original reports

Clinical Relevance of an Amplicon-Based Liquid Biopsy for Detecting *ALK* and *ROS1* Fusion and Resistance Mutations in Patients With Non–Small-Cell Lung Cancer

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abstract

PURPOSE Liquid biopsy specimen genomic profiling is integrated in non–small-cell lung cancer (NSCLC) guidelines; however, data on the clinical relevance for *ALK/ROS1* alterations are scarce. We evaluated the clinical utility of a targeted amplicon-based assay in a large prospective cohort of patients with *ALK/ROS1*-positive NSCLC and its impact on outcomes.

PATIENTS AND METHODS Patients with advanced *ALK/ROS1*-positive NSCLC were prospectively enrolled in the study by researchers at eight French institutions. Plasma samples were analyzed using InVisionFirst-Lung and correlated with clinical outcomes.

RESULTS Of the 128 patients included in the study, 101 were positive for *ALK* and 27 for *ROS1* alterations. Blood samples (N = 405) were collected from 29 patients naïve for treatment with tyrosine kinase inhibitors (TKI) or from 375 patients under treatment, including 105 samples collected at disease progression (PD). Sensitivity was 67% (n = 18 of 27) for *ALK/ROS1* fusion detection. Higher detection was observed for *ALK* fusions at TKI failure (n = 33 of 74; 46%) versus in patients with therapeutic response (n = 12 of 109; 11%). *ALK*-resistance mutations were detected in 22% patients (n = 16 of 74) overall; 43% of the total *ALK*-resistance mutations identified occurred after next-generation TKI therapy. *ALK G1202R* was the most common mutation detected (n = 7 of 16). Heterogeneity of resistance was observed. *ROS1 G2032R* resistance was detected in 30% (n = 3 of 10). The absence of circulating tumor DNA mutations at TKI failure was associated with prolonged median overall survival (105.7 months). Complex *ALK*-resistance mutations correlated with poor overall survival (median, 26.9 months *v* NR for single mutation; $P = .003$) and progression-free survival to subsequent therapy (median 1.7 *v* 6.3 months; $P = .003$).

CONCLUSION Next-generation, targeted, amplicon-based sequencing for liquid biopsy specimen profiling provides clinically relevant detection of *ALK/ROS1* fusions in TKI-naïve patients and allows for the identification of resistance mutations in patients treated with TKIs. Liquid biopsy specimens from patients treated with TKIs may affect clinical outcomes and capture heterogeneity of TKI resistance, supporting their role in selecting sequential therapy.

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ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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INTRODUCTION

Since the discovery of driver oncogenic alterations in non–small-cell lung cancer (NSCLC), the treatment landscape has grown exponentially. Specific tyrosine kinase inhibitors (TKIs) targeting different alterations have impressively improved outcomes of patients with advanced NSCLC compared with chemotherapy.[1,2] Molecular testing is recommended at the time of diagnosis[3] and it should be also considered at disease progression (PD) in patients receiving TKI treatment to assess the resistance mechanisms that may support the selection of subsequent therapies. Tumor biopsy is the preferred approach for molecular testing, but in up to 30% of cases, the tissue quality is inadequate.[4,5] Analysis of circulating tumor DNA (ctDNA) liquid biopsy specimens provides a noninvasive surrogate material for detecting somatic mutations and is currently integrated in NSCLC clinical guidelines.[6,7]

CONTEXT

Key Objective

To determine if liquid biopsy specimens are clinically relevant in patients with *ALK*- and *ROS1*-positive advanced NSCLC.

Knowledge Generated

In a multicenter cohort of 128 patients with *ALK*- and *ROS1*-positive advanced NSCLC, an amplicon-based ctDNA next-generation sequencing (NGS) liquid biopsy was feasible and clinically relevant; 67% of *ALK* and *ROS1* fusions were detected in liquid biopsy specimens at diagnosis, and *ALK*- and *ROS1*-resistance mutations were identified in 22% of patients at TKI failure. This increased to 29% of patients at progression to second-generation TKI therapy.

Relevance

This amplicon-based, NGS liquid biopsy can enable molecular diagnosis for *ALK/ROS1* fusions from specimens at diagnosis and, in patients treated with TKIs, allows for the identification of resistance mutations that may influence treatment selection and clinical outcomes.

