Original Article

Earlier extracranial progression and shorter survival in ALK-rearranged lung cancer with positive liquid rebiopsies

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Background: Liquid rebiopsies can detect resistance mutations to guide therapy of anaplastic lymphoma kinase-rearranged (ALK$^+$) non-small-cell lung cancer (NSCLC) failing tyrosine kinase inhibitors (TKI). Here, we analyze how their results relate to the anatomical pattern of disease progression and patient outcome.

Methods: Clinical, molecular, and radiologic characteristics of consecutive TKI-treated ALK$^+$ NSCLC patients were analyzed using prospectively collected plasma samples and the 17-gene targeted AVENIO kit, which covers oncogenic drivers and all *TP53* exons.

Results: In 56 patients, 139 instances of radiologic changes were analyzed, of which 133 corresponded to disease progression. Circulating tumor DNA (ctDNA) alterations were identified in most instances of extracranial progression (58/94 or 62%), especially if concomitant intracranial progression was also present (89%, P<0.001), but rarely in case of isolated central nervous system (CNS) progression (8/39 or 21%, P<0.001). ctDNA detectability correlated with presence of "short" echinoderm microtubule-associated protein-like 4 (*EML4*)-*ALK* fusion variants (mainly V3, E6:A20) and/or *TP53* mutations (P<0.05), and presented therapeutic opportunities in <50% of cases. Patients with extracranial progression and positive liquid biopsies had shorter survival from the start of palliative treatment (mean 52 *vs.* 69 months, P=0.002), regardless of previous and subsequent therapy and initial ECOG performance status. Furthermore, for patients with extracranial progression, ctDNA detectability was associated with shorter next-line progression-free survival (PFS) (3 *vs.* 13 months, P=0.003) if they were switched to another systemic therapy (49/86 samples), and with shorter time-to-next-treatment (TNT) (3 *vs.* 8 months, P=0.004) if they were continued

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on the same treatment due to oligoprogression (37/86). In contrast, ctDNA detectability was not associated with the outcome of patients showing CNS-only progression. In 6/6 cases with suspicion of non-neoplastic radiologic lung changes (mainly infection or pneumonitis), ctDNA results remained negative.

Conclusions: Positive blood-based liquid rebiopsies in ALK$^+$ NSCLC characterize biologically more aggressive disease and are common with extracranial, but rare with CNS-only progression or benign radiologic changes. These results reconcile the increased detection of *ALK* resistance mutations with other features of the high-risk *EML4-ALK* V3-associated phenotype. Conversely, most oligoprogressive patients with negative liquid biopsies have a more indolent course without need for early change of systemic treatment.

Keywords: Anaplastic lymphoma kinase-rearranged (*ALK+*); non-small-cell lung cancer (NSCLC); liquid biopsy; extracranial progression; tyrosine kinase inhibitor (TKI); treatment failure; overall survival

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Introduction

"Liquid biopsies" analyzing cell-free genetic material of the tumor in the patients' blood (Circulating tumor DNA; ctDNA) have emerged as a non-invasive method for the molecular profiling of various malignancies (1,2). Non-small-cell lung cancer (NSCLC), and in particular anaplastic lymphoma kinase-rearranged (*ALK*$^+$) tumors are emerging as a model disease in this regard (3,4). ALK$^+$ NSCLC patients can experience long-lasting responses under tyrosine kinase inhibitors (TKI) (5-7), with treatment failure frequently occurring due to resistance mutations in exons coding for the ALK kinase domain (8-10). Several proof-of-principle studies have recently shown that mechanisms of acquired resistance to ALK inhibitors can be efficiently captured by longitudinal ctDNA assays, which therefore represent a promising tool to guide the selection of tailored next-line therapies (11-16). The current study focusses on how the potential clinical utility of liquid rebiopsies in ALK$^+$ NSCLC depends on the anatomical pattern of treatment failure, and on the prognostic information that their results additionally provide about the subsequent disease course and patient survival. We present this article in accordance with the STROBE reporting checklist (available at http://dx.doi.org/10.21037/tlcr-21-32).

Methods

Study population, objectives, collection of clinical data, ethics

This study included all ALK$^+$ NSCLC patients treated with TKI between 2014 and 2019 in our institution with ctDNA results at the time of radiologic changes. There were two main study objectives: (I) relationship between the results of liquid rebiopsies and the anatomic pattern of disease progression; and (II) the survival [i.e., progression-free survival (PFS), time-to-next treatment and overall survival (OS)] of progressive patients according to the results of liquid rebiopsies. Blood samples had been prospectively collected from each patient at each visit every 8–12 weeks during the entire disease course, as previously published (17). Clinical data were systematically collected from the medical records with a cut-off in June 2020. There were no lost-to-follow-up cases, as all patients were treated in-house. In order to control for possible confounders, every case was annotated regarding baseline clinical characteristics and treatment in great detail, and these data were analyzed together with parameters of interest, including multivariable testing. Particular attention was given to the localization of disease progression, i.e., intra- *vs.* extracranial, and intra- *vs.* extrathoracic, as well as to parameters that could influence patient outcome, i.e., the ECOG performance status (PS), molecular characteristics as outlined in the next section, whether the ongoing systemic treatment was switched at the time of liquid biopsy or continued beyond disease progression, the type of TKI treatment in conjunction with molecular results, and the administration of local therapies in case of oligoprogression. For PFS, the progression date documented in the records was verified by the investigators with review of radiologic images, i.e., chest/abdomen CT and brain MRI-based restaging every 6–12 weeks, without

