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Review Comments (Round 1)

Reviewer A

Comment 1:
Authors present a prospective study assessing the role of quantitative monitoring by droplet digital PCR for plasma (ctDNA) ALK resistance mutations in patients being treated for NSCLC.Their results show multiple ALK mutations detected in patients, which develop and disappear during the course of treatment before disease progression. While no statistical analysis was performed, authors suggest a correlation between time of mutation detected and progression free survival.

General comments:
Authors present an interesting study that demonstrates the feasibility of plasma ctDNA monitoring for ALK resistant mutations in NSCLC. While there are serious limitations to this study including small sample size and lack of statistical analysis (which authors acknowledge), and insufficient analysis and discussion of findings, this observational study's most interesting finding is the unique dynamic profile of ALK mutations that appear and disappear before tumour progression. This reason for this is unexplained.However, the question in my mind is could it possibly demonstrate that ALK resistance may not follow the prototypic pattern of resistant mutations in other disease types e.g. EGFR? Unfortunately authors do not devote sufficient attention and discussion in addressing this. The correlation of time at which mutation was detected to PFS is interesting. Authors need to address whether that level of correlation is sufficient for clinical use and possibly suggest ways of improving it for clinical utility.

Reply 1:
Thank you for your comments and suggestion.

The EGFR_T790M mutation, which occurs during treatment with EGFR inhibitors in EGFR-mutated lung cancer, is associated with disease progression and is an indicator of resistance mutations. EGFR is dimeric or monomeric in the case of the EGFR_exon19 deletion type. However, the development of resistance mutations is associated with clinical drug resistance.

However, the pattern of occurrence of resistance mutations in ALK lung cancer is different. The most frequent fusion partner of ALK is EML4, which forms trimers [1]. This difference is important because resistance will not occur if the other two molecules are not mutated even if one molecule is mutated, at least theoretically. In this study, EML4-ALK (variant 1) was confirmed in case 1; however, in the other cases, the fusion partner and variant were unknown. Although this hypothesis has not been confirmed,

we believe that ALK multimer formation is one of the reasons why PFS does not correlate with chronology when the mutation is detected.

We would state that further studies with large sample sizes are required to confirm the observed correlation between the time at which the mutation was detected and progression-free survival. We have modified the last part of the Discussion section of our manuscript accordingly (see below).

Changes in the text:
The following text has been added to the Discussion section of the manuscript:
“Epidermal growth factor receptor (EGFR)_T790M mutations, which occur during treatment with EGFR inhibitors in EGFR-mutated lung cancer, are associated with disease progression as indicators of resistance mutations. EGFR is dimeric or monomeric in the case of the EGFR_exon19 deletion type. However, the development of resistance mutations is associated with clinical drug resistance.” (See Page 12, lines 240–244).

“Long-term studies with large sample sizes and accompanying statistical analyses are warranted to confirm the findings of the present study and apply the observed correlation between the time at which the mutation was detected and PFS in the clinic.” (See Page 13, lines 269–272).

Comment 2:
Comments:
Introduction:
1.Line 3 - The statement below needs modification as ALK gene rearrangement is only one of the underlying mutations in the development of NSCLC.

Anaplastic lymphoma kinase (ALK) gene rearrangement is
the underlying mutation in the development of NSCLC

Reply 2:
We have revised the text accordingly.

Changes in the text:
“Anaplastic lymphoma kinase (ALK) gene rearrangement is one of the underlying mutations associated with NSCLC development (1-3) and has been identified in 3%–8% of NSCLC cases (4).” (See Page 4, lines 59–61).

Comment 3:
2. Ensure attributions/citations are properly given to all statements made in the manuscript. E.g. in Second paragraph of introduction: i.different ALK inhibitors give different sensitivities....no citation given

ii. strobe reporting checklist....

Reply 3:
The manuscript has been revised to include the appropriate citations. We have also provided the Strobe Reporting Checklist as a separate document at the time of submission.

Changes in the text:
“Repeated biopsies during disease progression are crucial to elucidate the molecular mechanisms underlying the development of resistance to ALK inhibitors (1-2, 10). Different ALK mutations show different sensitivities to each TKI *in vitro*, and none of the TKIs share the same spectrum of activity against ALK mutants (1).” (See Page 4, lines 72–75).

“We present the following article in accordance with the STROBE reporting checklist (11).” (See Page 5, line 95)

Comment 4:
3.Study design: State prior duration of crizotinib therapy for patients retrospectively enrolled

Reply 4: The prior duration of crizotinib treatment in the patients enrolled retrospectively is shown in Figure 1.
No. 1 877 days, No. 2 498 days, No. 3 291 days, No. 4 460 days, No. 5 1,678 days, and No. 6 550 days).

