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ALK-gene rearrangement: a comparative analysis on circulating tumour cells and tumour tissue from patients with lung adenocarcinoma

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Background: A subgroup of anaplastic lymphoma kinase (*ALK*)-rearranged lung tumours can respond to ALK inhibitors. Until now, the ALK status in circulating tumour cells (CTCs) isolated from patients with lung cancer has not been characterised. We assessed the ALK status in CTCs detected in patients with lung cancer and correlated the results to the ALK status defined in the corresponding tumour tissue.

Patients and methods: A total of 87 patients with lung adenocarcinoma showing CTCs isolated using the isolation by size of epithelial tumour cell method were screened for their ALK status both in tumour samples and in CTCs. *ALK* break-apart fluorescence *in situ* hybridisation (FISH) and immunoreactivity analyses using an anti-ALK antibody (5A4 clone) were carried out on CTCs and compared with the results obtained in the corresponding tissue specimens.

Results: A total of five patients showed *ALK*-gene rearrangement and strong ALK protein expression in CTCs and in the corresponding tumour samples. Both ALK-FISH and ALK immunoreactivity analyses show negative results in CTCs and corresponding tumour samples for 82 patients.

Conclusions: We demonstrated that the ALK status can be determined in CTCs isolated from patients with lung cancer by immunocytochemistry and FISH analyses. These results favour non-invasive, *ALK*-gene status pre-screening on a routine basis on CTCs isolated from patients with lung cancer and open new avenues for real-time monitoring for adapted targeted therapy.

Key words: *ALK*-gene rearrangement, circulating tumour cells, lung carcinoma, fluorescence *in situ* hybridisation, immunocytochemistry, crizotinib

Introduction

Lung cancer is the most prevalent neoplasm and the major cause of tumour-related mortality worldwide [1–5]. Despite the recent advances in the management of resected lung cancers and the more effective treatment of metastatic tumours, the cure rate of patients with lung cancer remains low. However, the recent discovery of driver oncogenic mutations in lung carcinomas and the increasing development of targeted therapies show new encouraging results in advanced stage patients [6–8]. Among these therapies, gefibinib and erlotinib, tyrosine-kinase inhibitors raised against the epidermal growth factor receptor (EGFR), which exhibit an activating tyrosine mutation in 10%–20% of adenocarcinomas, are used [7, 9]. More Recently, genomic alteration involving the anaplastic lymphoma kinase (*ALK*) (2p23) and the echinoderm microtubule-associated protein like-4 (*EML4*) (2p21) genes was identified in a subset of patients with lung cancer having an outstanding favourable response to an ALK small-molecule inhibitor (crizotinib) [7, 10–13]. The *ALK*-gene rearrangement was found in 1%–7% of non-small-cell lung cancers (NSCLCs) according to most of the series without *KRAS*- and *EGFR*-associated mutations in most of the tumours [10, 12–14]. Specific histological features characterise this subset of ALK-positive lung adenocarcinomas, presenting with a solid or acinar growth pattern, a cribriform structure, the presence of mucous cells (signet-ring cells or goblet cells), abundant extracellular mucus, a lack of lepidic growth and a lack of significant nuclear pleomorphism [14]. Moreover,

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patients with tumours with *ALK* rearrangement were younger, were more frequently males, in most of the series, and were never smokers/former light smokers [12, 14].

Circulating tumour cells (CTCs) can be isolated in more than 40% of patients with lung cancer according to the series and methods [15–17]. Moreover, the prognosis of patients with lung cancer, both in late and early stages of the disease, correlates to the presence and the number of CTCs [15, 16]. CTCs can be isolated by different direct and indirect methods [18, 19]. Genomic alterations, particularly mutations occurring in the *EGFR* gene, have been demonstrated in CTCs isolated in NSCLC patients [20].

We recently demonstrated that CTCs can be isolated by different methods even in early-stage disease in patients undergoing surgery for lung carcinomas [15, 21]. Moreover, the presence and the number of CTCs were associated with worse prognosis [15]. Interestingly, using a direct method that isolated the CTCs according to their size (isolation by size of epithelial tumour cells, ISET), we defined cytopathological criteria of malignancy, which allowed good characterisation of CTCs with malignant features [22, 23]. In addition, by applying an immunocytochemistry (ICC) approach to CTCs isolated by ISET from NSCLC patients, our group and another group showed that a variable number of CTCs display a epithelio-mesenchymal transition (EMT) phenotype [17, 21, 24, 25].