formal RECIST reevaluation, as several studies have demonstrated very good agreement between real-world and RECIST-based assessments (18,19). For evaluating the potential clinical utility of liquid biopsies in case of anatomically restricted disease progression and continuation of the same systemic treatment, changes in tumor size were considered regardless of the RECIST threshold, because any significant increase of tumor lesions poses therapeutic dilemmas to the treating physicians, and no uniform definition of oligoprogression exists in the literature (20). Time-to-next-treatment (TNT) was calculated from the start of the respective treatment line until change of systemic therapy or death. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the ethics committee of the Heidelberg University (S-296/2016). Written informed consent was provided by every participant.

Molecular analyses

Diagnosis of NSCLC was performed at the Institute of Pathology Heidelberg from tissue specimens according to the criteria of the current WHO Classification (2015) for lung cancer (21). Newly diagnosed cases were screened for the presence of an ALK alteration by fluorescence *in situ* hybridization (FISH, ZytoLight SPEC ALK probe, ZytoVision GmbH, Bremerhaven, Germany) and reverse-transcription polymerase chain reaction until 2015, or by immunohistochemistry (D5F3 clone, Roche, Mannheim, Germany) and RNA-based next-generation sequencing (NGS, ThermoFisher Lung Cancer Fusion Panel, Waltham, MA, USA) thereafter, as previously described (22). Molecular workup at baseline also included DNA-based NGS covering all exons of *EGFR* as well as several other genes with potentially actionable alterations (e.g., *BRAF*, *MET*, *HER2*) and/or considered relevant for lung cancer biology (e.g., exons 4–10 of *TP53*), as published (23). For liquid biopsies, cfDNA was isolated from 2 mL plasma using the AVENIO cfDNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) and quantified with the Qubit dsDNA High Sensitivity Kit (ThermoFisher). Enrichment of the characteristic mononucleosomal fragment peak (160–200 bp) and absence of contaminating high molecular weight genomic DNA (24,25) were verified using the Bioanalyzer 2100 High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA). Sequencing libraries were constructed from 1.5–50 ng cfDNA (median 20.3 ng), using the AVENIO ctDNA Library Preparation Kit with the AVENIO Targeted and the AVENIO Surveillance Panel (both from Roche), which enrich a 17-gene (81 kb) and 197-gene (198 kb) target region, respectively, as described previously (16). Only genomic regions covered by both panels were considered for the data reported here, i.e., fusions in *ALK* intron 19, *RET* intron 11, *ROS1* introns 31–15; single nucleotide variants (SNV) in *ALK* exons 3–7/9/10/12/14/15/16/19–28, all exons of *TP53*, *KRAS*, *EGFR*, *MET*, *ERBB2*, *BRCA1/2*, selected exons of *BRAF* [3/8/10-18], *NRAS* [2–4], *APC* [2/3/5–7/9/10/12–16], *KIT* [2/3/5/8–20], *PDGFRA* [3–23], *RET* [2/5/7/9/10/12–19], *ROS1* [10/15/16/22/23/27/36–42], as well as copy number alterations in *MET*, *EGFR* and *ERBB2*. Equimolar amounts of 16 libraries were pooled and sequenced on an Illumina NextSeq550 using the High Output Kit V2 (300 cycles) according to the manufacturer's protocol (Illumina, San Diego, CA, USA). Automated raw data processing and data analysis was performed with the AVENIO ctDNA analysis software (Roche, version 2.0.0), which applies the bioinformatics pipeline from CAPP-Seq (26) with integrated digital error suppression (27). The variant allele frequency cut-off for a positive SNV call was 0.01%. Called variants were verified manually for sufficient coverage (i.e., ≥2,500 read counts) and visualized using the Integrative Genomics Viewer (IGV) (28) as published (16).