Changes in the text:
“Six patients were already being treated with the ALK TKI alectinib or crizotinib (median duration of crizotinib treatment prior to study enrolment in the retrospectively enrolled patients: 524 days [range, 291–1678]; Figure 1).” (See Page 6, lines 102–104)

Comment 5:
4.Patient characteristics requires more details e..g. table 1 should include specific histopathologic diagnosis, tumour grade, disease stage and where possible, time to progression (1st, 2nd, third), time to death etc

Reply 5:
We have addressed the details of patient characteristics by adding the histologic type and clinical stage details in Table 1. Time to disease progression is shown in Figure 1. Seven of the nine patients survived, and data on survival are not presented accordingly.

Changes in the text:

Table 1. Patient characteristics

Characteristics	Patients (%)
	n = 9
Sex	
Male	5 (56)
Female	4 (44)
Age at baseline, years	
Median	53
Range	37–80
Smoking history	
Current	1 (11)
Former	2 (22)
Never	6 (67)
Histology	
Adenocarcinoma	9 (100)
others	0 (0)
Clinical Stage	
IV	9 (100)
others	0 (0)
No. of treatments[a]	
1	1 (11)
2	4 (44)
3	1 (11)
≥4	3 (33)
No. of ALK TKI treatments	
1	3 (33)
2	3 (33)
3	2 (22)
4	1 (11)
No. of cytotoxic chemotherapy[b] treatments	
0	5 (56)
1	3 (33)
2	1 (11)

Data are presented as n (%) unless otherwise stated.

ALK, anaplastic lymphoma kinase; TKI, tyrosine kinase inhibitor

[a]Treatments include ALK TKI, chemotherapy, and immune checkpoint inhibitors

[b]Cytotoxic chemotherapy: pemetrexed +/-, cisplatin or carboplatin +/-, bevacizumab

+/-, and/or pembrolizumab +/-

Comment 6:
5. More details on clinical monitoring for disease progression is required in the text.Authors do note that the RECIST criteria was used. A concise patient specific summary should be given and reference can then be made to the RECIST criteria for more details.

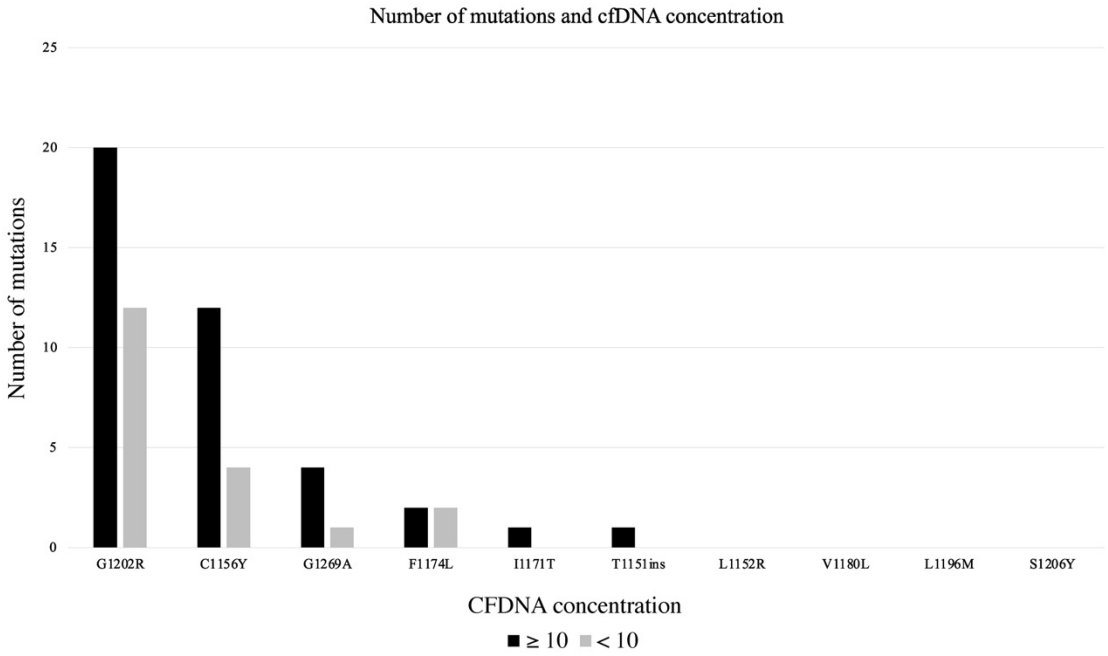
Reply 6:
For the retrospective cohort, we evaluated patients with CT imaging every 3 months in line with routine clinical practice. However, a fixed protocol for scheduling brain MRI scans is unavailable because this procedure is performed according to changes in symptoms, tumor markers, and other factors.