The assessment of *ALK*-gene rearrangement in CTCs isolated from patients with lung cancer has not been reported earlier. However, this may be a relevant clinical goal for non-invasive pre-screening of patients with lung cancer in avoiding potential morbidity related to the lung biopsy and the removal of tumour tissue. The aim of this work was (i) to assess the ALK status in CTCs having cytopathological criteria of malignancy isolated by ISET in 87 patients with lung adenocarcinomas and (ii) to compare the ALK status found in CTCs and in the corresponding tumour tissue. For this purpose, we used an assay based on a dual immunochemical and fluorescence *in situ* hybridisation (FISH) approach for *ALK*-gene rearrangement and applied it to both the CTCs and the corresponding tumour tissue samples.

Table 1. Main clinicopathological data of the 87 cases included in this study

Clinical and pathological parameters	Number of patients (%)
Overall	65 (100)
Age (years)	
Mean	66
Range	37–85
Gender	
Male	41 (63)
Female	24 (37)
Tobacco exposure (PY)	
Number	53 (81)
Average	38.2
Range	0–152
Tumour size (cm)	
Mean	3.9
Range	0.4–18
Histology	
Invasive adenocarcinoma (ADC)	65 (100)
Acinar predominant ADC	33 (51)
Papillary predominant ADC	21 (32)
Micropapillary predominant ADC	4 (6)
Lepidic predominant ADC	4 (6)
Solid predominant ADC with mucin production	3 (5)
Number of CTCs	
>50 CNHC-MF (range 51–500)	28 (43)
<50 CNHC-MF (range 14–49)	37 (57)
pTNM staging	
I	30 (46)
IA	12
IB	18
II	16 (25)
IIA	9
IIB	7
III	14 (21)
IIIA	12
IIIB	2
IV	5 (8)
TTF1 antigen expression	44 (67)
Neoadjuvant therapy	14 (21)

TNM, tumour node metastasis; PY, packs year; CNHC-MF, circulating non-haematological cells with malignant features.

methods

patients and samples

In a previous study using the ISET method for patients undergoing surgery for NSCLC, we detected CTCs having cytomorphological malignant features in 76 (37%) of 208 cases [15]. For the present work, we selected from this latter population 40 cases with a primary adenocarcinoma. In addition, 47 patients with lung adenocarcinoma included in the study between May 2011 and December 2011 had CTCs with malignant features. Among these 87 patients, 34 had blood samples (10 ml) collected and treated by ISET at different times: before surgery and at 7 and 15 days after surgery. All patients gave their informed consent to participate in this study. The main clinicopathological features of the selected 87 patients are summarised in Table 1. Tumours were classified according to the seventh pTNM classification and to the last histological classification of lung adenocarcinomas [26, 27]. FISH analysis was performed on the tumour samples using a break-apart probe for the *ALK* gene (Vysis LSI *ALK* Dual Color, Abbott Molecular, Abbott Park, IL; see Supplementary data, available at *Annals of Oncology* online). To be adequately interpreted, tumour cell nuclei should have at least one co-localisation signal. To be considered as *ALK* rearranged, at least 15% of interpretable tumour cell nuclei should harbour an abnormal probe hybridisation pattern [28]. Immunohistochemistry (IHC) was performed on deparaffinised sections using a primary antibody against the ALK protein (1:50, 5A4; Abcam, Cambridge, UK) incubated for 45 min at room temperature (Supplementary data, available at *Annals of Oncology* online). Targeted mutation analysis for (i) *EGFR* mutation hot spots, (ii) *KRAS* mutation hot spots and (iii) *BRAF* mutations was carried out from DNA extracted from frozen tumour tissue sections by pyrosequencing, as previously described [29, 30] (Supplementary data, available at *Annals of Oncology* online).

ICC and FISH on ISET filters

ICC and FISH were performed on CTCs isolated using the ISET method on unstained spots of the corresponding filters containing CTCs with malignant features detected by MGG staining on six spots [15]. Two spots were used for ICC and two spots were used for FISH per filter. For ICC, the spots were incubated with a primary antibody against the ALK protein (1:50, 5A4; Abcam, Cambridge, UK) for 30 min at room temperature. The reactions were visualised with 3,3′-diaminobenzidine, followed by counterstaining with haematoxylin. Cytoplasmic staining was considered positive for ALK [30] (Supplementary Data). FISH carried out on two or more spots used a break-apart probe for the *ALK* gene (Vysis LSI *ALK* Dual Color, Abbott Molecular) in accordance with the manufacturer's instructions. Cells showing split signals or alone 3′ signals were considered positive for *ALK* rearrangement [31]. Filters were examined independently and blinded to clinical, IHC, ICC data and tissue genotype. We tested the reproducibility of the ICC and FISH results for ALK detection on CTCs of 102 filters of 34 patients who underwent blood sampling before surgery and 7 and 15 days after surgery.

A total of 14–500 interpretable tumour cell nuclei were analysed for each patient. To be correctly interpreted, tumour cell nuclei should have at least one colocalisation signal. To be considered as *ALK* rearranged, at least 15% of interpretable tumour cell nuclei should harbour an abnormal probes hybridisation pattern.