Statistical methods

Survival data were analyzed according to Kaplan-Meier and compared between patient groups with the logrank test. The relative effects of liquid biopsy results and other clinical or molecular parameters on patient survival were analyzed with multivariable testing using a Cox regression model that included the liquid biopsy result (positive or negative), type of *EML4-ALK* variant, baseline *TP53* status, baseline ECOG PS, the number of treatment lines before liquid biopsy, and whether the treatment was switched or continued beyond progression after the liquid biopsy. Since primary endpoint of our study was the relationship between ctDNA results and radiologic changes, samples from different progression time-points of the same patient were analyzed as independent, in order to account for the fact that several factors can influence liquid biopsy positivity differently in each sample of the same patient (e.g., progression site, progression rate, tumor volume, preceding treatment), and that these factors act similarly across different patients. Duration of follow-up was calculated using the reverse Kaplan-Meier method (29). Categorical data were

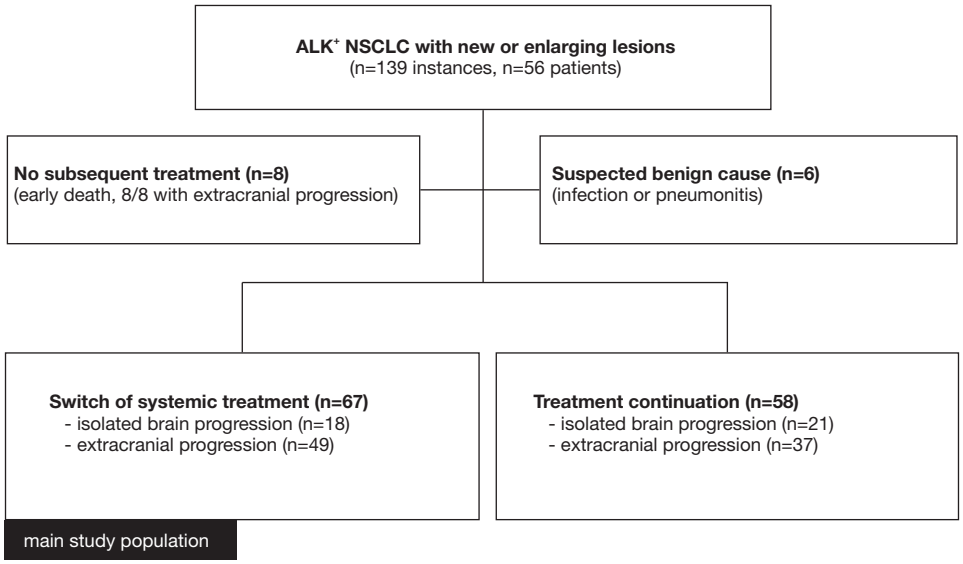

Figure 1 Flowchart of study patients.

compared with the chi-square, and numerical data with the Student's *t*-test. Confidence intervals for proportions were computed according to Clopper-Pearson (30). Statistical calculations were performed with SPSS version 24 (IBM Corp., Armonk, NY, USA) and plots generated with GraphPad Prism version 7 (GraphPad Software, La Jolla, CA, USA). All statistical tests were two-sided. P values <0.05 were considered statistically significant. The oncoprint plot (Figure S1) was generated using the cBioPortal (31,32).

Results

Cohort overview

Overall, in 56 TKI-treated ALK$^+$ NSCLC patients 139 instances of radiologic changes at the time of ctDNA profiling were identified, of which 133 corresponded to disease progression. The patient subsets and clinical characteristics are summarized in *Figure 1* and *Table 1*. The number of samples drawn at the time of radiologic changes varied per patient according to the clinical course, with a mean value of 2.46 (range, 1–7) for the entire cohort. An overview of all identified mutations alongside other patient characteristics is shown in Figures S1,S2 and Table S1. Mean coverage for ctDNA NGS was 14,730× and 4,385× pre- and post-deduplication, respectively. In 6/6 cases with a suspected benign cause for the recent radiologic changes, namely pneumonitis or lung infection based on the patients' history, clinical presentation and laboratory findings, the liquid biopsies were negative, and these were excluded from further analysis (*Figure 1*).

Liquid biopsy results according to the progression site

Among the remaining 133 cases, extracranial progression was noted in 71% (n=94), central nervous system (CNS) progression in 50% (n=67), and an isolated CNS progression in 29% of cases (n=39, *Figure 1*), with an overall percentage of liquid biopsy positivity at 50% (66/133, *Table 1*). The frequency of positive liquid biopsies was significantly higher with extracranial (62%) *vs.* isolated CNS progression (21%, P<0.001), and even higher with combined extra- and intracranial progression (89%, P<0.001, *Figure 2*). The infrequent cases with CNS-only progression and a positive liquid biopsy, tended to have a higher intracranial tumor load, i.e., multiple brain metastases including large lesions >1 cm in 8/8 patients (exemplary data shown in Figure S3). Apart from the higher intracranial tumor load, there was no other distinguishing radiologic characteristic of these cases, for example, radiologic evidence of meningeal carcinomatosis with isolated CNS progression was present in 2/8 cases with positive liquid biopsies *vs.* 2/31 cases with negative liquid biopsies. Baseline patient characteristics significantly associated with liquid biopsy positivity were presence of

