Clinical Studies

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Circulating Tumor Cells with Aberrant *ALK* Copy Number Predict Progression-Free Survival during Crizotinib Treatment in *ALK*-Rearranged Non–Small Cell Lung Cancer Patients

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Abstract

The duration and magnitude of clinical response are unpredictable in *ALK*-rearranged non–small cell lung cancer (NSCLC) patients treated with crizotinib, although all patients invariably develop resistance. Here, we evaluated whether circulating tumor cells (CTC) with aberrant *ALK*-FISH patterns [*ALK*-rearrangement, *ALK*-copy number gain (*ALK*-CNG)] monitored on crizotinib could predict progression-free survival (PFS) in a cohort of *ALK*-rearranged patients. Thirty-nine *ALK*-rearranged NSCLC patients treated with crizotinib as first ALK inhibitor were recruited prospectively. Blood samples were collected at baseline and at an early time-point (2 months) on crizotinib. Aberrant *ALK*-FISH patterns were examined in CTCs using immunofluorescence staining combined with filter-adapted FISH after filtration enrichment. CTCs were classified into distinct subsets according to the presence of *ALK*-rearrangement and/or *ALK*-CNG signals. No significant association between baseline numbers of *ALK*-rearranged or *ALK*-CNG CTCs and PFS was observed. However, we observed a significant association between the decrease in CTC number with *ALK*-CNG on crizotinib and a longer PFS (likelihood ratio test, $P = 0.025$). In multivariate analysis, the dynamic change of CTC with *ALK*-CNG was the strongest factor associated with PFS (HR, 4.485; 95% confidence interval, 1.543–13.030, $P = 0.006$). Although not dominant, *ALK*-CNG has been reported to be one of the mechanisms of acquired resistance to crizotinib in tumor biopsies. Our results suggest that the dynamic change in the numbers of CTCs with *ALK*-CNG may be a predictive biomarker for crizotinib efficacy in *ALK*-rearranged NSCLC patients. Serial molecular analysis of CTC shows promise for real-time patient monitoring and clinical outcome prediction in this population. *Cancer Res; 77(9); 2222–30. ©2017 AACR.*

Introduction

A new paradigm has recently emerged for the treatment of non–small cell lung cancer (NSCLC) with the discovery of molecularly defined subsets of patients who can be treated effectively by therapies targeted to a specific oncogenic driver alteration (1). The discovery of *anaplastic lymphoma kinase* (*ALK*)-rearrangements in NSCLC triggered their validation as a therapeutic target (2, 3). In 2011, crizotinib received FDA approval as the first ALK inhibitor for advanced *ALK*-rearranged NSCLC patients after demonstrating impressive clinical results in the 3% to 5% of *ALK*-rearranged NSCLC patients (3, 4). The latest update of the phase I clinical trial demonstrated high overall response rate (ORR, 60.8%) and a median progression-free survival (PFS) of 9.7 months (5). In two randomized phase III trials, crizotinib prolonged the PFS and increased the ORR in treatment-naïve *ALK*-rearranged patients as compared with standard first- or second-line chemotherapy (6, 7). However, treatment with crizotinib is marked by heterogeneity in the magnitude and duration of patient response. Response durations vary from a few months to several years, and the long-term effectiveness of crizotinib is invariably limited by the development of acquired resistance (8–16). Mechanisms of acquired resistance have been identified in approximately 30% to 40% of crizotinib-resistant patients and have been distinguished into two main categories: those affecting the *ALK*-gene itself including additional genetic alterations through secondary mutations, gene amplification or gain of copy number, or those independent of the *ALK*-gene *via* other therapy-induced adaptive responses (15, 17). New therapeutic agents have been developed to counteract resistance, such as

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second-/third-generation ALK inhibitors—ceritinib, alectinib, brigatinib, or lorlatinib—as well as combined treatment strategies with heat-shock protein 90 inhibitors (13, 18–23). Currently, there are no known predictive biomarkers allowing to identify those patients who will benefit from a long-term response to crizotinib from those whom are at risk of acquiring resistance shortly after starting crizotinib and would require alternate therapy promptly.

In current practice, predictive biomarkers for drug sensitivity and therapy selection are assessed either within the primitive tumor tissue taken at diagnosis or within tumor biopsies collected from metastasis during the course of the disease. Primitive tumor tissue can be difficult to obtain in patients with certain tumor types such as NSCLC where surgery is rarely a component of treatment. In NSCLC, genotyping and eligibility for targeted therapy are currently assessed using tumor biopsies, which can prove challenging to perform and obtain, are invasive, and may yield limited tissue quantities. Because tumors continually evolve and adapt to targeted therapies, it is becoming increasingly important in clinical practice to monitor the genetic changes occurring in tumors "in real-time" especially when selecting second- or third-line therapies. Subjecting patients to serial and repeated tumor biopsies for monitoring tumor evolution during treatment course and at the time of resistance is, in most cases, clinically not feasible. Therefore, finding alternative means to monitor tumor evolution and emergence of acquired resistance is a critical issue for improving the success rate of targeted therapies. Circulating tumor cells (CTC) can be obtained through a simple, noninvasive, and easily repeated blood draw (24–26). In contrast to tumor biopsies, CTCs have the potential to allow longitudinal monitoring of tumor features at different time-points during treatment, thereby guiding therapeutic decisions in a patient's clinical course. Another interesting characteristic is that CTCs are likely to be released from different metastatic sites and may have the potential to inform on the genetic heterogeneity of metastatic disease. Therefore, as such, CTCs may represent the metastatic disease as well as, if not better than a single-site tumor biopsy (25).