ALK and *ROS1* fusions occur in approximately 5% and 2%, respectively, of cases of advanced NSCLC.[8] Highly selective TKIs have enlarged the therapeutic arsenal. In patients positive for *ALK*, crizotinib[9] was the first TKI approved as frontline treatment, but second-generation TKIs (ie, alectinib, ceritinib, or brigatinib)[2] have improved frontline clinical outcomes, displacing crizotinib from the first-line TKIs, and third-generation TKIs, such as lorlatinib, with high activity in the crizotinib-resistance setting,[10] are being tested as first-line therapy (ClinicalTrials.gov identifier: NCT03052608). Unfortunately, PD remains inevitable. *ALK* mutations are one of the main mechanisms of resistance to TKI, identified in up to 30% of the cases at PD after crizotinib therapy.[11] Second- and third-generation TKIs can overcome resistance to crizotinib,[12] and a sequential strategy has been established as the standard of care after TKI failure.[3,13-15] Similarly, *ROS1*-positive NSCLC can develop crizotinib-resistance mutations in the *ROS1* kinase domain, conferring variable degrees of sensitivity or resistance to next-generation TKIs.[16-18] However, each TKI has a different spectrum of coverage for resistance mutations.[12] Thus, detecting specific resistance mutations may influence the choice of the TKI sequence.

Liquid biopsy already provides an alternative option to tissue for molecular profiling in treatment-naïve patients with NSCLC.[19] Although a few studies have recently reported the feasibility of using liquid biopsy specimens for profiling patients positive for *ALK*[20-22] and *ROS1*[23] alternations, larger, real-life, prospective cohorts are needed to assess the relevance of this strategy.

Here, we evaluated the clinical utility of targeted, amplicon-based, NGS liquid biopsy in a large prospective cohort of patients positive for *ALK/ROS1*. Also, we assessed the clinical relevance of the detection of *ALK/ROS1* fusions and resistance mutations on clinical outcomes and explored ctDNA as a potential predictive biomarker for efficacy of sequential TKIs.

PATIENTS AND METHODS

Study Population

Patients ≥ 18 years old with *ALK*- and *ROS1*-fusion–positive advanced NSCLC were prospectively enrolled between October 2015 and August 2018 at Gustave Roussy (CECCTC study no. 2008-A00585-50), at Centre Léon Bérard, and six other French institutions (LIBIL study, ClinicalTrials.gov identifier: NCT02511288). All patients provided written informed consent for biomedical research and the institutional ethics committees approved the protocol. *ALK* or *ROS1* fusion was determined by a validated test on tumor tissue (Data Supplement).

Sample Collection and ctDNA Analysis

Prospective samples were collected at any time point at diagnosis and/or at each disease radiologic evaluation (under response by RECIST v1.1[25] *v* progression). Patients receiving therapy with no previous sample collected at diagnosis were also enrolled; samples were collected at each radiologic evaluation. In blood samples, plasma was isolated and ctDNA analysis was centralized (Inivata, Cambridge, UK, and Research Triangle Park, NC) using InVisionFirst-Lung, which identifies single nucleotide variants, insertions and deletions, copy number variations, and fusions, with whole-gene and gene hotspots across a 36-gene panel (Data Supplement). Methods were as previously described[24] (Data Supplement). Fusion load was evaluated using fusion reads normalized to control single nucleotide polymorphism primers across all replicates at each time point. Relative change in fusion load was calculated between time 0 (T0) and subsequent time points (TX) to give an estimate of fusion load over time relative to T0.

Statistical Analysis

Outcomes assessment is summarized in the Data Supplement. Survival curves were estimated with the Kaplan-Meier method and were compared by the log-rank test. All *P* values were two-sided and values $< .05$ were considered statistically significant.

The presence of one *ALK* mutation was defined as "single ALK," the presence of two or more *ALK* resistance mutations was defined as "complex ALK"; the presence of other somatic mutations was defined as "others," "non-ALK," or non-ROS1," and the absence of mutations in blood, included in the 36-gene panel, was defined as "negative ctDNA." The prognostic value was measured in univariate analysis. For sensitivity analysis, referring to tissue data as the standard, the sensitivity was defined as true positive divided by the sum of true-positive and false-negative results.

Data were processed and analyzed using SPSS software, version 25.0.0 (IBM Corp., Armonk, NY). The numbers of all included patients and recorded variables were reported using descriptive statistics and the relationship between clinical characteristics and response was determined by Fisher exact test.

