Peer Review File
Article Information: https://dx.doi.org/10.21037/tlcr-22-912

Reviewer A

The manuscript by Kim et al. aimed to investigate whether circulating tumor DNA (ctDNA) in patients with non-small cell lung cancer (NSCLC) could be an alternative to tissue DNA in terms of variant detection. They also evaluated whether either cell-free DNA (cfDNA) or tumor mutational burden (TMB) could stratify treatment outcomes such as overall survival (OS). The results from the study will help us understand the feasibility and limitations of cfDNA-based next-generation sequencing (NGS) assays; however, there are several concerns in the current version of the manuscript for publication.

Major comments:

1. To interpret the survival data in Figures 1 and S5, the authors should consider the mixed patient backgrounds and treatments, as well as the information on patients with PD-L1 high, EGFR, and ALK, when comparing cfDNA high vs. low (Fig. 1) and TMB high vs. low. The authors should provide clarification on how to interpret the OS data despite these factors.

Reply 1:
We appreciate the review's insightful comments regarding the survival analysis of ctDNA. Considering the mixed patient backgrounds and treatments, we performed univariate Cox-regression analysis with the clinical variables of cfDNA concentration, TMB (ctDNA), smoking, PD-L1, EGFR, and ALK. And we confirmed that none of them were significantly correlated with patient survival. We have added the results of univariate analysis with clinical variables in Supplementary Table 2 and mentioned them in the Results section.
Changes in the text:
"Meanwhile, univariate analysis using clinical variables showed that although a variety of patients were enrolled in cohorts A and B, none of the patient characteristics significantly affected survival (S2 Table)". (Page 8, Line 193-195)

<Supplementary Table 2>

	Hazard ratio	P-value
Cohort A		
cfDNA concentration (high to low)	3.30 (95% CI: 1.00-10.94)	0.051
Tumor mutational burden, ctDNA (high to low)	1.70 (95% CI: 0.49-5.88)	0.41
Smoking (ex- or current smoker to non-smoker)	2.38 (95% CI: 0.51-11.03)	0.27
PD-L1		
1-50% to <1%	2.59 (95% CI: 0.50-13.37)	0.26
>50% to <1%	3.10 (95% CI: 0.63-15.40)	0.17
EGFR (mutant to wild-type)	0.49 (95% CI: 0.10-2.38)	0.38
ALK (mutant to wild-type)	0.04 (95% CI: 0.00-2809.74)	0.58

:

2. The study enrolled patients who were on treatment in the cohort B. The authors should explain how to interpret the OS data despite the uncontrolled baseline.

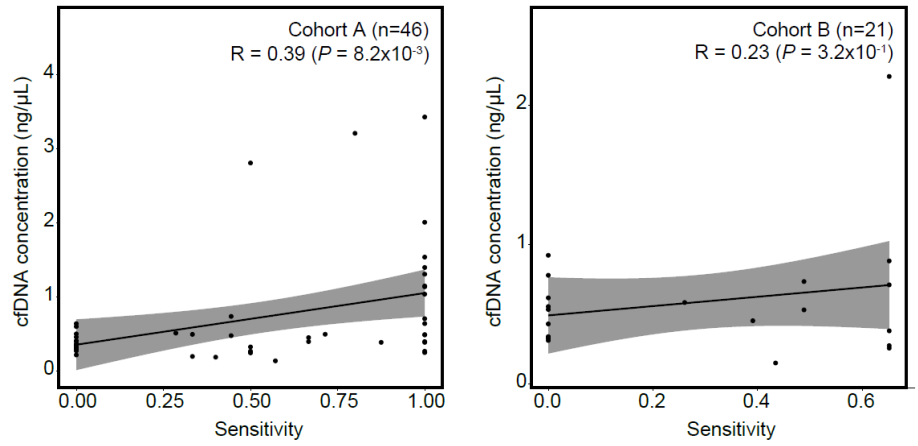
Reply 2:
We appreciate the reviewer's sharp comments and are sorry for the incorrect description. As noted by the reviewer, the date of enrollment was not suitable for baseline because patients in cohort B were enrolled in the study during the treatment period. Instead, we calculated overall survival (OS) as the time from confirmed progressive disease (PD) to death for cohort B patients. To clarify the analysis, we had corrected and added description of calculating OS in the Method section.
Changes in the text:
"Overall survival (OS) was defined as the time from study enrollment to death for cohort A and from confirmed PD of studied treatment to death for cohort B." (Page 7, Line 174-176)