Changes in the text:
The text has been revised as follows:

“The treatment response of each patient was evaluated through CT imaging approximately every 3 months, and additional CT scans were performed when the symptoms worsened. The standard Response Evaluation Criteria in Solid Tumors (version 1.0) was used based on the CT and other imaging findings.” (See Page 6, lines 119–122).

Comment 7:
Results:
Are there any discernible patterns between ctDNA variables e.g.ctDNA concentration, quantitative level of ALK mutations, the number of mutations detected. This should be stated even if negative.

Reply 7: Thank you for the comment. We created a new Supplementary Figure 3 that addresses this comment as follows:



There was no obvious relationship between the number of mutations and cfDNA concentration, and the sample size was too small to perform statistical analyses. This information has also been incorporated into the manuscript (see below).

Changes in the text:
"We also explored the possibility of a relationship between mutation number and cfDNA concentrations (Supplementary Figure 3). However, no apparent relationship was found, and the sample size was too small to perform statistical analyses." (See Page 10, lines 119–201)

Comment 8:
Conclusions:
A more detailed discussion of resistance mechanisms is warranted e.g. any explanation for why do resistance mutations appear and disappear; has this profile been previously noted in the literature? What is its implication for resistance monitoring in ALK+ NSCLC, how does this fit with the standard understanding of the role of drug resistant mutations in other mutations in NSCLC e.g. EGFR. Though no significant positive correlative finding was demonstrated, authors need to elucidate their reasoning by specifically address clinical correlates of their findings with the disease course in the discussion e.g. the role of ctDNA concentration, quantitative level of ALK mutations, the number of mutations detected. Was any discernible pattern detected?

Reply 8:
The appearance and disappearance of resistance mutations may be because EML4-ALK forms a trimer; therefore, genetic mutations inserted into one DNA allele are not fully resistant, and mutation-inserting cells may be killed by ALK-TKI.

Several profiles characterize the appearance and disappearance of mutations, but in our study, all of them have been reported multiple times during treatment or after disease progression. Thus, we believe that our study is the first to investigate the appearance and disappearance of gene mutations during the course of the disease.

When considering the options for drug modification in the context of resistance mutations, we believe that the selection of ALK-TKIs should be based not only on the type of mutation but also on the increase in the concentration of the mutation frequency over time and the clinical progression of the disease.

Resistance mutations were detected in samples with ctDNA levels exceeding 10 ng/mL. Digital PCR was used for absolute quantification. However, even in case no. 2, in which G1202R and G1269A were detected simultaneously, neither was detected in subsequent samples. Whether these two mutations were cis- or trans-splicing events could not be detected by digital PCR and were inconsistent with clinical efficacy. This was considered a limitation of this study.

A brief overview of the resistance mechanisms is now provided in the Discussion section, and we have addressed the issue of the correlation between cfDNA concentration and mutation number in response to comment 7.

Changes in the text:
“Other bypass mechanisms are also associated with TKI resistance. We did not study ALK amplification of the ALK kinase domain or activation of the bypass signaling pathway in this study as it was outside the scope of this study. Mechanisms of TKI resistance can be categorized into those associated with alterations that prevent the inhibition of the target receptor TK by TKIs, changes in tumor cell lineage, and activation of bypass +/- downstream signaling pathways that promote cell survival and proliferation (21)” (See Pages 12–13, lines 261–264).

Reference:
21. Cooper AJ, Sequist LV, Lin JJ. Third-generation EGFR and ALK inhibitors: mechanisms of resistance and management [published correction appears in Nat Rev Clin Oncol. 2022 Nov;19(11):744]. Nat Rev Clin Oncol. 2022;19:499-514.

Reviewer B
Comment 9:
1. A discussion about the assays potential benefits over currently available ctDNA NGS assays would strengthen the manuscript. Also, about half of patients with ALK TKI resistance have off targeted mechanisms of resistance and my understanding is that this assays can only detect ALK resistance mutations

Reply 9:
The digital PCR method used in this study was quantitative and more sensitive than NGS. As you have highlighted, digital PCR cannot detect ALK fusion partners nor can it determine whether the event was a cis- or trans-splicing event, even when two resistance mutations are detected at the same time. This is a limitation of the research assay method, and the Discussion section of the manuscript has been revised accordingly to include this aspect.

Changes in the text:
“In addition, although digital PCR is more sensitive than next-generation sequencing, it is limited by the fact that it cannot detect ALK fusion partners or cis- or trans-splicing occurred, even when two resistance patterns are detected simultaneously.” (See Page 13, lines 272–274).

Comment 10:
2. It would be helpful to have some additional clinical details about the patients treated on study. For the patients on treatment it would be helpful to know how long they had been on current treatment and the radiographic disease status since these factors may influence the rate of mutations and the amount of ctDNA. I would suggest including the treatment sequence (crizotinib alone, crizotinib followed by next generation ALK TKI, first-line next generation ALK TKI, etc) since this may impact the rate and type of mutations

Reply 10:
Additional clinical details regarding the patients are provided in the revised Table 1. Details regarding the treatment duration are presented in Figure 1. X-ray and CT imaging findings at the time of the appearance of the resistance mutation are not shown because a complete response was achieved, no primary lung cancer was observed, or no change was observed.