TABLE 1. Patients' Baseline Characteristics

Characteristic	*ALK* (n = 101)	*ROS1* (n = 27)
Age, median (range), years	52 (21-84)	54 (26-83)
Sex		
Male	42 (42)	13 (50)
Female	59 (58)	13 (50)
Missing		1
Smoking status		
Never	57 (58)	18 (69)
Smoker	42 (42)	8 (31)
Missing		1
Histology		
Adenocarcinoma	97 (96)	25 (93)
NSCLC, other	4 (4)	1 (7)
Squamous	—	—
Missing	—	1
Stage at diagnosis		
I-IIIA	11 (14)	3 (14)
IIIB-IV	70 (86)	19 (86)
Missing	20	5
Brain metastasis at baseline	42 (42)	9 (35)
Molecular diagnosis		
FISH (+)	78 (77)	19 (70)
IHC (+)	63 (62)	15 (56)
Other (+)	—	2 (7)
No. of prior systemic lines at inclusion, median (range)	2 (1-9)	2 (1-8)

NOTE. Data reported as No. (%) unless otherwise indicated.

Abbreviations: —, no cases; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; PD, progressive disease, NSCLC, non–small-cell lung cancer.

RESULTS

Of 128 enrolled patients, 101 had *ALK*-positive and 27 *ROS1*-positive NSCLC. Baseline characteristics are summarized in Table 1. Overall, 404 blood samples were collected: 29 in TKI-naïve patients (25 with *ALK* fusions, four with *ROS1* fusions) and 375 in patients receiving treatment (Data Supplement). A median of two samples was collected per patient (range, 1 to 13 samples).

Detection of ctDNA *ALK/ROS1* Fusions

Treatment-naïve cohort. Twenty-seven samples were eligible for analysis. In 18 patients, the fusion was detected in blood (n = 16 patients with *ALK*; n = 2 with *ROS1*), with a sensitivity of 67% for *ALK* and *ROS1* fusion (Data Supplement). In the *ALK* cohort, eight variant 1, two variant 2, and six variant 3 fusions were detected. In the *ROS1* cohort, one *CD74-ROS1* and one *SLC34A2-ROS1* fusion were detected.

Fusion detection in blood was associated with a high number of metastatic sites and visceral involvement (Data Supplement). The metastatic pattern was not related to *ALK* variants or *ROS1* partner gene. Plasma NGS was performed on 25 samples, of which 11 (44%) had concurrent gene aberrations: *TP53* mutation (24%; n = 6), followed by *NRAS*, *STK11*, and *CDKN2A* mutations, and *EGFR* amplification (Data Supplement). One patient had an *ALKL1196Q* mutation at diagnosis.

Radiologic response. A total of 143 samples collected at the time of confirmed objective response by RECIST, version. 1.1, were evaluable for fusion analysis (*ALK*, n = 109; *ROS1*, n = 34). Fusions were detected in 14 samples (10%): 12 of 109 (11%) for patients with *ALK* and two of 34 (6%) for *ROS1* patients (Data Supplement).

A total of 121 samples were collected at the time of PD on systemic therapies (eg, chemotherapy, TKIs; *ALK*, n = 96; *ROS1*, n = 15). Among them, 74 were collected at TKI failure, with a detection rate of 45% (n = 33 of 74) for *ALK* and 30% (n = 3 of 10) for *ROS1* fusions (Data Supplement). The detection rate was higher in patients with visceral and bone metastases (Data Supplement).

Detection of ctDNA Mutations at TKI Failure

ALK mutations were detected in 22% of samples (n = 16 of 74) collected at TKI failure, including five samples with single *ALK* mutations, three with complex *ALK* mutations, and eight with *ALK* and other genes mutations (Fig 1A).

ALK mutations were more frequently detected in patients with bone or liver progression (75% to 80%) compared with exclusive CNS or thoracic progression (10%). Detection of *ALK* mutations at isolated CNS relapse was 10% (n = 3 of 29); 0% after crizotinib (n = 0 of 11) versus 18% after therapy with a next-generation TKI (n = 3 of 17). In 55%, no mutations were detected (n = 16 of 29). *ALK* variant 3 was associated with *ALK* mutations (37%; n = 6 of 16), compared with variant 2 (13%; n = 2 of 16) and variant 1 (none).