Table 1 Characteristics and outcome of study patients

All instances of disease progression (N=133)	Liquid biopsy positive[1] (n=66)	Liquid biopsy negative (n=67)	P value
Age (median; IQR)	57; 14	56; 17	ns (0.44)
Sex, female, n [%]	37 [44]	33 [51]	ns (0.43)
Adenocarcinoma, n [%]	62 [94]	66 [99]	ns (0.16)
Never/light smokers, n [%][2]	55 [86]	50 [78]	ns (0.13)
ECOG PS at initial diagnosis, n [%][3]			
PS 0	37 [56]	49 [73]	0.0240
PS 1	29 [44]	16 [24]	
PS 2	0	2 [3]	
EML4-ALK fusion variant, n [%][3,4]			
"short" (mainly V3)	29 [54]	20 [35]	0.0484
"long" (mainly V1/V2)	25 [41]	37 [58]	
TP53 status at BL, n [%][5]			
Mutated	20 [33]	11 [19]	ns (0.068)
TP53 status at LB, n [%][5]			
Mutated	45 [69]	11 [19]	<0.001
No. of samples per patient, mean [SE]	2.5 [1.98]	2.7 [1.79]	ns (0.73)
Extracranial progression, next-line treated (n=86)[6]			
Treatment switch (n=49)	(n=32)	(n=17)	
Time from initial treatment to LB (months), median [SE]	19 [3]	16 [3]	ns (0.32)
Therapy lines from initial treatment until LB, mean [SE]	2.8 [0.3]	1.5 [0.2]	<0.001
Therapy lines after LB, median [SE]	2.2 [0.3]	1.6 [0.3]	ns (0.16)
Next-line PFS (months), median [SE]	3 [0.4]	13 [4]	0.003
OS from treatment start (months), median/mean [SE][7]	35/49 [9]	47/68 [10]	0.012
OS from LB (months), median/mean [SE]	13/16 [2]	n.r./35 [3]	<0.001
Follow-up from treatment start (months), median [SE]	35 [7]	41 [3]	ns (0.35)
Treatment beyond progression (n=37)	(n=18)	(n=19)	
Time from initial treatment to LB (months), median [SE]	27 [7]	20 [4]	ns (0.10)
Therapy lines from initial treatment until LB, median SE]	3.2 [0.4]	2.5 [0.2]	ns (0.13)
Therapy lines after LB, mean [SE]	2.2 [0.4]	2.1 [0.3]	ns (0.81)
Localization:intrathoracic only, n [%]	17 [94]	68% (13/19)	ns (0.11)
Time-to-next-treatment (months), median [SE]	3 [1]	8 [1]	0.004
OS from treatment start (months), median/mean [SE][7]	45/68 [10]	n.r./59 [6]	ns (0.44)
OS from LB (months), median/mean [SE]	19/20 [6]	27/27 [5]	ns (0.12)

Table 1 (*continued*)

Table 1 (*continued*)

All instances of disease progression (N=133)	Liquid biopsy positive[1] (n=66)	Liquid biopsy negative (n=67)	P value
CNS-only progression (n=39)	(n=8)	(n=31)	
Time from initial treatment to LB (months), [median] (SE)	27 [12]	22 [4]	ns (0.49)
OS from treatment start (months), median [mean] (SE)[7]	115/103 [16]	62/75 [6]	ns (0.19)
OS from LB (months), median [mean] (SE)	n.r./31 [5]	n.r./26 [3]	ns (0.55)

[1], "positive" liquid biopsy refers to the detection of any tumor single-nucleotide variant, gene fusion or copy number variation measurable with the AVENIO ctDNA Targeted kit (s. Methods). [2], smoking status available for 128/133 cases; light smoking refers to less than 10 pack-years. [3], r=0.42, P<0.0001 for the correlation between ECOG PS and presence of "short" *EML4-ALK* fusion variants. [4], *EML4-ALK* fusion typing available for 111/133 cases through RNA-NGS of tissue samples at initial diagnosis; "short" variants: V3 (E6:A20) in 44, and (E9:A20) in 5 typeable cases; "long" variants: V1 (E13:A20) in 34, V2 (E20:A20) in 24, (E17:A20) in 1, and (E18:A20) in 3 typeable cases. [5], *TP53* status at baseline by DNA-NGS of tissue samples collected at initial diagnosis was available for 119/133 cases; *TP53* status at LB was considered mutated if a *TP53* mutation had been detected either at baseline (by tissue analysis) or in the LB at progression; *TP53* status at LB was considered "wild-type" if a wild-type status had been determined at baseline (by tissue analysis) without subsequent detection of a *TP53* mutation in the LB. [6], extracranial progression in the lungs, pleura, liver, bone, adrenal glands, or other organs. [7], OS from the start of palliative systemic treatment for metastatic disease; treatment details shown in Figure S1. IQR, interquartile range; SD, standard deviation; SE, standard error of the mean (SE); PS, performance status; CNS, central nervous system; BL, baseline; LB, liquid biopsy; n.r., not reached.