We used the human NSCLC cell line H2228 obtained from ATCC (Manassas, VA) as an *ALK* rearrangement positive control [32]. Cells were cultured and maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum, as previously described [32]. Around 50 cells were mixed into 10 ml of a blood sample taken from healthy volunteers. Samples were then filtered using the ISET method, as described previously [23]. FISH using a break-apart probe and ICC with anti-ALK antibodies were then carried out as described earlier.

results

Positive ALK immunostaining was found in five tumours corresponding to adenocarcinomas with a solid predominant structure with mucin production. These five cases showed strong positive cytoplasmic staining (score 3+) for all tumour cells as defined previously, with membrane reinforcement in a couple of cells (not shown) [31]. FISH analysis performed on the same paraffin block on serial sections demonstrated *ALK*-rearranged adenocarcinomas (not shown). The other 82 tumours were negative for ALK immunostaining and for *ALK* rearrangement by using FISH analysis. Overall, ten tumours (12%) had an EGFR mutation (1 exon 18, 6 exon 19 and 3 exon 21 mutations) and 20 cases (23%) were KRAS mutated (18 codon 12 of exon 2 and 2 codon 13 of exon 2). The *BRAF* mutation was not detected. In addition, the five ALK-rearranged tumours had EGFR, KRAS and BRAF wild-type status.

Positive ALK immunostaining was found in CTCs isolated in five patients, corresponding to the patients having *ALK*-rearrangement in tumours (Figure 1A1 and B1 and supplementary Figure S1, available at *Annals of Oncology* online). The clinicopathological data of these five patients are detailed in Table 2. The anti-ALK ICC using the 5A4 clone showed strong cytoplasmic staining (score 3+) of 100% of the CTCs with membrane reinforcements in most of the cells (Figure 1A1 and B1 and supplementary Figure S1, available at *Annals of Oncology* online). *ALK* FISH was informative in these five cases (Figure 1A2 and B2 and supplementary Figure S1, available at *Annals of Oncology* online). All CTCs had abnormal signal patterns with at least three signals observed per cell in each case, consistent with either gene amplification or aneusomy (Figure 1A2 and B2 and supplementary Figure S1, available at *Annals of Oncology* online). Moreover, FISH confirmed the presence of an *ALK* translocation, all cases having break apart of 5′ and 3′ probes and multiple signals per cells (Figure 1A2 and B2 and supplementary Figure S1, available at *Annals of Oncology* online). None of the five cases had loss of either part of the FISH probe. Finally, for these five patients, CTCs stained with MGG showed CTCs with malignant features, as described previously (Figure 1A3 and B3 and supplementary Figure S1, available at *Annals of Oncology* online) [22]. The positivity of ALK-ICC and *ALK*-FISH were controlled for each patient with circulating non-haematological cells with malignant features isolated using ISET at 7 and 15 days after the first detection.

No positive immunostaining with the anti-ALK antibody and no *ALK* rearrangement using FISH analysis were demonstrated in the 82 other selected patients with lung cancer showing CTCs with malignant features on MGG staining (Figure 1, C1–C3). *ALK*-rearranged H2228 cells diluted in blood samples, than filtered using the ISET method demonstrated strong positive ALK immunostaining and *ALK* translocation (Figure 1D1–D3).

The reproducibility of the results for the detection of ALK by ICC and FISH was tested on CTCs of 102 filters of blood samples from 34 patients who underwent blood sampling before surgery, and 7 and 15 days after surgery. Of the 34 patients included, 5 patients had ALK-positive tumour tissue and 29 patients had ALK-negative tumour tissue. Positive results were consistently obtained by ICC and FISH for CTC from the three different blood samples obtained from each of the five patients with ALK-positive tumours. Negative results for ALK were consistently obtained by ICC and FISH on CTC from the three different blood samples obtained from each of the 29 patients with ALK-negative tumours.

discussion

In this study, we have shown, using a dual ICC–FISH assay, that the ALK status can be detected non-invasively in CTCs characterised by a cytomorphological approach in a subset of patients with lung cancer. Moreover, our results demonstrated a strict correlation between the ALK status determined in CTCs and in the corresponding tumour tissue samples in a series of 87 patients with lung adenocarcinoma. Five of these patients had clinicopathological characteristics previously reported to be associated with *ALK*-gene rearrangement in the Western population and showed *ALK*-gene rearrangement both in CTCs and in the corresponding resected tumour samples [28]. Conversely, we never found CTCs with an *ALK*-gene rearrangement in patients with a tumour without *ALK*-gene rearrangement, as demonstrated by FISH. Recent studies focused on the relevance for the prognosis of ALK-positive lung cancer patients, independent of ALK-targeted therapy [33–35]. Some of these studies demonstrated that in patients not treated with pre-ALK inhibitors, ALK-positive patients had