Using the CellSearch, a method based on the detection of EpCAM-positive epithelial cells, CTC levels were shown to have prognostic significance in several metastatic cancers, including NSCLC (25, 27). Longitudinal monitoring of changes in total CTC counts has also suggested a potential role for CTC in predicting whether a patient may benefit or not from anticancer treatment (27, 28). Few studies have, until now, demonstrated the feasibility of CTC assays for the detection of predictive biomarkers. Three groups, including our own, have reported *ALK*-rearrangement detection in CTCs using a size-based technique for CTC enrichment and FISH. The choice of this approach was justified by the higher number of CTCs recovered by this technique as compared with the CellSearch in NSCLC (29–31). Using ISET (isolation by size of epithelial tumor cells) and filter-adapted FISH (FA-FISH), we determined a cut-off value of four or more *ALK*-rearranged CTCs per 1 mL blood to predict whether *ALK*-rearrangement was present or not within the tumor tissue (29). In this study, several CTC subsets harboring distinct *ALK*-FISH patterns—including *ALK*-rearranged CTCs and CTCs with a gain of *ALK*-copy number (*ALK*-CNG)—were identified and were correlated with variable evolution on crizotinib treatment in the five examined patients (29). Based on this observation, we hypothesized that CTC subsets differing in *ALK*-FISH patterns might be associated with different clinical outcomes in *ALK*-rearranged patients treated by crizotinib. In the present study, we evaluated whether these CTC subsets monitored on crizotinib in an extended cohort of 39 *ALK*-rearranged patients could inform on treatment benefit. The association between *ALK*-rearranged and *ALK*-CNG CTC subsets and clinical parameters, including PFS and overall survival (OS), is presented herein.

Materials and Methods

Study design

The study (IDRCB2008-A00585-50) was conducted at Gustave Roussy (Villejuif, France), authorized by the French national regulation agency ANSM (Agence Nationale de Sécurité du Medicament et des produits de santé), and approved by the Ethics Committee and our Institutional Review Board. Stage IV *ALK*-rearranged NSCLC patients were recruited into the study between March 2011 and November 2014 and included 18 patients from our first study (29). Informed written consent was obtained from all patients. All patients received 250 mg crizotinib twice daily at initiation of therapy. Peripheral blood samples were collected at baseline and at an early time-point on crizotinib (usually 2 months depending on patient visits). All patients had a CT scan and/or MRI every 6 or 8 weeks. Consequently, CT scans and/or MRIs were performed at baseline and before the second time-point (median, 7 days before the second time-point; range, 1 to 49 days). *ALK*-rearrangement was tested in tumor by FISH, IHC, or reverse transcription (RT)-PCR (32).

Medical records were reviewed and clinical characteristics were collected retrospectively—including age, sex, smoking status, tumor histologic type, number of previous treatment lines before crizotinib, Eastern Cooperative Oncology Group (ECOG) performance status, number of metastatic sites, level of lymphocytes, PFS, and OS.

PFS was defined as the time between the date of the start of crizotinib and the date of clinical or radiological progression according to RECIST. OS was defined as the time between the date of the start of crizotinib and the date of death. Patients who were progression-free and alive at the time of analysis were censored.

CTC detection by CellSearch and enrichment by ISET

CTCs were enumerated using the CellSearch (Janssen Diagnostics, LLC) as previously reported (27, 33). CTC enrichment by ISET (Rarecells) was performed on 10 mL of blood as previously reported (29, 33).

Combination of immunofluorescent staining and *ALK* FA-FISH in ISET-enriched CTCs

Immunofluorescent staining (DAPI/CD45) and FA-FISH were performed on three ISET spots as previously reported (29, 34, 35). Scanning and analysis of immunofluorescent staining and FISH signals were performed on an ARIOL (Leica Biosystems Richmond Inc.) according to a previously reported sequential process briefly described in the Data Supplement (35). FA-FISH data came from the analysis of three independent ISET spots by combining immunofluorescent staining and *ALK* FA-FISH. Data presented result from the addition of the values of the three ISET spots. When a divergence $\geq$30% on one spot was observed compared with the two others, additional

spots were tested and data from spots with an inconsistent result were not considered.