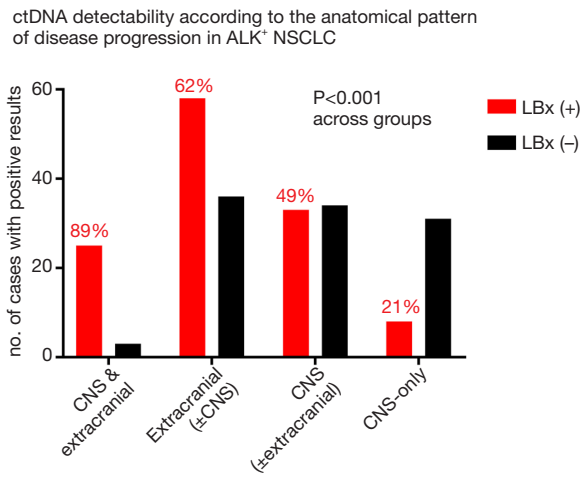

Figure 2 Liquid biopsy positivity according to the anatomical pattern of disease progression in ALK+ NSCLC. Liquid biopsies were positive in 8/39 (21%, 95% CI: 9–36%) instances of CNS-only progression, 33/67 (49%, 95% CI: 37–62%) instances of CNS (±extracranial) progression, 58/94 (62%, 95% CI: 51–72%) instances of extracranial (±CNS) progression, and 25/28 (89%, 95% CI: 72–98%) instances of concomitant extracranial and CNS progression.

"short" echinoderm microtubule-associated protein-like 4 (*EML4*)-*ALK* fusion variants (mainly V3, in 54% *vs.* 35% of liquid biopsy-positive *vs.* negative cases, P=0.0484, *Table 1*) as detected by tissue RNA-NGS at initial diagnosis, and a worse ECOG PS at initial diagnosis (PS 0–1 in 56–44% *vs.* 73–24% of subsequently liquid biopsy-positive *vs.* negative cases, P=0.024, *Table 1*). The initial ECOG PS and presence of "short" *EML4-ALK* variants were positively correlated (r=0.42, P<0.001). In addition, presence of *TP53* mutations at initial diagnosis as detected by tissue DNA-NGS, showed a trend for association with subsequent liquid biopsy positivity (baseline *TP53* mutations in 33% *vs.* 19% of subsequently liquid biopsy-positive *vs.* negative cases, P=0.068, *Table 1*). The correlation between mutated *TP53* status and liquid biopsy positivity became highly significant at the time of disease progression, when the *TP53* results of liquid biopsies were also considered (69% *vs.* 19%, P<0.001, *Table 1*). Among patients with *TP53* mutations detectable in tissue samples at baseline (n=13), some displayed *TP53* mutations in subsequent ctDNA samples (n=6) and some not (n=7), while other patients tested *TP53* wild-type at diagnosis and showed newly detectable *TP53* mutations at progression (n=9, Figure S2).

Outcome of progressive patients according to liquid biopsy results

Next, we analyzed patient outcomes in conjunction with liquid biopsy results. OS for progressive patients was shorter in case of positive *vs.* negative liquid biopsies both

from the time-point of liquid biopsy collection (13 *vs.* 28 months in median, P<0.001, *Figure 3A*), and from the start of palliative systemic treatment (58 *vs.* 67 months, P=0.005, *Figure 3B*). Subgroup analysis according to the site of disease progression, showed that this association between liquid biopsy results and OS pertained to cases with extracranial, but not with CNS-only progression (*Figure 3C,D* and *Table 1*).

Within the subset of cases with extracranial disease progression and immediate switch to a different systemic treatment, the PFS of subsequent treatment line (3 *vs.* 13 months in median, P=0.003, *Table 1* and *Figure 3E*), the OS from the time-point of liquid biopsy collection (13 months *vs.* not reached, P<0.001, *Table 1*), and the OS from start of palliative systemic treatment (35 *vs.* 47 months in median, P=0.012, *Table 1*) were shorter in case of positive compared to negative liquid biopsies. Similarly, within the subset with extracranial oligoprogression and continuation of the same systemic treatment, liquid biopsy positivity was associated with significantly shorter TNT (3 *vs.* 8 months, P=0.004, *Figure 3F* and *Table 1*). Of note, although liquid biopsies were drawn at comparable time-points after start of palliative systemic treatment in positive *vs.* negative cases (on average 19 *vs.* 16 months, P=0.32, *Table 1* and Figure S1), cases with positive liquid biopsy results had already gone through significantly more therapy lines (mean 2.8 *vs.* 1.5, P<0.001, *Table 1*) and died earlier than cases with liquid biopsy negative results, despite the heavier treatment of the former. In multivariable analysis, liquid biopsy positivity in cases with extracranial progression was associated with shorter OS from the start of palliative systemic treatment, independent of other established predictors of worse outcome, i.e., presence of "short" *ALK* fusion variants, presence of *TP53* mutations at diagnosis, initial ECOG PS, number of previous treatment lines, and treatment continuation or switch after the liquid biopsy (*Table 2*). The phenotype of liquid biopsy positivity at progression of ALK+ NSCLC according to the results of this study is summarized in *Figure 4*.