Comment 11:
3. In the retrospective cohort, were the sample collected a pre-specified time points or routine clinical care samples? Were any samples excluded due to quantity not sufficient or technical issues?

Reply 11:
This retrospective cohort study was not preplanned, and data from the cohort relied on extra samples collected at each visit during routine clinical care. No samples were arbitrarily excluded due to technical problems.

Changes in the text:
“No samples were excluded due to technical issues or insufficient quantity.” (See Page 9, line 179–180).

Comment 12:
4. One clinical question is the time between the time resistance mutation was first detect and radiographic or clinical progression. It would be helpful to have the interval in months from this case series (if available)

Reply 12:
In response to your previous comment, the X-ray and CT images at the time of appearance of the resistant mutation showed a complete response, no primary lung cancer, or no change.

The main finding of this study was that the time from initial mutation detection to disease progression could not be accurately estimated.

Comment 13:
5. It would be helpful if the future development plans for this assay were discussed

Reply 13:
The Bio-Rad digital PCR assay is not yet available for routine clinical use. Recent advances in next-generation sequencing technology have made it possible to obtain a sensitivity equivalent to that of the digital PCR method. We believe that liquid biopsy using next-generation sequencing will become the mainstream method in the future. However, we believe that the detection of resistance mutations may not correlate with clinical disease progression in ALK lung cancer, as shown in this study, which provides useful information for the clinical community.

Comment 14:
6. Did the cfDNA concentration and the sensitivity of the assay vary with disease status?

Reply 14:
All patients in this study had stage IV disease. Tumor volume and cfDNA concentration may be related, but data are insufficient to identify the presence of a correlation because the tumors may present with microscopic lesions. Previous reports have shown an association between the clinical stage and cfDNA or ctDNA concentrations; therefore, we can assume that sensitivity varies depending on the disease state.

Reviewer C
Comment 15:
Sasaki et al., longitudinally profiled ALK mutations in the plasma DNA of nine ALK+

NSCLC patients using established ddPCR assays. The authors detected known ALK resistance mutations such as G1202R, C1156Y, G1269A, F1174L, T1151ins, and L1171T. They also showed that shorter time to detectability of ALK mutations in patients on alectinib therapy is associated with shorter progression-free survival. Altogether, the manuscript is well written, but presented a small patient cohort and the findings were not particularly novel as others (PMID: 29376144, 31358542, 33161228, 34876698) have already performed longitudinal molecular profiling of ALK+ NSCLC in plasma DNA. On the other hand, there is benefit to publishing the manuscript due to the use of ddPCR specifically for detection of ALK resistant mutations. Prior to acceptance, some points have to be addressed/clarified by the authors as outlined below.
Methods
1. In p. 4, line 160: the template volume of 8.8 µL is specified. What is the amount of cfDNA in this volume? After cfDNA isolation, were the samples diluted to the same concentration so that the same amount of template DNA is used in the subsequent reaction?

Reply 15:
The amount of cfDNA contained in 8.8 µL of template DNA was 35.2–315 pg/µL. The amount of template DNA also varied from 0.7–6.3 ng/well because the digital PCR could not contain the same concentration of template DNA due to the volume of the PCR reaction solution.
If less elution buffer was used when extracting DNA with the QIAamp® Circulating Nucleic Acid Kit, all the template DNA amounts could have been matched, but in this study, extraction was performed with 150 µL of elution buffer per the pilot study conducted in EGFR lung cancer.

Comment 16:
2. Did the authors use technical replicates during ddPCR? Please specify the number of wells used for each sample during the ddPCR run. If indeed the authors used only one reaction, please provide a justification for this study design and acknowledge the limitations such a strategy introduces in data interpretation. Working with ctDNA assays is difficult enough due sensitivity issues so I am concerned with the lack of technical replicates, particularly in cases where copies/µL values are low. In cases where samples are limited, the possibility of using multiplexed probes is recommended in future studies.

Reply 16:
In this study, one well was used for each sample. The original plan was to aliquot the cfDNA and use the other half for amplicon next-generation sequencing targeting the exons of ALK. This study aims to verify the correlation between these two methods. However, because we were unable to fully analyze samples with low DNA concentrations during amplicon preparation at the time of next-generation sequencing, only the digital PCR aspects are shown in this study. If there were residual specimens,

we believe it is desirable to study each specimen in the two wells. As you have suggested, we will consider the use of multiplex probes in future studies.