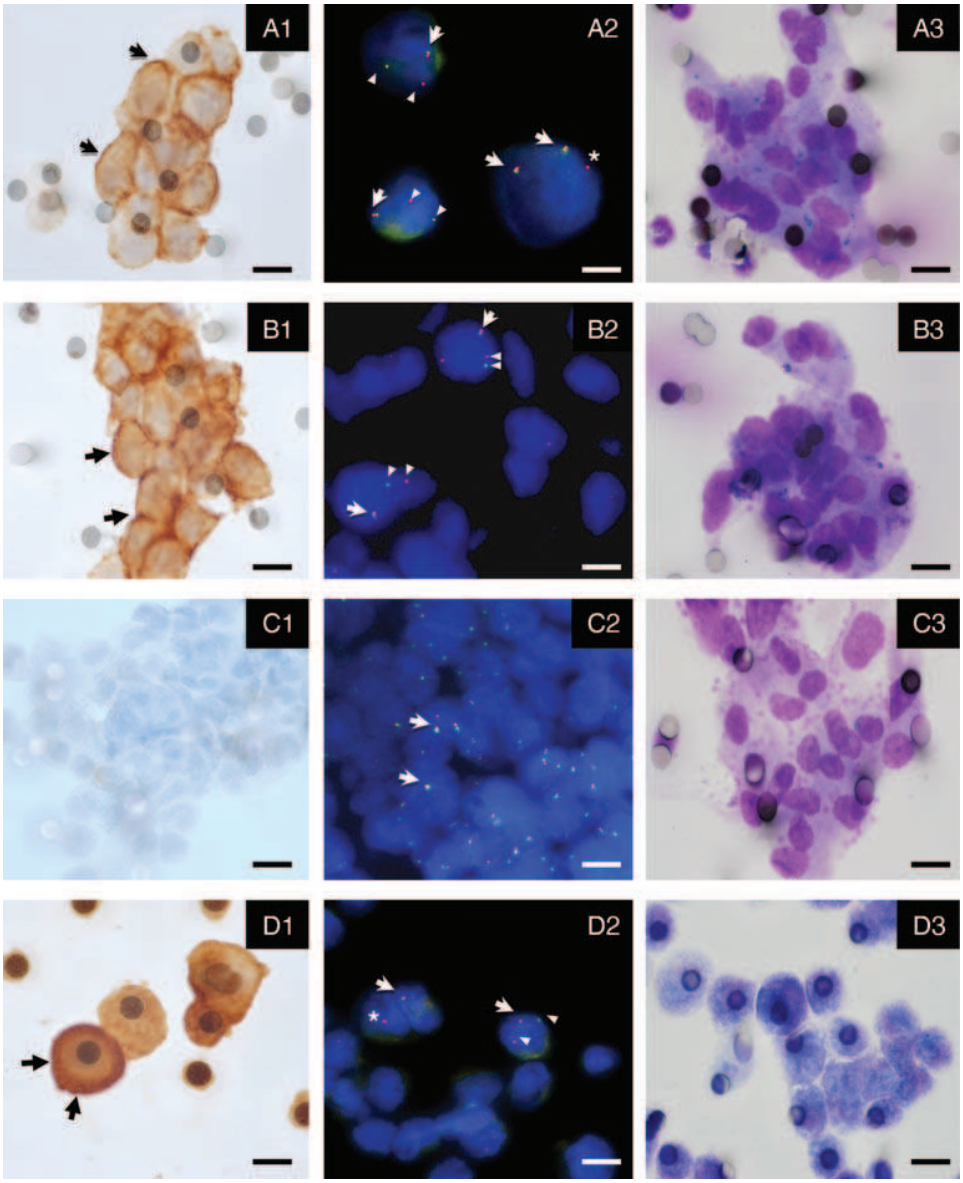

Figure 1. (A) Case 1 and (B) case 2. (A1 and B1) Circulating tumour cells showing an intense and cytoplasmic staining (score 3+) with some membrane reinforcements (arrows) (ALK immunostaining using 5A4mAb, immunoperoxidase; original magnification ×1000; bar: 16 µm). (A2 and B2) Circulating cell nuclei hybridised with a dual-color 2p23 LSI *ALK* locus-specific split probe. The two probes (3′, red; 5′, green) show a distinct separation of the red and green signals (arrowheads) indicating a rearrangement in the 2p23 *ALK*-gene locus. The probes give overlapping signals in nuclei without the rearrangement (arrows). Isolated 3′ signals (red) are also observed (asterisks) (original magnification ×1000; bar: 16 µm). (A3 and B3) Circulating cells showing malignant cytomorphological criteria isolated by the ISET method (original magnification ×1000; MGG staining; bar: 16 µm). (C) One patient with negative FISH ALK and negative IHC ALK in tissue tumour. (C1) Circulating tumour cells showing no staining (score 0) (ALK immunostaining using 5A4 mAb, immunoperoxidase; original magnification ×1000; bar: 16 µm). (C2) Circulating cells nuclei hybridised with a dual-color 2p23 LSI *ALK* locus-specific split probe. The two probes (3′, red; 5′, green) gave overlapping signals in nuclei without the rearrangement (arrows). No split signal was detected in these tumour cells (original magnification ×1000; bar: 16 µm). (C3) Circulating cells showing malignant cytomorphological criteria isolated by using the ISET method (original magnification ×1000; MGG staining; bar: 16 µm). (D) H22213 cells isolated by the ISET method. (D1) ALK immunostaining using 5A4 mAb (immunoperoxidase, original magnification ×1000) showing an intense and cytoplasmic staining (score 3+) with some membrane reinforcements (arrows). (D2) FISH using the dual-color 2p23 LSI *ALK* locus-specific split probe on the H2228 tumour cell line spiked into peripheral blood and further isolated by the ISET method. The two probes (3′, red; 5′, green) show distinct separation of the red and green signals (arrowheads) indicating a rearrangement in the 2p23 *ALK*-gene locus. The probes gave overlapping signals in nuclei without the rearrangement (arrows). Isolated 3′ signals (red) are also observed (asterisks) (original magnification ×1000; bar: 16 µm). (D3) H2228 cells stained with MGG after blood filtration (original magnification ×1000; MGG staining; bar: 16 µm).

Table 2. Clinicopathological data of the cases with FISH *ALK*-gene rearrangement and positive immunocytochemistry using an anti-ALK antibody in CTCs isolated by isolation by size of epithelial tumour cells (ISET) method

Case number	1	2	3	4	5
Sex	Male	Male	Female	Male	Female
Age (years)	45	48	47	52	43
Smoking status	Never smoked	Never smoked	Never smoked	Never smoked	Never smoked