3. In line 127, the study collected blood samples several times before tumor progression in the cohort B. An important question is whether cfDNA/ctDNA can detect relapse earlier than tumor markers and imaging. It could strengthen the importance of the study if the authors added a time course of cfDNA/ctDNA along with changes in tumor marker levels and image findings.

Reply 3:
We appreciate the valuable suggestion to improve our study. Unfortunately, although we collected serial blood samples during the treatment of patients in cohort B, we could not finally perform the targeted gene panel sequencing analysis on all serial blood samples because of time and cost limitations. Instead, we applied panel sequencing to blood samples at study enrollment and the disease progression and compared the results between these samples. At the suggestion of the reviewer, we have added a description of the importance of serial ctDNA sequencing in the Discussion section. And this serial ctDNA sequencing study will be conducted in the future.
Changes in the text:
"The serial ctDNA analysis may be used in treatment monitoring along with tumor markers or imaging and in early detection of disease progression. This serial ctDNA sequencing analysis was left for future work." (Page 13, Line 350-352)

4. Does the concentration of cfDNA in Figure 1B refer to the one in the blood as used in Figure 1A? In Figure 1A, the unit for the concentration is ng/uL. Is the unit of ug/uL correct in Figure 1B?

Reply 4:
We appreciate the detailed review and comment. As the reviewer pointed out, the concentration of cfDNA in Figure 1A and Figure 1B are identical, and the unit in Figure 1B was typing mistake. We have corrected the unit for the concentration in Figure 1B to ng/μL.
Changes in the text:
<Figure 1B>

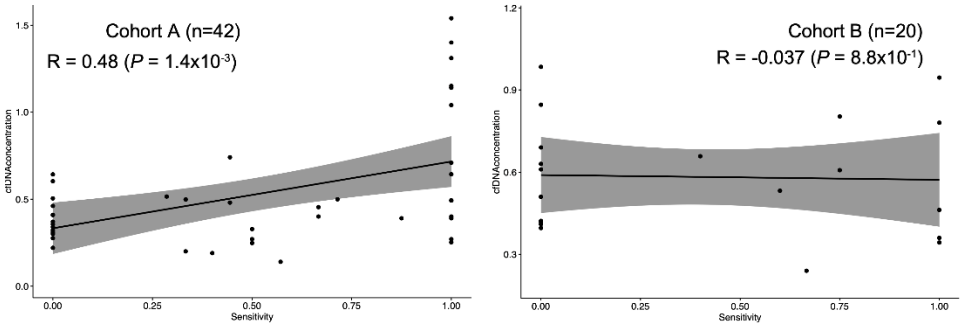


5. The interpretation and messages from Figure 2B data should be clarified. If a fixed amount of DNA was used for sequencing, why does the sensitivity differ across samples with different cfDNA concentrations? Instead, if variable amounts of DNA were used according to cfDNA concentrations, please validate its rationale. The coefficients suggest weak correlations between two variables. However, the effects from three outliers on the left (~3-4 ug/ul of cfDNA) and one on the right (~2 ug/ul) should be carefully examined. Without these outliers, cfDNA concentration might not be correlated with sensitivity. The precision of commercially available liquid biopsy platforms for detecting major driver mutations such as KRAS is 100% at 30 ng cfDNA inputs. In the left figure, some samples with zero sensitivity show at least more than 0.5 ug/ul (=500ng/ul). Although the definition of the concentration is unclear at the moment, one could assume that the authors were able to prepare 30 ng of cfDNA inputs at least, which was sufficient for sequencing in general.