Combination of immunofluorescent staining and FA-FISH using *ALK* break-apart probe combined with the chromosome 2–specific centromeric probe of ISET-enriched CTCs

Immunofluorescent staining of filters, FA-FISH, and scanning were performed as described below. The detection of the aneusomy of chromosome 2 was performed using the Vysis *ALK* Break Apart rearrangement Probe Kit combined with XCyting Centromere Enumeration Probe XCE 2 in blue (MetaSystems GmbH; 1:1). The FA-FISH method was established using *ALK*-rearranged NCI-H2228 cell line spiked into peripheral blood samples of healthy donors (Supplementary Fig. S1). The amplification was defined either by a cluster of isolated red signals (10) or ≥6 copies of *ALK* and a ratio of *ALK* to centromere copies superior to 2.5, whereas a ratio inferior to this with a copy number superior to 2 defines a CNG (36).

Scanning and image analysis of combined immunofluorescent staining and *ALK* FA-FISH in ISET-enriched CTCs

Scanning was performed on an ARIOL (Leica Biosystems Richmond Inc.) according to a sequential process, which includes: (i) scanning of filters after fluorescent staining, (ii) automated selection of $DAPI^+/CD45^-$ cells, (iii) FISH scanning in $DAPI^+/CD45^-$ cells, and (iv) analysis of FISH signals in $DAPI^+/CD45^-$ cells. Specific FISH scanning settings have been established to detect a maximum number of FISH spots in filter-enriched CTCs, including 30 z-stacks, a distance of 0.6 μm between two z-stacks, and a multi-exposure protocol consisting of three separate exposure times for the green and red fluorochromes (35). Filters were analyzed by experienced operators (E. Pailler and M. Oulhen). The *ALK*-status was validated by a cytogeneticist (N. Auger). The dual-color FISH assay using the Vysis *ALK* Break Apart rearrangement Probe Kit (Abbott Molecular Inc.) was performed as we previously reported (29). The *ALK* Break Apart Kit consists of two probes adjacent to the 3' (red) and 5' (green) ends of *ALK*. In cells with a native status of *ALK*, the overlapping of the 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 greater than two signal diameters is considered as a split), or an isolated 3′ (red) signal.

Cell lines

The NCI-H2228 cell line was obtained from American Type Culture Collection (ATCC CRL-5935, batch 3527839, obtained in March 2008). NCI-H2228 cells have been authenticated by us and repeatedly controlled for the presence of *ALK*-rearrangement by FISH testing. Recent FISH experiment for controlling the *ALK*-rearranged status of NCI-H2228 cells was performed on March 2016. NCI-H2228 cells were cultured in RPMI medium 1640 (Thermo Fisher Scientific Inc.) supplemented with 10% FBS (Thermo Fisher Scientific Inc.) and maintained in a humidified incubator in 5% CO_2 at 37°C. NCI-H2228 cells were spiked at various dilutions into blood from healthy donors filtered on ISET, as done for patient samples.

Statistical analysis

Statistical analyses were performed using R software version 3.3.0 for Mac (packages MASS v-7.3.45 and survival v-2.39.3 were used; ref. 37) and SAS/STAT software version 9.4 of the SAS system for Windows (SAS Institute Inc.). *ALK*-rearranged and *ALK*-CNG CTC counts were considered as distinct scores, and descriptive statistics were calculated separately. Wilcoxon tests were used to compare CTC counts (i.e., *ALK*-rearranged, *ALK*-CNG) between groups, for each covariate. Linear regressions were performed to assess the correlation between CTC number at baseline and on crizotinib, for each gene alteration independently. Cox proportional hazard models were used to estimate HRs and 95% confidence interval (CI) for PFS and OS. Each continuous covariate was scored as 0 and 1, < median and ≥ median, respectively, except for age at baseline where a cut-off was applied (0: <55 y/o; 1: ≥55 y/o). A stepwise backward/forward procedure was applied to the full multivariate model in order to objectively select the optimal predictors (38). For each statistical analysis, including Cox models, a P value ≤0.05 was considered significant.

Results

Detection of CTCs with aberrant *ALK*-FISH patterns at baseline and on crizotinib

Thirty-nine metastatic *ALK*-rearranged NSCLC patients treated by crizotinib as first ALK inhibitor were examined. Patient characteristics are presented in Table 1. Median age was 53 years (range, 25–81). A majority of patients were never or light smokers (67%) with adenocarcinoma tumor histology (97%). All patients had received one or more prior treatments. The mean duration of

Table 1. Demographics and clinical parameters

Clinical parameters	Patients ($N = 39$) N (%)
Age at baseline (y/o)	
Median	53
Range	25–81
<55	20 (51)
≥55	19 (49)
Sex	
Female	18 (46)
Male	21 (54)
Smoking status (PY)	
<15	26 (67)
≥15	13 (33)
Tumor histologic type	
Adenocarcinoma	38 (97)
Mixed adenocarcinoma and SCC	1 (3)
Number of previous treatment lines	
1	19 (49)
≥2	20 (51)
ECOG PS at baseline	
≤1	33 (85)
>1	6 (15)
Number of metastatic sites	
1	16 (41)
≥2	23 (59)
Brain metastases at baseline	
Absent	28 (72)
Present	11 (28)
Liver metastases at baseline	
Absent	26 (67)
Present	13 (33)
Level of lymphocytes at baseline[a]	
$<1.10^6$/L	10 (33)
$\geq 1.10^6$/L	20 (67)