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
pTNM stage	IIA	IIA	IIIB	IV	IV
Histology	Adenocarcinoma with solid architecture	Adenocarcinoma with solid architecture	Adenocarcinoma with solid architecture	Adenocarcinoma with solid architecture	Adenocarcinoma with solid architecture
Status for *EGFR*, *KRAS*, *BRAF* mutations	Wild-type	Wild-type	Wild-type	Wild-type	Wild-type
ALK FISH (tumour)	Positive (40% of cells)	Positive (50% of cells)	Positive (60% of cells)	Positive (40% of cells)	Positive (50% of cells)
ALK IHC (tumour)	Positive (100% of cells)	Positive (100% of cells)	Positive (100% of cells)	Positive (100% of cells)	Positive (100% of cells)
Number of CNHC-MF	≥50 cells (70–90 cells)	≥50 cells (60–150 cells)	≥50 cells (70–100 cells)	≥50 cells (60–100 cells)	≥50 cells (80–120 cells)
ALK FISH (CTCs)	Positive (100% of cells)	Positive (100% of cells)	Positive (100% of cells)	Positive (100% of cells)	Positive (100% of cells)
ALK ICC (CTCs)	Positive (100% of cells)	Positive (100% of cells)	Positive (100% of cells)	Positive (100% of cells)	Positive (100% of cells)
Follow-up (5 years)	Alive (no relapse)	Alive (no relapse)	Deceased	Deceased	Deceased

TNM, tumour node metastasis; CNHC-MF, circulating non-haematological cells with malignant features.

a shortest survival and were associated with a higher risk of metastasis [33, 35]. Moreover, ALK-positive patients were more resistant to *EGFR* tyrosine kinase inhibitor treatment than ALK-negative patients [33]. Conversely, a recent study demonstrated that patients with wild-type *EGFR* ALK-positive lung adenocarcinoma had a better outcome [34]. In the present work, the 5-year follow-up of the five *EGFR* wild-type ALK-positive patients showed no recurrence for the 2 stage II patients who underwent surgery, whereas the 3 stage IIIb/IV patients died within 6 months after the diagnosis. No adjuvant therapy, in particular no targeted therapy against *ALK* rearrangement, was administered in these patients.

The rationale for developing a non-invasive assay to detect *ALK*-gene rearrangement through CTCs isolation and characterisation is based on clinical considerations. Treatment with crizotinib has to be restricted to tumours with a proven *ALK*-gene rearrangement, which implies a systematic pre-screening of tumour samples with the reliable technical approaches. However, tumour tissue from patients with lung cancer is not always available or in a sufficient amount to perform both the pathological examination and an increasing list of immuno/molecular analyses aimed at stratifying patients for the use of targeted therapies. At variance with free tumour DNA/RNA in plasma, which may be derived from apoptotic cells and lacks the tumour cell invasive properties, CTCs may represent a "liquid biopsy" and constitute the ideal target for non-invasive theranostic tests.