Reply 5:

We appreciate the reviewer's comments. First, we could not use a fixed amount of DNA because we obtained and used a constant amount of blood samples from the patients who had variable cfDNA concentrations. Since the cfDNA concentrations can vary by systemic tumor burden, condition of patients, or other various patient's factors, we wanted to analyze the influence of cfDNA concentration on the accuracy of ctDNA sequencing.

We removed outliers that the reviewer has mentioned to examine the correlation between sensitivity and cfDNA concentration. In Cohort A, there were 4 outliers with 3~4 ng/ul, and we re-calculated correlations after excluding these outliers and we found that correlation was stronger and with increased statistical power. On the other hand, correlation was weaker with less statistical power after 1 outlier removed in Cohort B. We found out that there is no huge difference between with or without outliers, and we can still see the significant correlation in Cohort A.



Reviewer-only Figure 1. Correlation between sensitivity and cfDNA concentration excluding outliers

We are sorry about the miswriting on the cfDNA concentration unit, and we have corrected it from ug/ul to ng/ul. In fact, many of the samples in our study had less than 30 ng of DNA. Also, the accuracy of ctDNA analysis can vary by sequencing method, type of analysis platform, cancer types, and targeted genes. Therefore, it is difficult to simply compare the precision of the platforms directly with each other.

Changes in the text:

No change in the manuscript for this reply.

6. Since the authors raised a concern regarding false-positive results as in line 299, two patients with EGFR T790M only by liquid biopsy in Fig. 2B are interesting. Did they respond to osimertinib? Otherwise, is the data presented strong enough to say they are true-positive?

Reply 6:

Despite being concerned about false positives in ctDNA sequencing, the two patients with EGFR T790M only detected by liquid biopsy could be considered true variants because of the sufficient evidence with more than 1% VAF from ctDNA sequencing. In addition, one (B-075) of the two patients repeated the EGFR mutation test on the biopsy tissue using a PCR-based commercial test product, and was finally confirmed to have the EGFR T790M mutation. This patient was eventually treated with osimertinib, and the tumor size decreased after starting osimertinib. Unfortunately, another patient found additional HER-2 amplification and was subsequently treated with a drug targeted for HER-2 amplification (Unknown response to osimertinib). We have added a description of osimertinib treatment and response for the one patient as follows.

Changes in the text:

"And one of the two patients actually treated with osimertinib and had a response with decreasing tumor size." (Page 11, Line 276-277)

Minor comments:

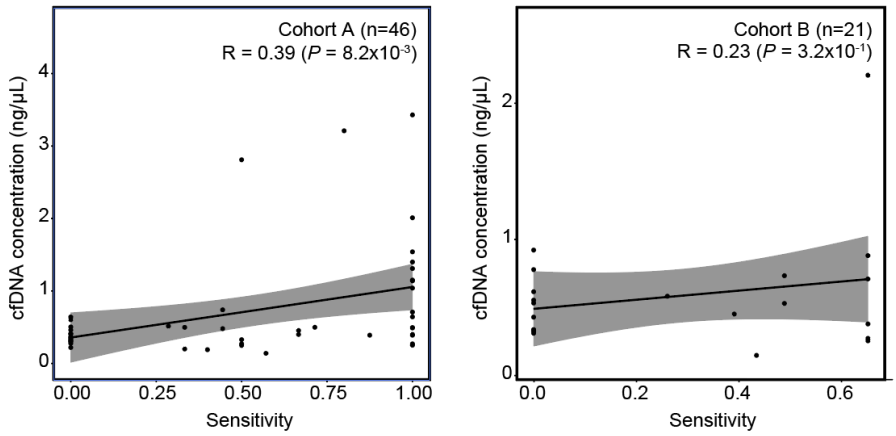
1. In Figures 1B, S3, and S5, the authors should add the numbers of samples.