Abbreviations: PS, performance status; PY, pack-year; SCC, squamous cell carcinoma; y/o, years old.
[a]$N = 30$.

crizotinib therapy was 15.4 months (median, 10.2 months; range, 0.8–48.1)—7 of 39 patients being censored as they were continuing treatment at the time of analysis. One patient (P6) received discontinuous crizotinib treatment because of hepatic toxicity. All 39 patients had a blood sample for *ALK*-rearrangement detection in CTCs at baseline. Among them, 29 patients had a blood sample drawn at an early time-point (usually 2 months, depending on patient visit) of crizotinib therapy. Ten patients were not monitored at this early time-point due to medical follow-up in another hospital. *ALK*-rearrangement was tested by FISH in tumor biopsies for all 39 *ALK*-rearranged patients. When useful to assess or confirm positivity, FISH was combined to IHC or RT-PCR (Supplementary Table S1). *ALK*-rearrangement was evaluated in ISET filtration-enriched CTCs from 3 mL blood using a recently developed two-step method that combined two-color (DAPI/CD45) immunofluorescent staining, FA-FISH, and semi-automated microscopy analysis (35).

Two main subsets of CTCs with aberrant *ALK*-FISH patterns were detected: *ALK*-rearranged CTCs and *ALK*-CNG CTCs (Supplementary Table S1, Fig. 1A). The *ALK*-rearranged CTC subset included CTCs with variable numbers of splits of the red and green signal (3′ and 5′) and of *ALK*-native copies (3′/5′≥2). In contrast to our previous study performed on a limited number of 18 *ALK*-rearranged patients (29), *ALK*-rearranged CTCs with an isolated red signal (3′) were rarely observed in 10 patients of this extended cohort (Fig. 1A). The number of *ALK*-native copies (3′/5′≥2) was highly variable in CTCs harboring only a CNG (Fig. 1A). Using a specific third-color FISH probe for the chromosome 2 centromere (Fig. 1B), the same number of *ALK*-copies and centromeres were observed, confirming that these CTCs harbored a gain of chromosome 2 (i.e., a polysomy of chromosome 2) and not an amplification of the *ALK*-gene.

At baseline, the median numbers of *ALK*-rearranged CTCs and *ALK*-CNG CTCs were 14 per 3 mL (range, 1–119) and 12 per 3 mL (range, 1–53), respectively (Supplementary Tables S1 and S2). On crizotinib, median numbers of *ALK*-rearranged CTCs and *ALK*-CNG CTCs were 13 per 3 mL (range, 3–60) and 15 per 3 mL (range, 2–177), respectively (Supplementary Tables S1 and S2). Using CellSearch, median values of zero CTCs per 7.5 mL were detected both at baseline and on crizotinib (range, 0–713; range, 0–544), respectively (Supplementary Tables S1 and S2). Linear regressions showed a significant correlation between the number of *ALK*-rearranged CTCs at baseline and on crizotinib ($R^2 = 0.278$; $P = 0.003$), whereas no correlation was observed in *ALK*-CNG CTC numbers ($R^2 = 0.017$; $P = 0.503$; Fig. 1C). These results suggested that the number of *ALK*-CNG CTCs on crizotinib was independent from CTCs numbers at baseline and therefore possibly affected by treatment.

Association between CTC subsets and clinical parameters or outcome

Associations between *ALK*-rearranged or *ALK*-CNG CTC counts at baseline and clinical parameters are presented in Supplementary Table S3. A significant association was observed between the numbers of *ALK*-CNG CTCs and the smoking status (P=0.002). All other clinical parameters were not significantly associated with *ALK*-rearranged or *ALK*-CNG CTC counts at baseline. After a median follow-up of 16.1 months (mean, 19.4 months), 33 of the 39 (84.6%) evaluable patients had disease progression by RECIST criteria. Median PFS was 6.9 months (mean, 11.2 months; 95% CI, 2.4–9.7 months). Twenty-five (64.1%) patients died, resulting in a median OS of 16.7 months (mean, 19.9 months; 95% CI, 11.4–32.9 months).

For univariate analysis, counts of *ALK*-rearranged and *ALK*-CNG CTCs at baseline and on crizotinib were assigned into low/high groups according to their respective medians (Table 2, Supplementary Table S2). We did not observe a significant association between baseline *ALK*-rearranged CTCs or *ALK*-CNG CTCs and PFS (Table 2) or OS (data not shown). No clinical parameter was significantly associated with PFS (Table 2) or OS (data not shown).