In this study, we have used the ISET approach to isolate CTCs, as we and others have shown that this method displays a high sensitivity for CTC isolation in NSCLC patients [15–17]. As previously pointed out, CTC isolation using ISET is dependent on cellular size and independent of any cellular marker. Thus, tumour cells expressing epithelial markers as well as those having lost epithelial antigens, due to EMT, are efficiently isolated using ISET [17, 21, 24, 25]. Moreover, ICC and molecular analyses, including FISH, can be developed in CTCs isolated and characterised using ISET [17, 18, 21, 23–25]. Interestingly, we demonstrated the reproducibility of the results for the detection of ALK by ICC and FISH on a subgroup of 34 patients tested before surgery and, 7 and 15 days after surgery, and including five ALK-positive patients for tumour tissue. Consistent results were obtained blindly in the three samples obtained from each patient. These results confirm the technical reproducibility of ICC and FISH analysis for ALK on filters and show the feasibility of a kinetic real-time follow-up of patients by CTC analysis.

Reliable assessment of *ALK*-gene rearrangement in lung tumour tissues is recognised as a diagnostic and technical challenge [31, 36]. The ALK status on tumour samples can be evaluated by using FISH, IHC and/or the reverse transcriptase-polymerase chain reaction (RT–PCR) [31, 35–39]. FISH is the diagnostic method used as an eligibility criterion in current clinical trials with crizotinib [38]. IHC with antibodies specific for the human ALK protein (antibody clone ALK1) is diagnostic for an *ALK* rearrangement in a subset of anaplastic large cell lymphomas, having such sensitivity and specificity that genetic tests are considered unnecessary [38]. In NSCLCs, the expression of the ALK protein from the rearranged *ALK* gene is lower. However, the development and use of new ALK-specific antibodies has provided very interesting results. In this study, we have used the anti-ALK antibody, clone 5A4, which has been recently shown to accurately type 20/20 NSCLC tumour tissues [36]. In the five ALK-rearranged cases, we noted that no more than 50% of tumour cells were *ALK*-FISH positive in the tumour, whereas 100% of these cells were ALK positive by IHC. However, IHC for ALK was heterogeneous in certain areas of the tumours, and some cells were only faintly stained (1+) whereas others were strongly stained (3+). Thus, as described in a recent study, a correlation between IHC and FISH for the *ALK*-gene rearrangement can be observed in only

some areas of tumours [40], raising the issue of a better and more appropriate comparison between FISH-positive and "IHC 3+ only"-positive cells. As the preliminary data, the present study shows that ICC performed on CTCs may be a promising tool to detect *ALK* rearrangement as well as other genomic alterations, such as *EGFR* mutations. In this regard, some *EGFR* mutations can be demonstrated by IHC in a subset of lung adenocarcinomas using specific antibodies [31, 41]. We can speculate that such *EGFR* mutations could also be demonstrated using the ICC approach on CTCs in this subset of lung cancers. It is noteworthy that all CTCs detected in the present study were *ALK*-FISH positive and strongly positive by ICC using a specific antibody against ALK. We can, thus, speculate that CTCs harbouring this specific genomic alteration have facilitated migration and represent an aggressive set of tumour cells. *ALK*-gene rearrangement can also be detected by RT–PCR [30, 36]. RT–PCR is a challenging approach requiring high-quality RNA to afford amplification of multiple transcripts with variable sizes [36]. Finally, a quantitative real-time PCR approach has been recently developed to quantify *ALK* transcripts and obtained encouraging results [36]. In the present study, we did not try to look for *ALK* rearrangement using an RT–PCR approach, as we thought that the quantity and quality of the RNA we could potentially extract from CTCs isolated by ISET would not be sufficient for the test because the commercial buffer used for blood dilution before filtration contains formaldehyde. However, this strategy will be tested in the future in using a new ISET buffer developed to isolate fresh CTCs with unchanged sensitivity when compared with fixed CTCs.

The use of non-invasive CTC-based tests could allow in the future implementation of real-time molecular theranostic follow-up of patients to identify potential new genomic alterations involved in resistance to targeted therapies [42]. In this regard, the emergence of acquired resistance to crizotinib is a new challenge in the clinical care of ALK-positive lung cancer patients [11, 43, 44]. In fact, new genomic alteration(s) may occur during crizotinib therapy and can make the initial targeted treatment inefficient. Thus, real-time monitoring could be developed in aiming to detect the potential additional genomic alterations through molecular tests for CTCs isolated by ISET and diagnostically characterised by a morphological approach.