The alterations detected by liquid biopsies were sensitive to routinely available drugs in only 26% (17/66) of positive cases (Table S1). Detailed analysis of individual clinical courses showed that the great majority of cases (15/17 or 88%) had subsequently received ALK TKI with activity against the potentially druggable *ALK* mutations that were detected (Table S1).

Discussion

Main finding of the present study is the worse outcome of ALK+ NSCLC patients with positive liquid biopsies at the time of disease progression (*Figure 4*). At first, this might appear counterintuitive, because liquid rebiopsies are mainly employed to detect actionable genetic alterations and facilitate use of effective next-line targeted therapies (12). However, our results suggest that in most cases ctDNA detectability does do not go hand-in-hand with better therapeutic options. Even with the relatively narrow profiling focused on mutations of oncogenic drivers employed in this study, therapeutic opportunities emerged for less than half of liquid biopsy-positive cases, while genetic changes not sensitive to currently available therapies were more frequent (Figure S1 and Table S1). Moreover, ctDNA detectability correlated with presence of the high-risk molecular features "short" *EML4-ALK* fusion variant (mainly variant 3) and *TP53* mutations, which were independently associated with worse patient outcome in several retrospective analyses and the randomized phase 3 trial ALTA-1L (33-38). The association between presence of *EML4-ALK* V3 and detectability of *ALK* resistance mutations has also been noted by other investigators (39) and stands in apparent contradiction to the adverse phenotype of V3-positive patients (40). This dissonance is reconciled by the results of the present study, which show that treatable *ALK* resistance mutations constitute a minor only fraction of acquired genetic alterations associated by *EML4-ALK* V3 (Figure S1). Frequent are also coexistence of multiple *ALK* mutations, which are usually associated with resistance to currently available TKI (41), and newly acquired *TP53* mutations at the time of disease progression, which impair prognosis to an extent comparable to that of *TP53* mutations present already at baseline (42). In addition, copy number alterations captured across the genome by shallow whole genome sequencing also accumulate faster in patients with *EML4-ALK* V3 and/or *TP53* mutations, and are similarly associated with worse outcome (16). Nevertheless, it should be noted that the adverse prognostic impact of positive liquid rebiopsies goes beyond that of classical molecular risk factors in ALK+ NSCLC: in multivariable analysis, liquid biopsy positivity was associated with shorter overall survival independently of the *EML4-ALK* variant and the baseline *TP53* status. Moreover, this adverse implication of positive liquid