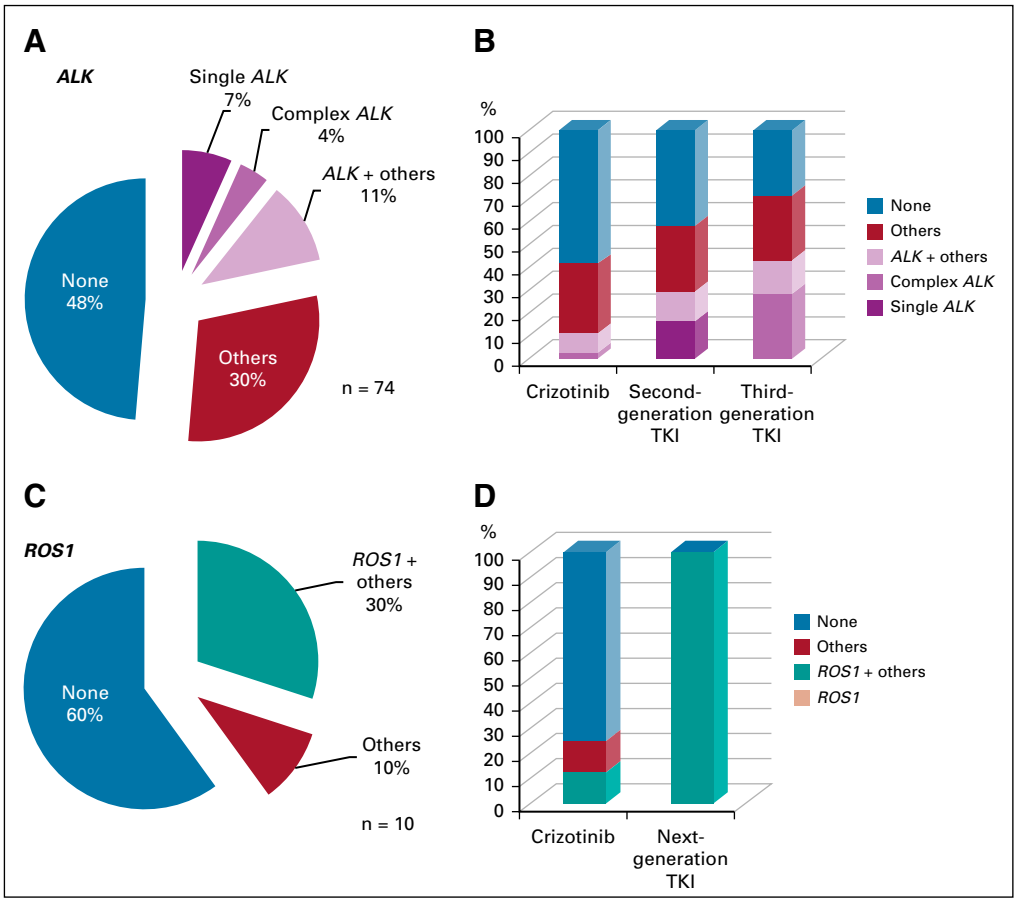

FIG 1. Somatic mutations detected in liquid biopsies at progressive disease to tyrosine kinase inhibitor (TKI; A, C) and according to exposure to prior TKI (B, D) in *ALK* (A, B) and *ROS1* (C, D) positive patients.

At PD, *ALK* mutations with third-generation TKIs were detected in 43% (n = 7) of samples compared with 29% in samples from patients treated with second-generation TKIs (n = 31) and 11% with crizotinib (n = 36; Fig 1B). *ALK* G1202R was detected as a single mutation (n = 3) or concurrent with other *ALK* mutations (n = 4) and was the most common resistance mutation (n = 1 after crizotinib therapy; n = 6 after treatment with next-generation TKIs).

Non-*ALK* mutations were detected in 41% of samples (n = 30 of 74); 27% (n = 8 of 30) were concurrent with *ALK* mutations. *TP53* was the most common (n = 26 of 74), 54% (n = 14 of 26) as the unique mutation, 27% (n = 7 of 26) concurrent with *ALK* mutations, and in 19% of samples (n = 5 of 26) associated with other mutations. Other mutations detected included *KRAS* plus *PI3KCA*, *PTEN* plus *PI3KCA*, *MET, STK11*, and *CDKN2A* (Data Supplement).

In 21 patients, 57 longitudinal samples during sequential therapies were available. In five patients, de novo *ALK* mutations emerged after TKI (Data Supplement).

Paired tissue and liquid biopsy specimens with resistance mutations were available in six cases (in all cases, the *ALK* fusion was confirmed in tissue samples, Table 2). The three cases with single *ALK* mutation had 100% tissue- and liquid-sample concordance (n =2 *ALK* G1202R; n =1 *ALK* L1196M). The other three cases had complex *ALK* mutations, one was concordant between tissue and liquid biopsy specimens (*ALK* F1174V and *ALK* L1198F). However, some discordance was observed in two cases: one case with *ALK* G1202R plus *ALK* E1154K in the tissue biopsy specimen and *ALK* G1202R plus *ALK* I1268V in the liquid biopsy specimen; and one case with more mutations detected in the liquid biopsy specimen (tissue: *ALK* G1202R plus *ALK* F1174L; liquid: *ALK* G1202R plus *ALK* F1174L plus *ALK* C1156Y plus *ALK* G1269A plus *ALK* S1206F plus *ALK* T1151M).