In conclusion, our study has shown the feasibility of detection of *ALK*-gene rearrangement in CTCs isolated by ISET and characterised as CTCs with malignant features. We found consistent results by using the ICC and FISH molecular approaches and, importantly, we also found consistent results in CTCs when compared with tumour tissues in the 87 tested patients. Although studies of a larger cohort are needed to extensively validate these data, our results open the way for a CTC-based theranostic approach for evaluation of non-invasive ALK status pre-screening of patients with lung cancer.

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disclosure

P.P.-B. is the inventor of ISET, Chief Scientific Officer and shareholder of the company Rarecells. The remaining authors have declared no conflicts of interest.

references

1. Goya T, Asamura H, Yoshimura H et al. Prognosis of 6644 resected non-small cell lung cancers in Japan: a Japanese lung cancer registry study. Lung Cancer 2005; 50: 227–234.
2. Jemal A, Siegel R, Ward E et al. Cancer statistics, 2008. CA Cancer J Clin 2008; 58: 71–96.
3. Naruke T, Tsuchiya R, Kondo H et al. Prognosis and survival after resection for bronchogenic carcinoma based on the 1997 TNM-staging classification: the Japanese experience. Ann Thorac Surg 2001; 71: 1759–1764.
4. Pfannschmidt J, Muley T, Bulzebruck H et al. Prognostic assessment after surgical resection for non-small cell lung cancer: experiences in 2083 patients. Lung Cancer 2007; 55: 371–377.
5. van Rens MT, de la Riviere AB, Elbers HR et al. Prognostic assessment of 2,361 patients who underwent pulmonary resection for non-small cell lung cancer, stage I, II, and IIIA. Chest 2000; 117: 374–379.
6. Hirsch FR, Wynes MW, Gandara DR et al. The tissue is the issue: personalized medicine for non-small cell lung cancer. Clin Cancer Res 2010; 16: 4909–4911.
7. Mino-Kenudson M, Mark EJ. Reflex testing for epidermal growth factor receptor mutation and anaplastic lymphoma kinase fluorescence in situ hybridization in non-small cell lung cancer. Arch Pathol Lab Med 2011; 135: 655–664.
8. Pao W, Girard N. New driver mutations in non-small-cell lung cancer. Lancet Oncol 2011; 12: 175–180.
9. Bria E, Milella M, Cuppone F et al. Outcome of advanced NSCLC patients harboring sensitizing EGFR mutations randomized to EGFR tyrosine kinase inhibitors or chemotherapy as first-line treatment: a meta-analysis. Ann Oncol 2011; 22: 2277–2285.
10. Gerber DE, Minna JD. ALK inhibition for non-small cell lung cancer: from discovery to therapy in record time. Cancer Cell 2010; 18: 548–551.
11. Sasaki T, Jänne PA. New strategies for treatment of ALK-rearranged non-small cell lung cancers. Clin Cancer Res 2011; 17: 7213–7218.
12. Shaw AT, Solomon B. Targeting anaplastic lymphoma kinase in lung cancer. Clin Cancer Res 2011; 17: 2081–2086.
13. Shaw AT, Yeap BY, Solomon BJ et al. Effect of crizotinib on overall survival in patients with advanced non-small-cell lung cancer harbouring ALK gene rearrangement: a retrospective analysis. Lancet Oncol 2011; 12: 1004–1012.
14. Yoshida A, Tsuta K, Nakamura H et al. Comprehensive histologic analysis of ALK-rearranged lung carcinomas. Am J Surg Pathol 2011; 35: 1226–1234.
15. Hofman V, Bonnetaud C, Ilie MI et al. Preoperative circulating tumor cell detection using the isolation by size of epithelial tumor cell method for patients with lung cancer is a new prognostic biomarker. Clin Cancer Res 2011; 17: 827–835.
16. Krebs MG, Sloane R, Priest L et al. Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. J Clin Oncol. 2011; 29: 1556–1563.
17. Krebs MG, Hou JM, Sloane R et al. Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. J Thorac Oncol 2012; 7: 306–315.
18. Paterlini-Brechot P, Benali NL. Circulating tumor cells (CTC) detection: clinical impact and future directions. Cancer Lett 2007; 253: 180–204.
19. Yu M, Stott S, Toner M et al. Circulating tumor cells: approaches to isolation and characterization. J Cell Biol 2011; 192: 373–382.
20. Maheswaran S, Sequist LV, Nagrath S et al. Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 2008; 359: 366–377.