Comment 17:
3. In p. 5, line 175: is the cutoff value 0.06 copies/L correct? The dynamic range of ddPCR is >0.25 copies/μL, according to the Bio-Rad's technical bulletin.

Reply 17:
The cutoff value was 0.25; 0.06 copies/μL was the lowest value detected.

The ddPCR conditions were as follows: Fragmented normal genomic DNA and artificial genetic DNA were used to set the threshold values. For each probe, three reactions were performed using 2000 copies of fragmented normal genomic DNA, three reactions were performed using a mixture of 2000 copies of fragmented normal genomic DNA and 100 copies of each artificial gene DNA, the target of the probe detection, three for each mutation, and one negative control reaction. The reaction was performed three times for each mutation and once for the negative control.

After measuring the fluorescence of the droplets, the 2D amplitude was selected using QuantaSoft™ Software to check the distribution of negative and positive droplets in channels 1 and 2. The cutoff value was set at the upper limit of the 95% confidence interval (TotalConfMax) of the copy number (copies/μL) measured using 2000 copies of fragmented normal genomic DNA. If the cutoff value was less than 0.25 (copies/μL), 0.25 (copies/μL) was set as the cutoff value in accordance with Bio-Rad's instruction manual.

Comment 18:
4. Supplementary Table 2: the volume unit should be in μL instead of mL

Reply 18:
As you pointed out, it was μL, not mL. We have corrected this accordingly.

Comment 19:
Results
1. The x-axis in figure 1 needs a label, is day 0 the day of diagnosis or the start of therapy? Should the x-axis label be "Days from diagnosis" or "Duration of therapy"? Day 0 does not necessarily mean therapy baseline, correct?

Reply 19:
The x-axis represents treatment duration. The figure legends have been modified accordingly.

Changes in the text:
“The x-axis represents the duration of treatment in days.” (See Page 22, line 6)

Comment 20:
2. Figure 2 gives a summary of the detected mutations longitudinally. I wonder if there would be benefit in illustrating this as a patient kinetics plot as was done previously (PMID: 29376144, 33161228). In this way we could see the quantitative changes in the abundance of mutation per patient, and potentially see when precisely the emergence of resistance mutations occurs. It would also be possible see the increase in mutation abundance at disease progression and conversely, the reduction at therapy response. It would also be more informative to show the specific abundance of each mutation rather than color-code it.

Reply 20:
We also refer to PMIDs 29376144 and 33161228 for references. In this study, we did not quantify wild types such as EML4-ALK and determined that it was not possible to illustrate the variant frequency. As you pointed out, this is a measurement system with low concentrations of cfDNA. Thus, we considered the important point to be “whether it was measurable by this research assay.”

Comment 21:
3. Is it known whether metastatic disease has spread in cases where cfDNA amounts are high? The dissemination of metastasis could explain the increase in cfDNA load.

Reply 21:
All the patients in this study had stage IV disease. However, for example, in case No. 1, the specimen was collected nine times when they almost achieved complete remission. As shown in Figure 2, the cfDNA concentration decreased slightly during this period. As previously reported, tumor volume and cfDNA concentration may be related, but data were insufficient to find a correlation because the tumors may present with microscopic lesions.

Comment 22:
4. Was the correlation used in Figure 3 Pearson or Spearman? I also do not think it is necessary to display the r2 value (an indicator of linearity) when the correlation is already indicated.

Reply 22:
Thank you for pointing this out. Spearman’s correlation coefficient was used. We have addressed this issue in the revised Figure 3 legend. In addition, we removed the r2

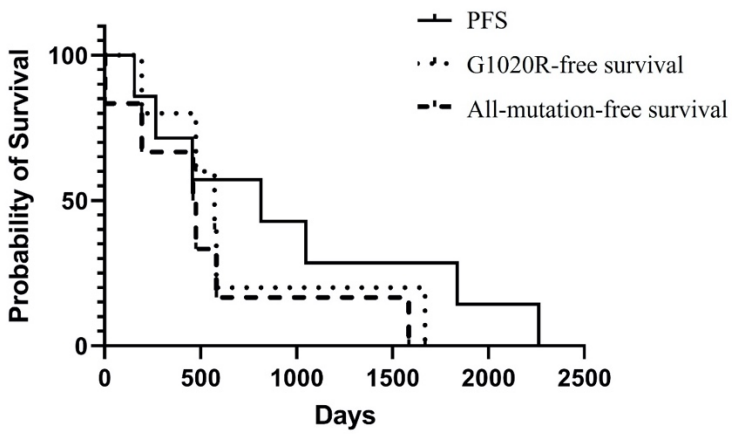
values.

Changes in the text:
“The correlation analyses were performed using Spearman’s rank correlation coefficient.” (See Page 23, line 8–9).