In the *ROS1* cohort, among the 10 samples collected at the time of PD, three (30%) had the *ROS1* G2032R-resistance mutation. All cases had concurrent mutations (*CTNNB1, TP53*, and *TP53* plus *CDKN2A).* The ctDNA somatic mutations evidenced at TKI failure are depicted in Figure 1C and 1D. Three patients had sequential samples for assessing the emergence of mutations; in one case, we observed the emergence of *ROS1* mutation at crizotinib failure (*SLC34A2-ROS1* fusion; Data Supplement).

Clinical Outcomes in *ALK*-Positive Patients According to Liquid Biopsy Specimens

The absence of mutations in ctDNA was associated with improved overall survival (OS; n = 74 samples; n = 55

TABLE 2. Clinical Efficacy of TKIs According to the ALK-Resistance Mutations Detected in Liquid Biopsy Specimens

						TKI					
Patient ID	**PD Site**	**Variant**	**Time Point**	***ALK* Resistance mutations**	***ALK* G1202R**	**Crizotinib**	**Alectinib**	**Ceritinib**	**Brigatinib**	**Lorlatinib**	**CTx**
CTC861	Liver	V3	After crizotinib	Complex *ALK*[a]	*G1202R*	✓	—	PFS < 3 mo[b]	—	—	—
CTC 1105	Bone	V3		Complex *ALK*[a]		✓	—	—	PFS < 3 mo[b]	—	—
BB 01-127	Adrenal, brain, liver	V2		Complex *ALK*[a]		✓	—	—	PFS 3-6 mo[c]	—	—
RC01-020	Brain, bone	–	After brigatinib	Complex *ALK*[a]	*G1202R*	✓	—	✓	✓	PFS ≥ 6 mo[d]	—
LY 01-045	Brain, lung, nodal, pleural	–	After lorlatinib	Complex *ALK*[a]		✓	—	✓	✓	✓	PFS < 3 mo[b]
RC01-020	Brain	–		Complex *ALK*	*G1202R*	✓	—	✓	✓	✓	PFS < 3 mo[b]
CTC861	Liver	V3		Complex *ALK*	*G1202R*	✓	—	✓	✓	✓	PFS 3-6 mo[c]
CTC 925	Nodal	–	After crizotinib	Single *ALK*[a]		✓	—	PFS ≥ 6 mo[d]	—	—	—
CTC 435	Pleural	V3	After ceritinib	Single *ALK*	*G1202R*	✓	—	✓	—	PFS ≥ 6 mo[d]	—
CTC 534	Liver	–		Single *ALK*		✓	—	✓	—	PFS ≥ 6 mo[d]	—
RC01-020	Brain	–		Single *ALK*		✓	—	✓	PFS ≥ 6 mo[d]	—	—
GA 01-034	Brain	–	After alectinib	Single *ALK*[a]		✓	✓	—	—	PFS < 3 mo[b]	—
LY 01-045	Brain, lung, nodal	–	After brigatinib	Single *ALK*		✓	—	✓	✓	PFS ≥ 6 mo[d]	—
CTC861	Liver	V3		Single *ALK*[a]	*G1202R*	✓	—	✓	✓	PFS 3-6 mo[c]	—
CTC 1342	Bone	V3		Single *ALK*[a]	*G1202R*	✓	—	✓	✓	PFS < 3 mo[b]	—
BB 01-127	Brain, liver	V2		Single *ALK*[a]		✓	—	✓	✓	PFS < 3 mo[b]	—

Abbreviations: ✓, patient has previously received this TKI; –, fusion not detected or not tested; —, TKI not received; CTx, chemotherapy; ID, identifier; PFS, progression-free survival; TKI, tyrosine kinase inhibitor.

[a]De novo mutations (in blood).

[b]Median PFS < 3 months.

[c]Median PFS after next therapy between 3 and 6 months.