21. Hofman V, Ilie MI, Long E et al. Detection of circulating tumor cells as a prognostic factor in patients undergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch Assay™ and the isolation by size of epithelial tumor cell method. Int J Cancer 2011; 129: 1651–1660.
22. Hofman V, Long E, Ilie M et al. Morphological analysis of circulating tumour cells in patients undergoing surgery for non-small cell lung carcinoma using the isolation by size of epithelial tumour cell (ISET) method. Cytopathology 2012; 23: 30–38.
23. Vona G, Sabile A, Louha M et al. Isolation by size of epithelial tumor cells: a new method for the immunomorphological and molecular characterization of circulating tumor cells. Am J Pathol 2000; 156: 57–63.
24. Hou JM, Krebs M, Ward T et al. Circulating tumor cells as a window on metastasis biology in lung cancer. Am J Pathol 2011; 178: 989–996.
25. Lecharpentier A, Vielh P, Perez-Moreno P et al. Detection of circulating tumour cells with a hybrid (epithelial/mesenchymal) phenotype in patients with metastatic non-small cell lung cancer. Br J Cancer 2011; 105: 1338–1341.
26. Lababede O, Meziane M, Rice T. Seventh edition of the cancer staging manual and stage grouping of lung cancer: quick reference chart and diagrams. Chest 2011; 139: 183–189.
27. Travis WD, Brambilla E, Noguchi M et al. International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol 2011; 6: 244–285.
28. Rodig SJ, Mino-Kenudson M, Dacic S et al. Unique clinicopathologic features characterize ALK-rearranged lung carcinoma in the western population. Clin Cancer Res 2009; 15: 5216–5223.
29. Ogino S, Kawasaki T, Brahmandam M et al. Sensitive sequencing method for KRAS mutation detection by pyrosequencing. J Mol Diagn 2005; 7: 413–421.
30. Richman SD, Seymour MT, Chambers P et al. KRAS and BRAF mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or Irinotecan: results from the MRC FOCUS Trial. J Clin Oncol 2009; 27: 5931–5937.
31. Hofman P, Ilie M, Hofman V et al. Immunohistochemistry to identify EGFR mutations or ALK rearrangements in patients with lung adenocarcinoma. Ann Oncol 2011 Nov 18 [Epub ahead of print] doi:10.1093/annonc/mdr535.
32. Koivunen JP, Mermel C, Zejnullahu K et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 2008; 14: 4275–4283.
33. Lee JK, Park HS, Kim DW et al. Comparative analyses of overall survival in patients with anaplastic lymphoma kinase-positive and matched wild-type advanced nonsmall cell lung cancer. Cancer 2011 Nov 15 [Epub ahead of print] doi:10.1002/cncr.26668.
34. Wu SG, Kuo YW, Chang YL et al. EML4-ALK Translocation Predicts Better Outcome in Lung Adenocarcinoma Patients with Wild-Type EGFR. J Thorac Oncol 2012; 7: 98–104.
35. Yang P, Kulig K, Boland JM et al. Worse disease-free survival in never-smokers with ALK+ lung adenocarcinoma. J Thorac Oncol 2012; 7: 90–97.
36. Just PA, Cazes A, Audebourg A et al. Histologic subtypes, immunohistochemistry, FISH or molecular screening for the accurate diagnosis of ALK-rearrangement in lung cancer: a comprehensive study of Caucasian non-smokers. Lung Cancer 2011 Dec 6 [Epub ahead of print] doi:10.1016/j.lungcan.2011.11.004.
37. Paik JH, Choi CM, Kim H et al. Clinicopathologic implication of ALK rearrangement in surgically resected lung cancer A proposal of diagnostic algorithm for ALK-rearranged adenocarcinoma. Lung Cancer 2011 Nov 28 [Epub ahead of print] doi:10.1016/j.lungcan.2011.11.008.
38. Sasaki T, Rodig SJ, Chirieac LR et al. The biology and treatment of EML4-ALK non-small cell lung cancer. Eur J Cancer 2010; 46: 1773–1780.
39. Popat S, Gonzalez D, Min T et al. ALK translocation is associated with ALK immunoreactivity and extensive signet-ring morphology in primary lung adenocarcinoma. Lung Cancer 2012; 75: 300–305.
40. Yi ES, Boland JM, Maleszewski JJ et al. Correlation of ICH and FISH for ALK gene rearrangement in non-small cell lung carcinoma: ICH score algorithm for FISH. J Thorac Oncol 2011; 6: 459–465.
41. Kitamura A, Hosoda W, Sasaki E et al. Immunohistochemical detection of EGFR mutation using mutation-specific antibodies in lung cancer. Clin Cancer Res 2010; 16: 3349–3355.
42. Krebs MG, Hou JM, Ward TH et al. Circulating tumour cells: their utility in cancer management and predicting outcomes. Ther Adv Med Oncol 2010; 2: 351–365.
43. Katayama R, Khan TM, Benes C et al. Therapeutic strategies to overcome crizotinib resistance in non-small cell lung cancers harboring the fusion oncogene EML4-ALK. Proc Natl Acad Sci USA 2011; 108: 7535–7540.
44. Heuckmann JM, Hölzel M, Sos ML et al. ALK mutations conferring differential resistance to structurally diverse ALK inhibitors. Clin Cancer Res 2011; 17: 7394–7401.