Comment 23:
5. Alternatively, Kaplan Meier plots could be implemented (instead of simple scatter plots as shown) to see whether you could establish a significant difference in PFS based on the time after alectinib treatment that you detected a resistance mutation.

Reply 23:
As suggested, a Kaplan–Meier curve was created as shown below. Although the sample size was too small to be statistically significant, we showed that the resistant mutation appeared earlier than disease progression. To represent events in which resistant mutations were first detected in the scatterplot, followed by repeated detection of mutations until an event such as exacerbation occurred, Figure 3 has been retained as scatterplots, and the Kaplan–Meier method is provided in Supplementary Figure 4.

Supplementary Figure 4:



	Median (days)	HR (95%CI) to PFS
PFS	814	
G1202R-free survival	573	0.64 (0.19-2.16)
All-mutation-free survival	468.5	0.56 (0.18-1.80)

Changes in the text:
“In addition, the resistance mutations appeared earlier than disease progression (Supplementary Figure 4).” (See Page 10, lines 209–210).

Comment 24:

6. How many patients are represented by the data points in Figure 3? You may want to include this information in the figure caption.

Reply 24:
The data points in Figure 3 represent six patients. The figure caption has been revised accordingly, along with the figure itself, demonstrating which patients are included in the figure.

Changes in the text:
“Figure 3. Correlation between progression-free survival and time from alectinib treatment initiation (or treatment modification) to detection of resistance mutations in six patients.” (See Page 22, lines 21–22).

Comment 25:
7. For patients with multiple resistance mutations, were there trends for which mutation comes relatively early or late in the course of the therapy?

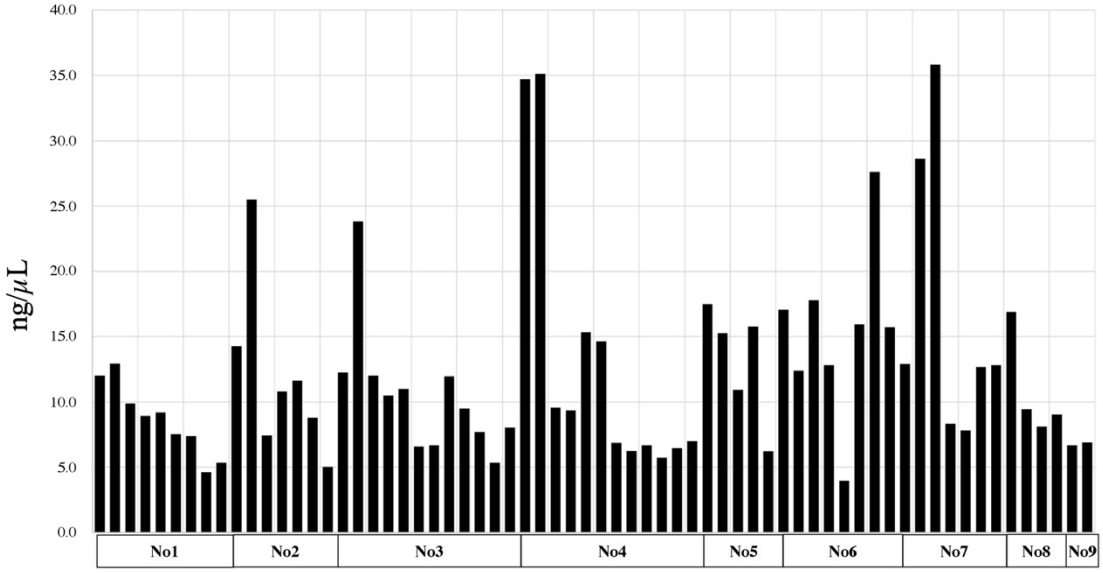
Reply 25:
In patients with multiple resistance mutations, there were no trends regarding the mutations that occurred earlier or later during the course of treatment. Of the 15 patients with multiple resistance mutations, 14 had the G1202R mutation, and the C1156Y mutation was the most common secondary resistance mutation in eight of these samples. Five of these patients received alectinib, and three received crizotinib. Multiple mutations were not detected in the samples before treatment initiation. The sample size was not large enough to allow for a statistical analysis of these data.

Comment 26:
8. Supplementary Figure 1: it would be better to group the samples per patient in order to see the cfDNA concentration variability among longitudinal samples and between patients.

Reply 26:
To observe the variation in cfDNA concentration, we included a line for cfDNA concentration in Figure 2. In the revised manuscript, we have included Supplementary Figure 2, which clarifies the relationship between cfDNA concentration and the course of treatment.

Changes in the text:
“Figure 2 and Supplementary Figure 2 show the cfDNA concentrations in the same patients over time and across different patients.” (See Page 10, lines 198–199).