[d]Median PFS after next therapy ≥ 6 months.

patients; Fig 2). The median OS was 58.5 (95% CI, 26.9 to not reached [NR]) months if one or more *ALK* mutations were detected versus 44.1 (95% CI, 21.7 to NR) months if non-*ALK* mutations were detected versus 105 (95% CI, 105.7 to NR) months in patients negative for ctDNA ($P = .001$). This effect was observed regardless of the number of lines of TKI received (more than TKIs *v* two or fewer TKIs; $P = .01$). This observation also held true when we assessed this outcome in the population exclusively treated with TKI as first-line therapy (n = 29; $P = .04$). The patients' characteristics according to the ctDNA mutations are described in the Data Supplement.

The presence of complex *ALK* mutations was associated with poor OS (median, 26.9 months; 95% CI, 13.9 months to NR) compared with single *ALK* mutation (median, NR; 95% CI, 57.0 months to NR; $P = .003$). This effect was also observed in the subgroup treated with upfront TKI ($P = .038$). The median OS in the four patients with emergence of ctDNA *ALK* G1202R was 59.5 (95% CI, 26.9 to NR) months.

The group with absence of ctDNA mutations had a median progression-free survival (PFS) to the therapy on which their disease progressed of 14.8 (95% CI, 8.1 to 23.1) months versus 9.6 (95% CI, 6.6 to 19.9) months if there was one or more *ALK* mutation or 7.8 (95% CI, 4.5 to 11.7) months if there were non-*ALK* mutations at TKI failure ($P = .31$). The median PFS of the four cases with emergence of *ALK* G1202R was 2.7 (95% CI, 2.03 to NR)