Due to the noncorrelated counts of *ALK*-CNG CTCs at baseline and on crizotinib, we considered the dynamic change of these CTC counts under treatment as a possible parameter to evaluate. Patients were assigned into two groups: 16 patients presented stable or increased numbers of *ALK*-CNG CTCs (mean increase, 27; range, 0–169), whereas 13 patients presented a decrease of the same cells (mean decrease, −8; range, −18 to −2; Fig. 2A). PFS according to the dynamic change of *ALK*-CNG CTC numbers is presented in Fig. 2B. We observed a statistically significant association between the dynamic change of *ALK*-CNG CTC numbers on crizotinib and PFS on univariate analysis (likelihood ratio test, P=0.025; Table 2). Median PFS was 14.0 months (95% CI, 2.3 months to NA) for patients presenting a decrease in *ALK*-CNG CTC numbers, and was 6.1 months (95% CI, 2.5–9.8 months) for patients with a stable or increased numbers of the same cells (Fig. 3). The dynamic change of *ALK*-CNG CTC numbers was not correlated with OS (Table 2, Fig. 3), which was most likely affected by subsequent treatment lines in this population after crizotinib resistance (Supplementary Table S4). No significant association between the dynamic change of *ALK*-rearranged CTCs on crizotinib and PFS (Table 2) or OS was observed on univariate analysis (data not shown).

Multivariate Cox proportional hazards regression analysis, using all the clinical covariates and CTC numbers, was significant for PFS (Table 2) but not for OS (data not shown; likelihood ratio test P values: 0.038 and 0.491, respectively). Smoking status, number of treatment lines, number of metastatic sites, and dynamic change of *ALK*-CNG CTC counts had significantly non-zero coefficients ($P \leq 0.05$). This result was reinforced by a stepwise backward/forward optimization applied on the full model, which selected the same four covariates and improved the model (likelihood ratio test, $P = 0.005$; Table 2; ref. 38). In this model, the dynamic change of *ALK*-CNG CTCs levels under crizotinib remained the most significant factor associated with the duration of PFS (HR, 4.485; 95% CI, 1.543–13.030, P = 0.006; Table 2). The Cox model for OS remained non-significant after the same optimization procedure (likelihood ratio test P value: 0.209).

Discussion

Herein, we examined aberrant *ALK*-FISH patterns in CTCs collected at initiation of crizotinib therapy from 39 *ALK*-rearranged patients. CTCs were classified into two subsets, including *ALK*-rearranged and *ALK*-CNG CTCs. Baseline CTC numbers in these two subsets were not predictive of crizotinib benefit. However, in 29 of these 39 patients monitored at an early time-point of crizotinib therapy, we observed a significant association between the dynamic evolution of the numbers of *ALK*-CNG CTCs on crizotinib and median PFS of patients. These results demonstrate for the first time that molecular analyses of CTCs can identify a