Reply 1:

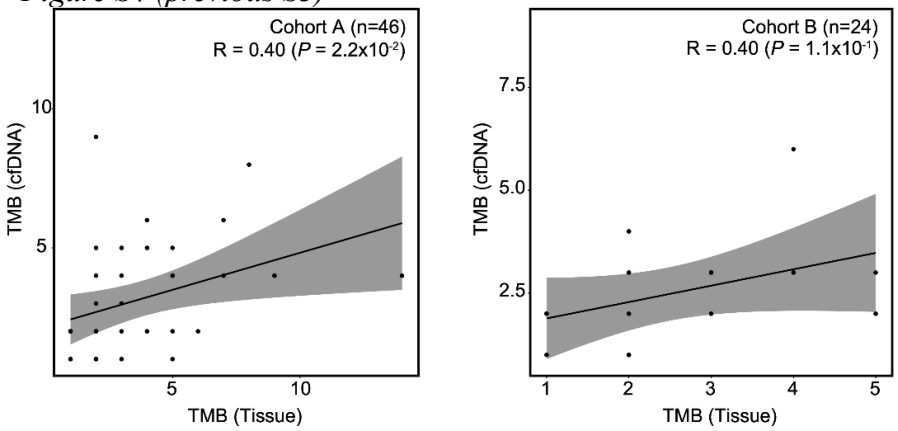
We now have added the number of samples in Figure 1B, S4 (previous S3) and S6 (previous S5).

Changes in the text:

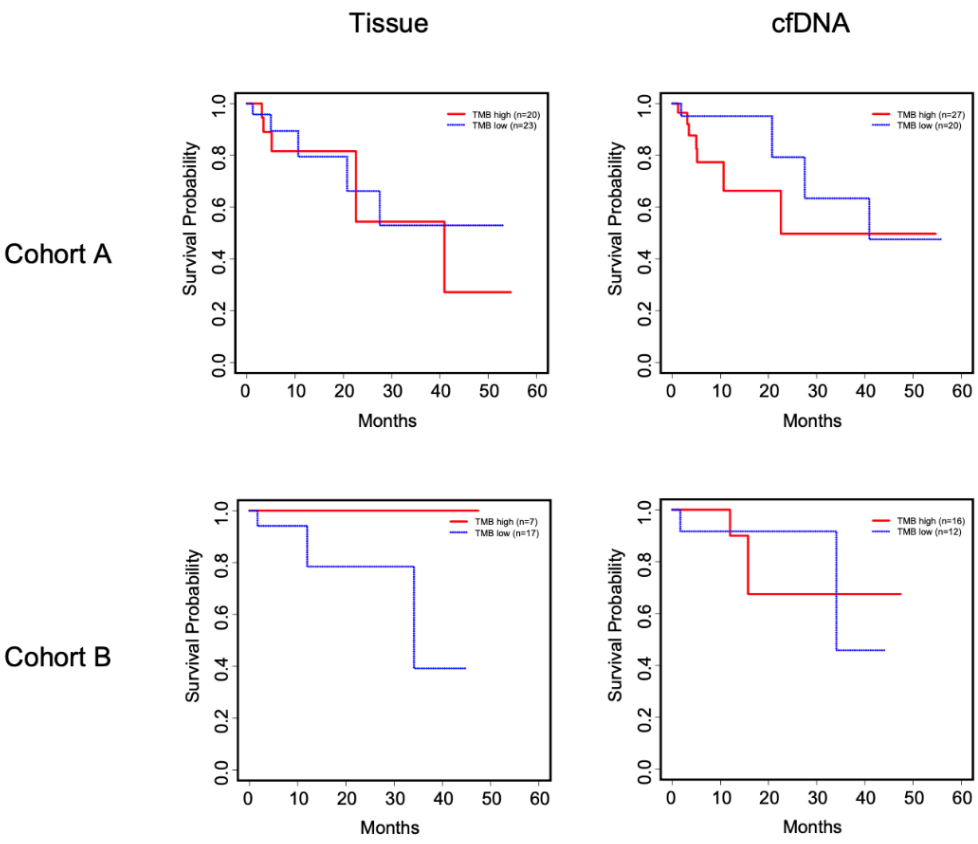
<Figure 1B>



<*Figure S4 (previous S3)*>



<*Figure S6 (previous S5)*>



2. According to the description in line 216, the left and right panels in Fig. S3 represent 46 and 24 samples, respectively. However, the dots seem to be less than expected. Are there any overlaps among the data? The authors should consider a better data presentation.