Supplementary Figure 2:



Comment 27:
Discussion
1. In addition to resistance mutations, there are other bypass mechanisms of resistance to TKIs. These mechanisms are obviously outside the scope of the work but a brief discussion on this would improve the manuscript, and perhaps comment on the limitations of using the detection of just 10 ALK mutations for tumor monitoring. A future outlook would be to use a larger panel that covers more genes for molecular profiling (PMID: 29376144, 31358542, 33161228, 34876698).

Reply 27:
ALK amplification of the ALK kinase domain and activation of the bypass signaling pathway were not included because they were outside the scope of this study. As you indicated, a future perspective could be to investigate molecular profiling using a panel that covers a larger number of genes. We have revised the Discussion section accordingly.

Changes in the text:
“Other bypass mechanisms are also associated with TKI resistance. We did not study ALK amplification of the ALK kinase domain or activation of the bypass signaling pathway in this study as it was outside the scope of this study. Mechanisms of TKI resistance can be categorized into those associated with alterations that prevent the inhibition of the target receptor TK by TKIs, changes in tumor cell lineage, and activation of bypass +/- downstream signaling pathways that promote cell survival and proliferation (21).” (See Pages 12–13, lines 258–264).

“We used 10 ALK mutations for tumor monitoring in this study. Future studies should investigate the clinical utility of molecular profiling using a panel incorporating a larger number of genes (22,23).” (See Page 13, lines 274–276)

References:
21. Cooper AJ, Sequist LV, Lin JJ. Third-generation EGFR and ALK inhibitors: mechanisms of resistance and management [published correction appears in Nat Rev Clin Oncol. 2022 Nov;19(11):744]. Nat Rev Clin Oncol. 2022;19:499-514.

22. Dietz S, Christopoulos P, Yuan Z, et al. Longitudinal therapy monitoring of ALK-positive lung cancer by combined copy number and targeted mutation profiling of cell-free DNA. EBioMedicine. 2020;62:103103.

23. Angeles AK, Christopoulos P, Yuan Z, et al. Early identification of disease progression in ALK-rearranged lung cancer using circulating tumor DNA analysis. NPJ Precis Oncol. 2021;5:100.

Comment 28:
2. In p. 6, line 243-244: were the allele frequencies/copies relatively low that maybe the fluctuation is due to the limit of detection of ddPCR?

Reply 28:
As you have mentioned, this is an important limitation of our analysis because it was based on samples with low copy numbers. We have revised the limitations section of the Discussion to emphasize this point.

Changes in the text:
“Finally, as our analysis is based on samples with low copy numbers, the fluctuations observed may be attributed to the limit of detection of ddPCR.” (See Page 13, line 276–278).

Comment 29:
3. As mentioned above, the authors may also include a short outlook statement on the possibility of using multiplexed ddPCR assays for detecting multiple mutations, in cases when the input template is limited.

Reply 29:
We have revised the Conclusion section of the manuscript to address this issue, including a caveat regarding the correlation between genetic mutations and clinical disease progression.

Changes in the text:
“In the future, multiplexed ddPCR assays may play a role in detecting multiple mutations when the input template is limited. However, our data indicate that the detection of genetic mutations does not correlate with clinical disease progression.” (See Page 14, lines 286–289).

Comment 30:
4. What could be possible explanations why L1196M, L1152R, V1180L, and S1206Y were undetectable? Are these mutations rare based on literature? Do these mutations come later after treatment with multiple TKIs?

Reply 30:
L1196M is listed in OncoKB (https://www.oncokb.org/) and is considered crizotinib resistant; L1152R, V1180L, and S1206Y were not detected in this study but are rare in the literature and databases, which is possibly why they were not detected.

Review Comments (Round 2)

Reviewer A

Comment 1:

Authors have made requested changes and clarification to the questions raised. I am satisfied with the manuscript and recommend acceptance.

Reply 1:

Thank you for your comment. We are happy that you are satisfied with our revised manuscript.

Reviewer B

Comment 2:

The authors have responded to my previous comments

Reply 2:

Thank you for your input. We are pleased that no further changes are required from your perspective.

Reviewer C

Comment 3:

General comment: I maintain that the manuscript is well written and the main message as well as the potential translational application of the technology employed here could have clinical impact. However, there are technical issues that were brought up in the

initial review that were not adequately addressed in the revision/rebuttal letter.

Reply 3:

Thank you for your positive comments. We have responded to your detailed comments on a point-by-point basis below.

Comment 4:

Reply 15: This information should be mentioned in the methods section. Using different input amounts of cfDNA for this analysis could be problematic particularly because the initial amount of plasma used for cfDNA isolation was also varied (the authors only mentioned maximum of 5 mL plasma, and I interpreted this to be 5 mL or less). Indeed, ddPCR provides absolute quantification but by not setting a constant input value (cfDNA or plasma) false negative findings would most likely occur. This should be considered as an important limitation of the study.