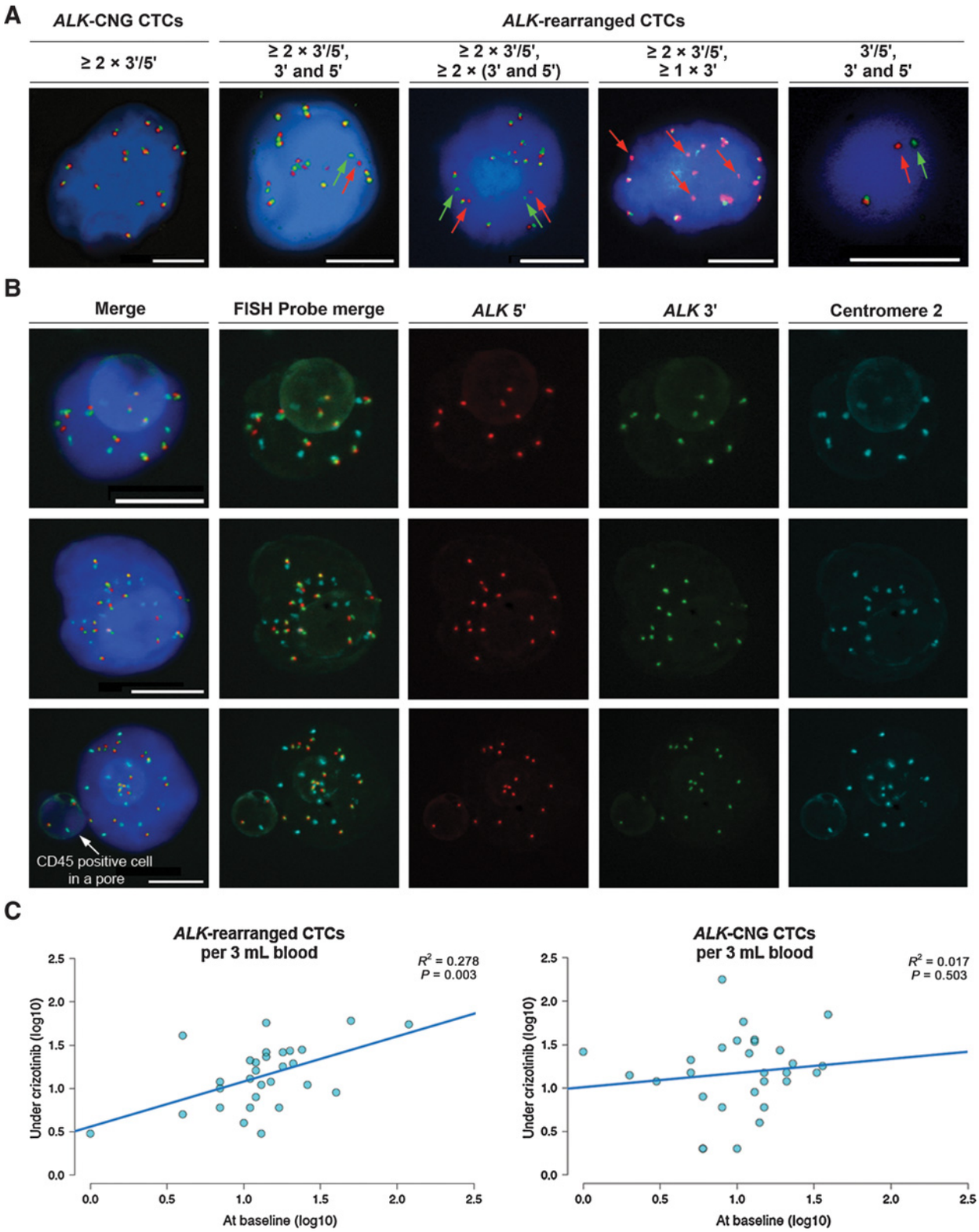

Figure 1.

Detection of CTCs harboring *ALK* gene aberrations at baseline and on crizotinib therapy in *ALK*-rearranged NSCLC patients. **A,** Examples of *ALK*-rearranged CTCs and CTCs with *ALK*-CNG detected by combined fluorescent staining and FA-FISH. Red and green arrows show the *ALK* 5′ and the *ALK* 3′ probes, respectively. Scale bars, 10 μm. **B,** Examples of *ALK*-rearranged and *ALK*-CNG CTCs detected by combined fluorescent staining and three-color FA-FISH for *ALK* gene and chromosome 2 centromeric detection. Scale bars, 10 μm. **C,** Linear regressions for *ALK*-rearranged and *ALK*-CNG CTCs at baseline and on crizotinib therapy.

Table 2. Univariate and multivariate cox regression analysis for prediction of PFS

	Univariate analysis			Multivariate analysis	
Variables	**HR (95% CI)**	***P* value**	**LR test *P* value**	**HR (95% CI)**	***P* value**
Age at baseline (y/o), ≥55 vs. <55	0.602 (0.292–1.244)	0.171	0.165	0.120 (0.013–1.110)	0.062
Sex, M vs. F	1.375 (0.686–2.755)	0.369	0.368	0.467 (0.129–1.685)	0.245
Smoking status (PY), ≥15 vs. <15	1.430 (0.685–2.987)	0.341	0.351	57.705 (3.823–870.900)	0.003
Number of previous treatment, ≥2 vs. 1	0.924 (0.464–1.840)	0.822	0.822	0.250 (0.082–0.759)	0.014
ECOG PS at baseline, >1 vs. ≤1	1.926 (0.784–4.732)	0.153	0.181	2.741 (0.419–17.920)	0.293
Number of metastatic sites, ≥2 vs. 1	1.535 (0.760–3.102)	0.233	0.227	7.003 (1.709–28.700)	0.007
Metastases at baseline, liver | brain | both vs. none	1.124 (0.811–1.558)	0.481	0.487	1.153 (0.648–2.053)	0.628
ALK-rearranged CTCs at baseline, ≥14 vs. <14	0.831 (0.417–1.658)	0.600	0.600	3.285 (0.611–17.650)	0.166
ALK-rearranged CTCs on crizotinib, ≥13 vs. <13	1.676 (0.729–3.853)	0.224	0.221	0.762 (0.142–4.086)	0.751
ALK- CNG at baseline, ≥12 vs. <12	1.017 (0.511–2.024)	0.961	0.961	0.325 (0.087–1.216)	0.095
ALK- CNG on crizotinib, ≥15 vs. <15	1.817 (0.810–4.077)	0.147	0.147	0.100 (0.013–0.767)	0.027
Dynamic change of *ALK*-rearranged CTCs, stable/increase vs. decrease	2.059 (0.838–5.058)	0.115	0.102	0.603 (0.112–3.241)	0.556
Dynamic change of *ALK*-CNG CTCs, stable/increase vs. decrease	2.768 (1.097–6.988)	0.031	0.025	7.331 (1.044–51.499)	0.045
				Optimized Model	
Smoking status (PY), ≥15 vs. <15				3.034 (1.210–7.607)	0.018
Number of previous treatment, ≥2 vs. 1				0.461 (0.176–1.205)	0.114
Number of metastatic sites, ≥2 vs. 1				3.413 (1.257–9.265)	0.016
Dynamic change of *ALK*- CNG CTCs, stable/increase vs. decrease				4.485 (1.543–13.030)	0.006

Abbreviations: LR, likelihood ratio; PS, performance status; PY, pack-year; y/o, years old.

predictive biomarker of early progression on crizotinib treatment in *ALK*-rearranged NSCLC patients. Further studies are needed to confirm our results and evaluate whether these findings may be extended to other ALK inhibitors. Although our data should be interpreted in the context of a small cohort, our study offers a promising perspective on the potential use of CTCs, which are accessible by a simple blood test, to monitor tumor genetic characteristics and to serve as a predictive biomarker of treatment efficacy among this lung cancer population.