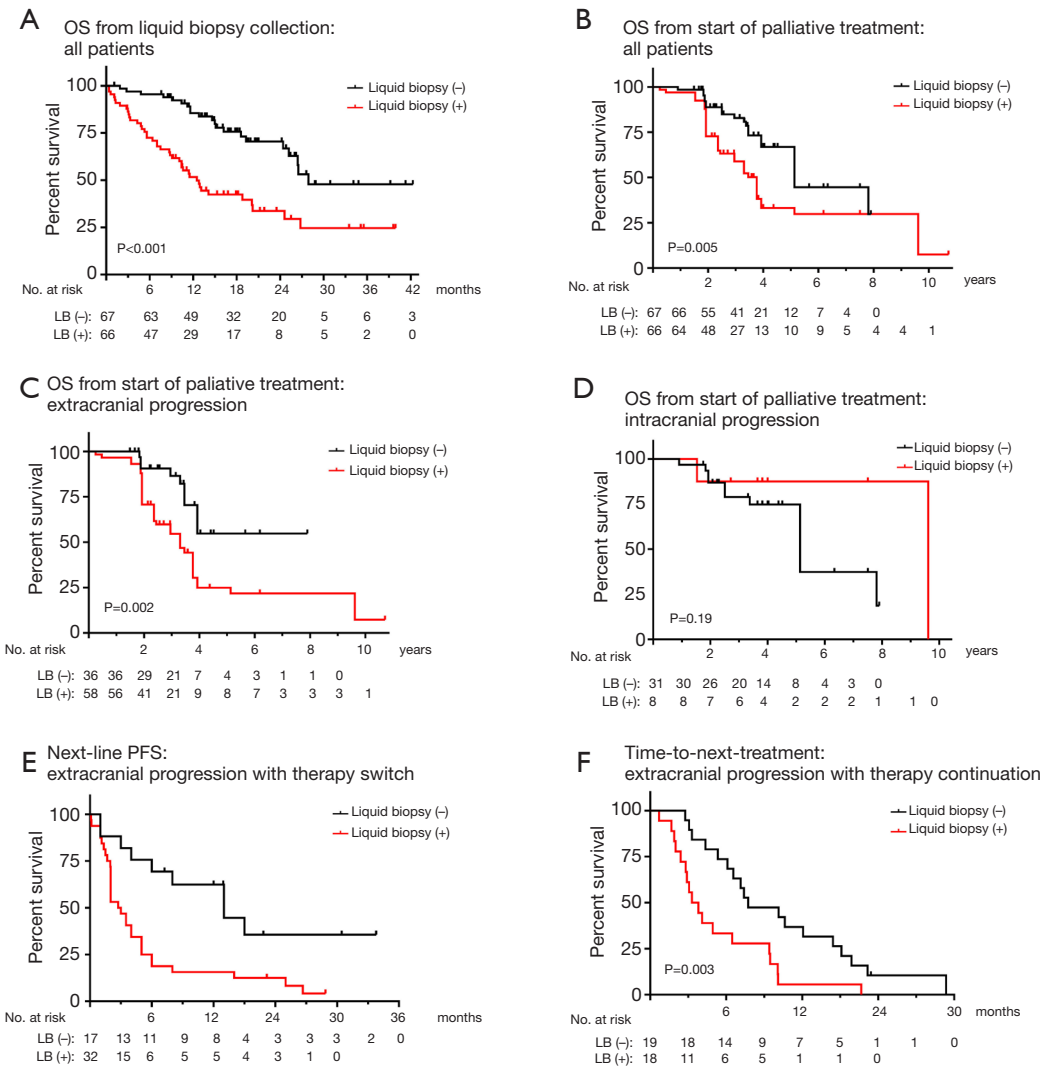

Figure 3 Patient survival according to the results of liquid biopsies at disease progression. (A) The median overall survival (OS) from the time-point of liquid biopsy collection was 13 months (95% CI: 9.6–15.4 months) in case of positive *vs.* 28 months (95% CI: 25–29 months) in case of negative liquid biopsies (logrank P<0.001). (B) The median OS from start of palliative systemic treatment for stage IV disease was 45 months (95% CI: 39–52 months) in case of positive *vs.* 62 months (95% CI: 48–75 months) in case of negative liquid biopsies (logrank P=0.005). (C) The median OS from start of palliative systemic treatment with extracranial progression was 40 months (95% CI: 29–51 months; mean 52 months, 95% CI: 41–63 months) in case of positive *vs.* not-reached (mean 69 months, 95% CI: 56–83 months) in case of negative liquid biopsies (logrank P=0.002). (D) The median overall survival (OS) from start of palliative systemic treatment with intracranial only progression was 115 months (95% CI: not available) in case of positive *vs.* 62 months (95% CI: 46–77 months) in case of negative liquid biopsies (logrank P=0.19). (E) The median progression-free survival (PFS) for next-line treatment with immediate switched to another systemic treatment was 13 months (95% CI: 5–21 months) in case of negative *vs.* 3 months (95% CI: 2–4 months) in case of positive liquid biopsies (logrank P=0.003). (F) The median time-to-next-treatment (TNT) with continuation of the same systemic treatment beyond disease progression was 8 months (95% CI: 6–9 months) in case of negative *vs.* 3 months (95% CI: 2–5 months) in case of positive liquid biopsies (logrank P=0.004).

rebiopsies was also independent of an impairment in the initial ECOG PS (*Table 2*), which is a recognized feature of the adverse V3 phenotype likely caused by more extensive metastatic spread already at diagnosis (43,44). This worse outcome of patients with positive liquid rebiopsies fits nicely with the adverse prognostic implications of ctDNA

Table 2 Relationship of overall survival with liquid biopsy positivity and other patient characteristics

Characteristics	OS from start of palliative systemic treatment	
	HR; P value	95% CI
LB positivity	2.70; 0.048	1.01–7.23
"short" *EML4-ALK* variant (mainly V3)	3.52; 0.011	1.33–9.32
TP53 mutation at initial diagnosis	2.92; 0.007	1.34–6.33
ECOG PS at treatment start	1.77; 0.25	0.67–4.68
Treatment lines before LB	0.88; 0.26	0.70–1.10
Treatment switch *vs.* continuation after LB	0.63; 0.11	0.35–1.18

PS, performance status; HR, hazard ratio; 95% CI, 95% confidence interval. The relationship of overall survival (OS) from the start of palliative systemic treatment with liquid biopsy (LB) positivity and other patient characteristics was analyzed in cases with extracranial progression with a multivariable Cox regression (n=79 cases with values available for all parameters).