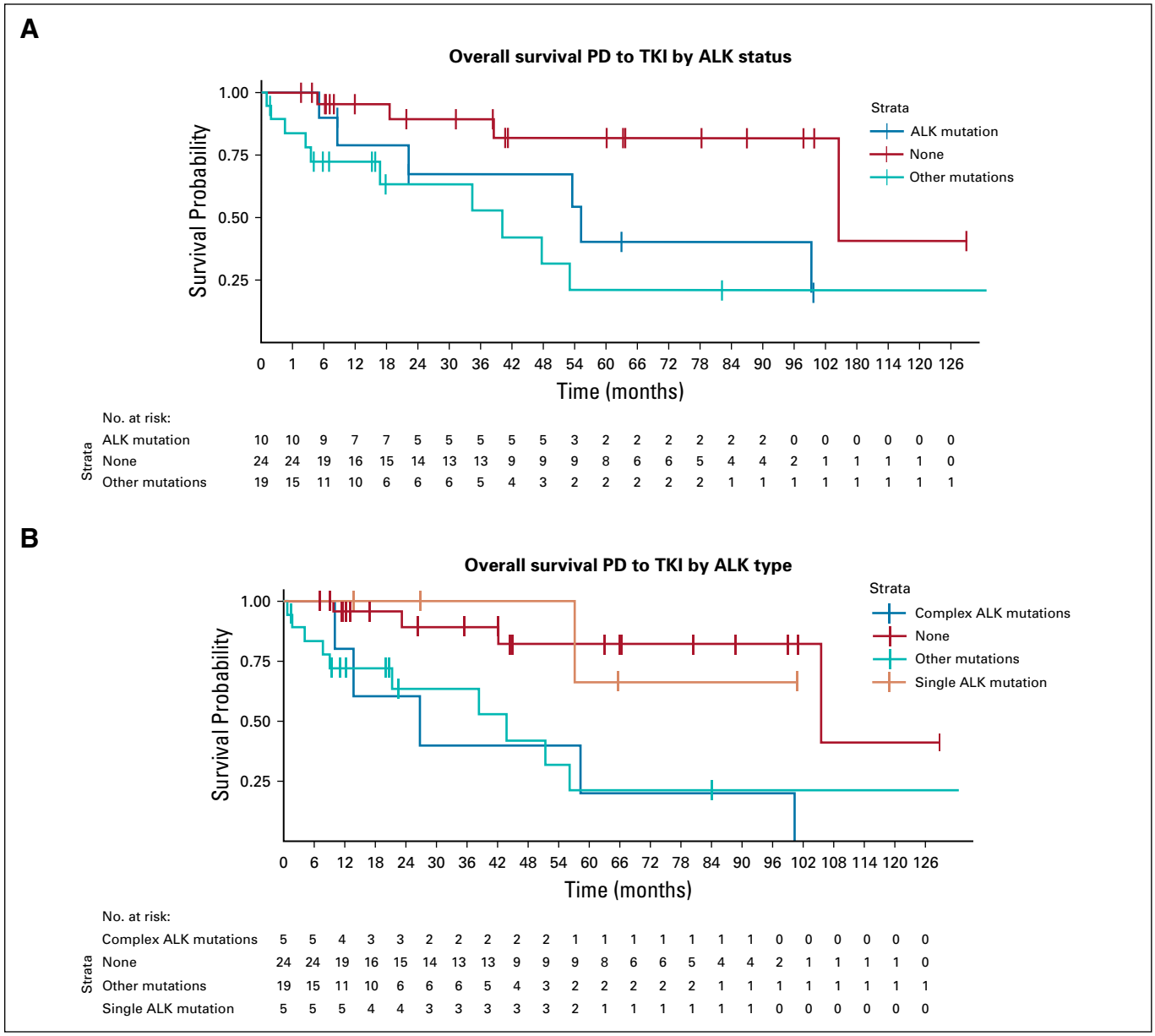

FIG 2. Overall survival according to the presence of *ALK* resistance mutations or other mutations in the liquid biopsy in *ALK* positive population. (A) PD while receiving TKI therapy, by *ALK* status. (B) PD while receiving TKI therapy, by *ALK* type. PD, disease progression; TKI, tyrosine kinase inhibitor.

months versus 8.6 (95% CI, 5.6 to 10.6) months in the remaining population ($P = .05$).

We then studied the PFS to the subsequent therapy according to ctDNA mutations detected in 56 samples. The median PFS was 20.7 (95% CI, 6.3 to NR) months in the negative ctDNA group versus 8 (95% CI, 2.8 to NR) months for non-*ALK* mutations versus 2.8 (95% CI, 1.2 to NR) months in the group with one or more *ALK* mutation detected ($P = .03$).

We further explored the PFS specifically in the subgroup harboring *ALK* mutations (n = 16; Data Supplement; Table 2). The *ALK* complex mutations were associated with poor efficacy, with a median PFS of 1.7 (95% CI, 0.9 to NR) months. In contrast, the *ALK* single mutation was more commonly associated with longer PFS, with a median of 6.3 (95% CI, 1.8 to NR) months ($P = .003$). The median PFS to the sequential therapy of the four cases with emergence of *ALK* G1202R was 3.7 (95% CI, 1.2 to NR) months versus 8.3 (95% CI, 4.9 to NR) months in overall population ($P = .15$).

The ctDNA dynamics were evaluated at TKI baseline and longitudinally in 34 patients. See the Data Supplement for a representation of the association between the fusion and the highest allelic frequency of any mutation detected in blood in *ALK*-positive patients and therapeutic response. The detection of fusion in blood and higher level of allelic frequency were correlated with PD at the time of response assessment.

The fusion load was calculated in nine patients. The clearance of the fusion was well correlated with the clearance of other somatic mutations detected in the same sample (Data Supplement) in response to therapy.

DISCUSSION

Herein, we report the clinical relevance of a targeted, amplicon-based NGS assay in a large, prospective, real-world cohort of 128 *ALK/ROS1*-positive patients. At the time of diagnosis, the sensitivity was 67%. At progression, *ALK*- and *ROS1*-resistance mutations were reported in 22% versus 30% of patients, respectively. We also describe the clinical relevance of liquid biopsy specimens on patient outcomes and the potential role of ctDNA as a predictive biomarker.

In our cohort, the sensitivity of amplicon-based NGS for the detection of *ALK* fusion was in line with the limited data reported to date on other blood-based approaches, such as hybrid-capture liquid biopsy (55%),[26] RNA exosomes (63%),[27] and reverse transcriptase polymerase chain reaction in platelets (65%).[27] In the treatment-naïve population with *ROS1*, we found a sensitivity of 67%; a single study recently reported a detection rate of 50% in plasma at the time of PD, with no data for treatment-I patients.[23] Sensitivity was correlated with higher number of metastatic sites and visceral involvement, reflecting the impact of tumor burden. Our sensitivity data are comparable with the detection rate of other mutations, for which liquid biopsy specimens are routinely used in cases where adequate tissue is unavailable.[26]

We showed the potential of ctDNA as a surrogate biomarker for therapeutic response, including a novel method for evaluating the relative fusion change: *ALK/ROS1* fusion detection was higher in patients at TKI failure than in those whose disease was responding to TKI treatment (49% *v* 11%). ctDNA clearance was correlated to radiologic response, which should be further explored.