In our first study, ISET-filtration, FA-FISH, and manual microscope analyses were used to detect *ALK*-rearrangement in CTCs captured in 1 mL blood (one ISET spot) from 18 *ALK*-positive and 14 *ALK*-negative patients (29). All *ALK*-positive patients had four or more *ALK*-rearranged CTCs per 1 mL blood, whereas no or only one *ALK*-rearranged CTC was detected in *ALK*-negative patients (29). One ISET spot contains on average 5,000 white blood cells (mainly granulocytes), around 70 CD45-negative cells, and a small number of CTCs. Consequently, the manual analysis of one ISET spot to identify *ALK*-rearranged CTCs is laborious, time-consuming and may be operator-dependent. In order to analyze larger blood volumes (i.e., more ISET spots per blood sample), limit intrasample variations (each spot representing an independent FA-FISH test), and reduce interoperator differences, we made technical changes and automated the microscope and imaging analyses. Also, in order to reduce the number of cells to be analyzed and render the analysis accessible by a scanner, we combined immunofluorescent staining and FA-FISH, and developed a specific automated FISH scanning protocol so as to limit FISH scanning to CTC candidates ($DAPI^+/CD45^-$ cells; ref. 35). The latter procedure was optimized for filters using 30 z-stacks spaced 0.6 μm apart as well as a multiexposure protocol for optimal capture and definition of FISH signals (35). With this new semiautomated FISH analysis method, we were able to analyze larger blood volumes (three independent ISET spots), limit intrasample variations, and subsequently improve the precision of the assay. Using this improved technical approach, approximately 80% of the patients included had at least four *ALK*-rearranged CTCs per 1 mL blood. This discrepancy from prior observations in our first study could be explained by a difference between spots of the same sample or a possible underestimation of some FISH signals (due to insufficient number of z-stacks in the case of very large CTCs) during the semiautomated analysis. Moreover, it must also be acknowledged that, in some cases, an automated protocol cannot completely match the manual and case-per-case microscope adjustment performed by experienced operators or cytogeneticists. However, in spite of this consideration, the method used here increased the feasibility, precision, and reliability of *ALK*-FISH testing in filtration-enriched CTCs and

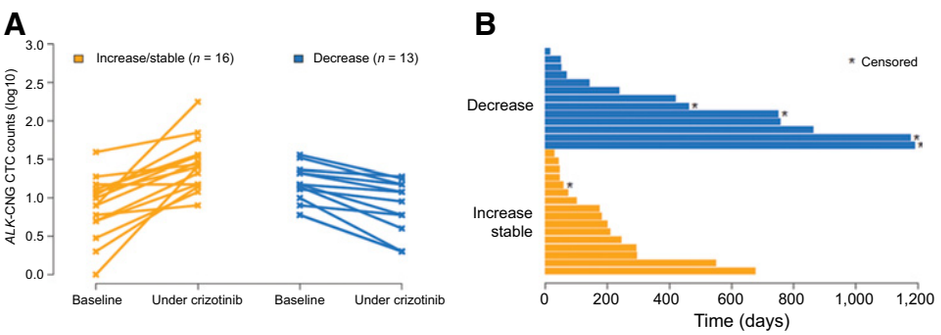

Figure 2.
Correlation between the dynamic change of the numbers of CTCs with an *ALK*-CNG on crizotinib and survival in *ALK*-rearranged patients. **A,** Dynamic change of the numbers of *ALK*-CNG CTCs on crizotinib. **B,** PFS according to the dynamic change of the number of *ALK*-CNG CTCs.

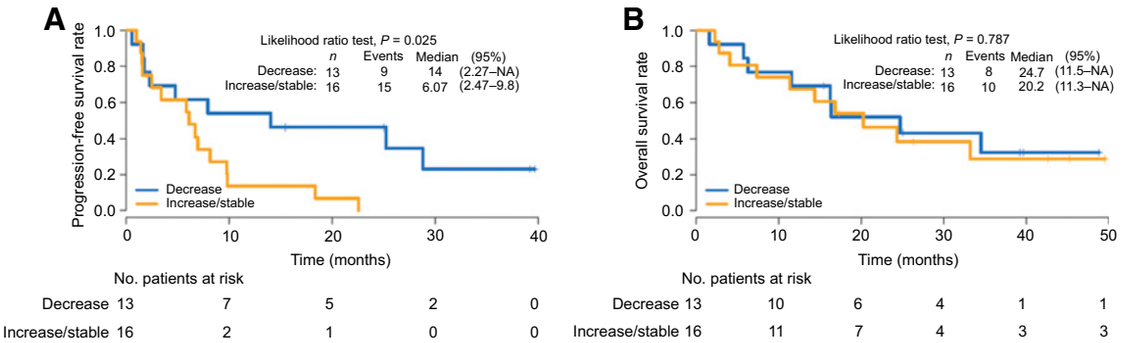

Figure 3.
Kaplan–Meir curves for PFS (**A**) and OS (**B**) of patients with a stable/increase or decrease of *ALK*-CNG CTCs on crizotinib treatment.

may contribute to progress in the validation and qualification of CTC assays in *ALK*-rearranged NSCLC. Finally, it is worth noting that other noninvasive approaches, such as circulating free DNA assays, are currently available for detecting gene rearrangements and gain of copy numbers, although the sensitivity and precision of these strategies need to be further evaluated and compared with those of CTC assays.

