucinous	+++	++	− to +	−
P17	Lung (PT)	Solid	± to ++	++ to +++	−	−
P18	Node (MS)	Mucinous	++	++	± (FS)	+ (FS)

Table A1. Epithelial and Mesenchymal Marker Expression in Tumor Tissue by Immunohistochemistry

Abbreviations: (−) negative; (±) weak staining in < 50% of tumor cells; (+) weak staining in > 50% of tumor cells; (++) moderate staining; (+++) strong staining; FS, focal staining; MS, metastatic site; N/A, not available; PT, primary tumor.
*All tumor specimens were biopsies, and invasive adenocarcinoma subtype was determined according to International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification (Travis WD, et al: Proc Am Thorac Soc 8:381-385, 2011).