Concurrent non-*ALK* mutations were associated with poor outcomes, consistent with reports from studies using tissue testing.[28,29] This emphasizes the clinical relevance of comprehensive genomic profiling to test for fusion and also mutations in other genes. Interestingly, we reported an *ALK* mutation at diagnosis that is typically detected at TKI failure; this has been previously reported in preclinical models.[30] Its impact on clinical outcome remains unknown.

We found *ALK* mutations in 22% of samples at TKI failure, which is comparable to the 24% presented by Shaw et al.[35] However, this is lower than the 30% reported in tissue[12] and 50% in liquid biopsy specimens in smaller cohorts of patients treated with second-generation TKIs.[20,21]

As previously reported, we detected more *ALK*-resistance mutations after next-generation TKIs (≤ 43%). Interestingly, the complex *ALK* mutations, potentially associated with compound mutations after sequential exposure to TKIs,[31] were detected in 45% of samples after crizotinib therapy and more likely were related to a polyclonality PD.[32] In addition, liquid biopsy specimens can reveal molecular heterogeneity, particularly in cases of complex *ALK* mutations that were not observed in tissue testing.

The *ALK* G1202R mutation was the most commonly detected (44%), generally after second-generation TKI, but also seen after crizotinib. *ALK*-resistance mutations were most frequently associated with *ALK* variant 3, including all cases of *ALK* G1202R, as previously reported from tissue biopsy specimens.[33] We observed the emergence of some de novo mutations in longitudinal analyses during sequential TKI therapy, including the emergence of *ALK* G1202R after crizotinib, or the accumulation of *ALK* mutations during sequential TKI therapy, as reported by Yoda et al.[31] We identified *ROS1*-resistance mutations in 30% of cases, comparable to the 33% recently reported by Dagogo-Jack et al.[23]

At TKI failure, no mutation was detected in approximately half of the liquid biopsy specimens, as previously reported[34,35]; this can be related to the sites of metastatic disease (ie, in case of isolated CNS or thoracic PD), with lower detection rate reported.[36] Systemic bone and liver PD was associated with higher detection than was isolated CNS or thoracic PD (≤ 80% *v* 10%). Thus, the pattern of metastatic disease

should be considered when interpreting data from liquid biopsy specimens in the context of resistance. More prospective data are required to draw definitive recommendations.

Other non-*ALK* and non-*ROS1* mutations were detected in 30% of *ALK* and 10% of *ROS1* samples at resistance. One hypothesis is that they could represent bypass mechanisms in some cases, reported as the second main cause of TKI resistance.[12,37] *KRAS, PI3KCA*, or *PTEN* mutations identified potentially could be related to bypass mechanisms.

In an exploratory analysis in the *ALK* population, the absence of ctDNA mutations was associated with improved outcomes; this could be related to a lower tumor burden or a less heterogeneous tumor. In contrast, the complex *ALK* mutations were associated with the worst survival outcome, probably reflecting polyclonal and resistant tumors or compound mutations. In the *ROS1* population, similar findings were observed. Patients with absence of ctDNA mutations at TKI failure had an improved outcome, and all patients with *ROS1* G2032R experienced rapid progression (< 3 months) to subsequent TKI therapy.

Our study has some limitations. Although the amplicon-based NGS approach used is highly sensitive for mutations, it is limited to the detection of known fusion partners, which cover 90% to 95% of the *ALK/ROS1* population.[38] Second, the sample size is limited and the heterogeneity of the samples collected in different time points is high, though, to our knowledge, it is the largest, real-world, prospective cohort of *ALK/ROS1* studied. In addition, in the *ALK* population, only 51% of the samples were collected after next-generation TKI, the current standard of care; however the information derived from cases after crizotinib therapy was also informative (ie, detection of *ALK* G1202R, uncommon after crizotinib therapy, and complex mutations that did not respond to the next TKI). Finally, patients were included either as they were diagnosed with advanced NSCLC or at any time of treatment, which may be associated with a bias in recruitment of patients with long survival. Prospective clinical trials are required in context of next-generation TKI therapies[39] that showed promising activity against the *ALK*G1202R and *ROS1*G2032R mutations, which are resistant to the majority of other available TKIs.

In conclusion, our clinical experience of an amplicon-based, NGS liquid biopsy in a large, real-world, prospective cohort of *ALK/ROS1*-positive patients with NSCLC provide evidence of the clinical utility of this approach at the time of diagnosis as well as at the time of PD for detection of resistance mutations. Liquid biopsy specimens in TKI-treated patients capture heterogeneity of TKI resistance, supporting the role of liquid biopsy in selecting sequential therapy.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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