In *ALK*-rearranged NSCLC treated by crizotinib as first-line treatment, the median PFS is 9.7 months, but the duration and magnitude of clinical benefit vary widely between patients ranging from a few months to several years (5). The biological mechanisms underlying the heterogeneity in crizotinib responses are unknown (17). Extensive analyses of diagnostic tumor biopsies according to *ALK*-FISH patterns performed prior to crizotinib therapy have failed to identify informative parameters associated with treatment benefit (39, 40). Our results are in agreement with these data in demonstrating that baseline numbers of *ALK*-rearranged and *ALK*-CNG CTCs were not predictive of crizotinib benefit. One of the earliest mechanistic hypotheses for the heterogeneous responses to crizotinib was the differential sensitivity of the different *EML4-ALK* variants (41–43). Recently, in a retrospective study of 35 patients, patients positive for *ALK*-variant 1 in tumor specimens were reported to have a significantly longer PFS to crizotinib than patients with nonvariant 1, a result that differed from *in vitro* studies showing that *ALK*-variant 2 was the most sensitive to ALK-inhibitors, variant 1 having an intermediate sensitivity (42, 44–46). Here, we report that the dynamic change on treatment of a CTC subset with an aberrant *ALK*-CNG can identify patients who will experience a durable response to crizotinib. In tumor biopsies from *ALK*-rearranged patients, *ALK*-CNG is frequent but in most cases limited to three or four *ALK*-copies. The presence of *ALK*-CNG tumor cells was also associated with aggressive clinical outcome in several cancers such as neuroblastoma, breast, and colorectal cancer, with most *ALK*-CNG co-occurring with other gene aberrations such as *MYCN*, *MET*, or *EGFR* (36, 47–50). Hyperploid tumor cells harboring a high number of *ALK*-copies are rarely found in tumor biopsies from *ALK*-rearranged patients but are a large contingent of CTCs. Herein, we have clarified the FISH status of these CTCs by three-color FISH experiments using a chromosome 2 centromere probe and have shown unambiguously that CTCs harbor a gain of the native *ALK*-gene and not an amplification. The rarity of these hyperploid cells in tumor biopsies from *ALK*-rearranged patients might explain why these have been unexplored.

The results of our study could appear unintuitive to the extent that one might expect an association between therapeutic outcome and *ALK*-rearranged CTCs rather than *ALK*-CNG CTCs. Cells bearing *ALK*-CNG have been identified in two cases of 11 resistance biopsy specimens, whereas another study reported high-level amplification of *ALK*-fusion in one case of 15 patients (9, 10). Consistent with earlier studies, it was recently reported that only a minority of *ALK*-rearranged patients (~20%) developed *ALK* resistance mutations on crizotinib therapy, implying that other mechanisms, such as gain or amplification of *ALK*-gene, upregulation of bypass signaling pathways (e.g., EGFR, KIT, MEK/ERK), or histologic transition (e.g., epithelial–mesenchymal transition, EMT), may be dominant resistance mechanisms (15, 17). Such pathways or biological processes may be activated in hyperploid tumor cells with aberrant *ALK*-CNG, which could be more prone to genomic instability and therefore to acquiring tumor-promoting alterations. Therefore, our results suggest that the increased migration under crizotinib therapy of CTCs with *ALK*-CNG—possibly harboring resistance alterations in other pathways than ALK and increased metastatic potential (due to this secondary activation)—is associated with poor benefit and a risk of progression shortly after starting treatment. Resistance to ALK-inhibitor is heterogeneous, and single-site biopsies may be unable to represent the full scope of resistance alterations. Issued from different metastatic sites, CTCs are enriched in cancer cells with specific biological characteristics, such as EMT properties or tumor-initiating cell capacity, and may be a valuable tool to evaluate tumor heterogeneity and monitor resistance aberrations in real-time.

We report for the first time that serial molecular analysis of CTCs could identify a predictive biomarker of therapeutic efficacy in *ALK*-rearranged NSCLC and could help to stratify patients at risk of early resistance. Although further confirmatory studies are needed, our results offer a new and important perspective on the potential use of CTCs in replacing biopsies for patient monitoring in real time and clinical outcome prediction in this population.

Disclosure of Potential Conflicts of Interest

D. Planchard is a consultant/advisory board member for Astrazeneca, BMS, MSD, Novartis, Pfizer, and Roche. J.-C. Soria is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Circulating Tumor Cells with Aberrant *ALK* Copy Number Predict Progression-Free Survival during Crizotinib Treatment in *ALK* -Rearranged Non–Small Cell Lung Cancer Patients

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