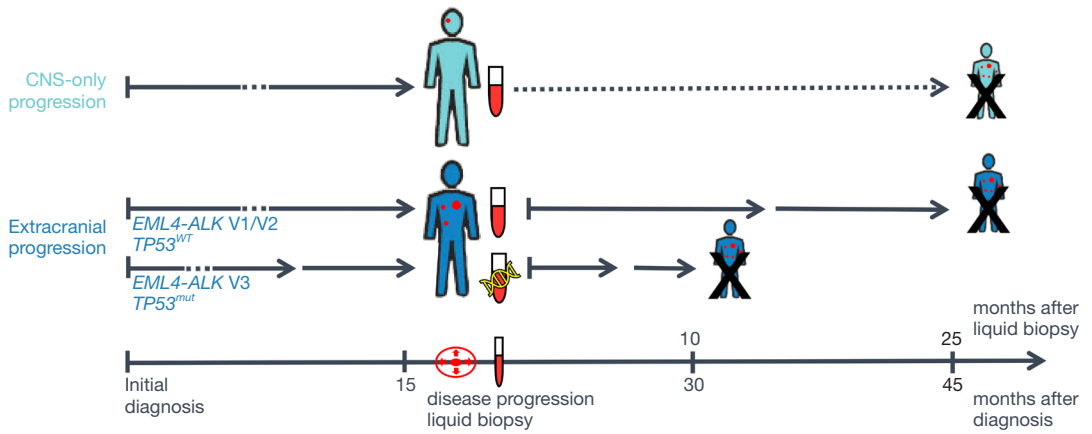

Figure 4 The phenotype of ALK+ NSCLC patients with positive liquid biopsies at disease progression. Baseline characteristics, clinical course, and outcome of ALK+ NSCLC patients with positive and negative liquid rebiopsies according to the findings of this study. Values on the time axis are based on the results shown in *Table 1*.

detectability already noted for oncogene-driven NSCLC at initial diagnosis (45-48), and under ongoing TKI treatment (48-50).

The partial only concordance between baseline tissue-based and subsequent ctDNA-based *TP53* results (Figure S2) is an additional argument for a combined tissue/liquid biopsy approach to molecular profiling in ALK+ NSCLC, as considerable spatial and temporal heterogeneity has been described for *TP53* and other mutations in lung cancer (51,52), while at a purely technical level liquid biopsies can both capture some tissue-NGS negative cases, as well as miss some positive ones (53).

Another important characteristic of liquid biopsy-positive patients in our study was faster progression through treatment lines (*Figure 4*), regardless of whether ongoing systemic treatment was switched immediately (*Figure 3E*) or continued beyond oligoprogression (*Figure 3F*). Conversely, most patients with anatomically restricted progression and

negative liquid biopsies appear to have a more indolent course. If this finding is confirmed by other studies, it could prove very useful for the stratification of oligoprogressive ALK+ NSCLC patients in clinical practice: for negative cases, continuation of current TKI treatment with or without local therapies could be a reasonable option, and was associated with a TNT >6 months in the majority of our patients, even without ablative measures in several cases (*Table 1* and Figure S1). On the other hand, for positive cases, the liquid biopsy results not only identify patients with more aggressive disease and need for earlier change of systemic therapy (*Figure 3F*), but may also guide selection of the most suitable next-line compound based on the exact alteration detected.

For patients with isolated CNS progression, ctDNA detectability was low in our study, approximately one-third of that with extracranial progression (21% *vs.* 62%, *Figure 2*). These results together with similar findings of other investigators across various oncogene-driven NSCLC (54), collectively show that the intracranial compartment is largely inaccessible for blood-based liquid biopsies. Consequently, the clinical utility of plasma ctDNA assays in the setting of CNS-only progression is limited, for example their results were not associated with survival in our study (*Figure 3D*). Analysis of cerebrospinal fluid is preferrable to that of blood for molecular profiling of primary and metastatic CNS tumors, as other investigators have noted (55-58). Notwithstanding, our results show that blood-based liquid biopsies can be positive in some cases with extensive brain involvement, including multiple brain metastases and large (>1 cm) lesions (Figure S3). As brain metastases are known to disrupt the blood brain barrier, cases with more and larger intracranial lesions can be reasonably assumed to have a more disrupted blood brain barrier, which could facilitate export of ctDNA in the circulation.

Main limitation of our work is its retrospective character, therefore its results warrant confirmation in a larger, prospective study. At the same time, the findings appeared to be consistent in various analyses, for example, the worse prognosis of liquid biopsy-positive patients was evident regardless of whether treatment was switched or continued beyond progression. Also, we tried to control potential confounders, for example potentially relevant parameters were systematically collected for all patients (Figure S1), compared for cases with liquid biopsy-positive and negative results (*Table 1*), and included in multivariable testing (*Table 2*). Overall, the findings of the present study argue for ctDNA detectability at progression as a feature of biologically more aggressive disease, which is in line with observations in the treatment-naive setting (45,47). However, the exact values of PFS, TNT and OS observed in our cohort, although typical for an ALK+ NSCLC cohort, are not directly generalizable, and will certainly also be affected by the availability of more effective targeted therapies, like upfront lorlatinib (59), in the future. Also, the favorable outcome of oligoprogressive patients with negative liquid biopsies warrants further investigation in a larger cohort.

In summary, positivity of blood-based liquid rebiopsies in ALK+ NSCLC appears to characterize extracranial progression and biologically more aggressive disease. These results reconcile the increased detection of *ALK* resistance mutations with other features of the high-risk clinical phenotype associated with *EML4-ALK* V3 (22,39), especially in case of concomitant *TP53* comutations (33). Conversely, most oligoprogressive ALK+ NSCLC patients with negative liquid biopsies appear to have a more indolent course without need for immediate change of systemic treatment.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of the Heidelberg University (S-296/2016), and written informed consent was obtained from all participants.

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