Reply 4:

We have revised the manuscript text (Methods and the limitations section of the manuscript), per your request.

Changes in the text:

Methods section

"The amount of cfDNA contained in 8.8 μL of template DNA varied between 35.2–315.0 pg/μL. The amount of template DNA also varied from 0.7–6.3 ng/well because the digital PCR could not contain the same concentration of template DNA due to the volume of the PCR reaction solution." (see Page 9, lines 170–173).

Discussion section

"Our study was also limited by the use of different input amounts of cfDNA and plasma in the analyses, which could increase the chance of false negative results." (see Page 14, lines 288–289)

Comment 5:

Reply 16: This similarly remains as a limitation of the study. The authors mentioned that they aliquoted 60 μL of isolated DNA and 8.8 μL is needed for 1 reaction. Given that there are 3 assays, it is unclear to me how there would be insufficient material for at least duplicate measurements.

Reply 5:

Thank you for your comment. As part of the data collection for this study, we simultaneously conducted research to confirm *ALK* exon sequences using next-generation sequencing (NGS). However, we could not obtain sufficient gene

amplification with amplicon-based NGS and, therefore, did not include those data in this study. If we had only examined the samples using the ddPCR method described in this study, we would have been able to perform duplicate analyses. Unfortunately, we no longer have any remaining samples.

We have revised the limitations section of the Discussion to highlight the limitation raised.

Changes in the text:

"We also only used one well for each sample, meaning technical replicates were not performed." (see page 14, lines 289–290).

Comment 6: Reply 17: addressed adequately

Reply 6: Thank you.

Comment 7: Reply 18: This remains unchanged in the revised manuscript.

Reply 7: We are sorry for this oversight and have correct it in the revised files.

Comment 8: Reply 19: addressed adequately

Reply 8: Thank you.

Comment 9:

Reply 20: I might have been misunderstood. I did not mean measuring the allele fraction of the *EML4-ALK* fusion. I meant for patients with a certain *ALK* mutation occurring in multiple plasma samples, it might be useful to look at the quantified copies detected and correlate this with therapy response or progression. For example, in Fig. 2, patient 6 was positive for the G1202R mutation in 6 samples. Does the highest mutation count correlate disease progression? Or did the authors mean that the mutation counts are too unreliable for this kind of analysis?

Reply 9:

The appearance of the G1202R mutation did not correlate with disease progression. This suggests that the impact of *ALK* fusion gene partners and their variants, as well as the emergence of resistant mutations, might be significant. However, this study had a small sample size, which limited the ability to confirm this relationship.

Comment 10: Replies 21-30: addressed adequately

Reply 10: Thank you.

Additional major comments:

Comment 11:

1. On page 14, line 483, the authors mentioned that "digital PCR is more sensitive than next-generation sequencing." This is not accurate and there are literature reporting the non-inferiority or even the superiority in sensitivity of NGS-based assays to digital PCR (PMID: 34246791, 24705333).

Reply 11:

We have revised this sentence to remove the text you have quoted.

Changes in the text:

"In addition, digital PCR is limited by the fact that it cannot detect *ALK* fusion partners or cis- or trans-splicing, even when two resistance patterns are detected simultaneously." (see Page 14, lines 289–291)

Comment 12:

2. The authors concluded that "detection of genetic mutations does not correlate with clinical disease progression." This is in contrast to majority of literature on ctDNA. An alternative explanation for the emergence of mutations prior to disease progression is perhaps the higher sensitivity of liquid biopsy technologies, which enables the capture of molecular progression prior to detection of disease progression by imaging techniques.

Reply 12:

We agree with you and have revised the text accordingly.

Changes in the text:

"Although our data indicate that the detection of genetic mutations do not correlate with clinical disease progression, the absence of a correlation may be the result of the higher sensitivity of liquid biopsy technologies, enabling the capture of molecular progression prior to the detection of disease progression by imaging." (see Page 15, lines 304–308)

Minor comments:

Comment 13:

1. p. 5, line 100: instead of "mutations", use "genetic alterations" as ALK rearrangements are structural variations and not mutations.

Reply 13:

We have made this change.

Changes in the text:

"*ALK* genetic alterations were initially described in 2007 in a Japanese subset (7%) of patients with NSCLC harboring a fusion oncogene *EML4-ALK* formed by the rearrangement of *EML4* with *ALK* (5)." (see Page 5, lines 78–80)

Comment 14: 2. Italicize all gene names.

Reply 14: This has now been actioned.

Comment 15: 3. Figure 3 legend: "The blue and red dots indicate the days…" this should only be "blue dots."

Reply 15: Thank you for your comment. We have revised the text as requested.

Changes in the text:

"The blue dots indicate the days when any of the resistance mutation was detected after treatment with alectinib, and the red dots indicate the days when they were first detected." (see Page 23, line 5)