Reply 2:

The number of dots in Figure S3 or others may not be correspondent to the n number shown in the figure due to duplicate values. For example, there are 3 samples showing 1 TMB from tissue and 2 TMB from cfDNA in Figure S3. We have a small amount of data and the TMB range is not dynamic, so our correlation plot may cause confusion. Unfortunately, we have not found any better presentation method for our correlation analysis without using dots. Instead, we have added the explanation about redundant values to clarify the figure contents. We hope that putting sample sizes in the plot and additional explanation would help the reviewer and prospective readers to understand clearly.

Changes in the text:

"Some dots in the figure contain redundant data where different samples have the same value." (Figure S4, previous S3)

3. For better interpretation of OS data, the authors should add information regarding post-relapse therapies.

Reply 3:

We totally agree with the reviewer's comments. But, unfortunately, some patients were transferred to hospice care without post-relapse therapies as their disease progressed and worsened. And several patients were referred to a local hospital without post-relapse therapies in our hospital. Although we were able to confirm the patient's survival and date of death through government agencies, we were unable to obtain treatment records for all patients. However, at least the patients treated at our hospital were treated through standard chemotherapy even after disease progression.
Changes in the text:
No changes in the manuscript for this reply.

Reviewer B

The authors tried to present this study as a clinical validation study between the detection and mutation profiles between tumor tissue and cfDNA (including ctDNA).

There are several points that needs to be clarified:
1. The definition of Cohort A and Cohort B.
In Section 2.1, Cohort A was defined as newly diagnosed with metastatic or recurrent NSCLC, while in Section 2.2, Cohort A became subjects before the second cycle of chemo and after confirming disease progression?
It was even more confusing that in Table 1, about 10% of the subjects in Cohort A were treated with targeted therapy, and again without stating how long subjects had been treated before samples were taken, or indeed they are not baseline.
Patients on cytotoxic therapy may have upsurge of cfDNA, which was not considered in the analysis.

Reply 1-1:
We appreciate the reviewer's detailed and insightful comments. First of all, we apologize for the incorrect description of the blood samples in Cohort A due to several words being omitted during the manuscript editing. In fact, we also obtained blood samples at the start of treatment, so the three blood samples were taken at baseline, before second cycle of chemotherapy, and after confirming disease progression. We are again sorry for the confusing sentences, and actually, the baseline blood samples were taken in Cohort A. We have corrected the description as follows.
Changes in the text:
"For Cohort A patients, three blood samples were obtained at starting treatment (baseline), before the second cycle of chemotherapy, and after confirming disease progression via imaging tumor evaluation." (Page 6, Line 118-120)

In Section 2.1, Cohort B was just metastatic or recurrent NSCLC received treatment without stating how long has the treatment been given or the median duration of treatment, while in Section 2.2, Cohort B had blood samples collected up to six times during follow up or when tumor progression was progressed.
The confusion in the above made the validity of results difficult to be ascertained, and there appeared to be much ambiguity as to the time points when blood samples were taken for the two cohorts, which were indeed crucial for the results to be valid and analyzable.

Reply 1-2:

We are sorry again for the ambiguous descriptions and confusing explanations. An attempt was made to describe an initial study plan to analyze serial cfDNA sequencing by sequentially taking blood samples, but the lack of explanation confused the meaning of the results of subsequent studies. Actually, we obtained multiple blood samples for the study patients, but we finally used the blood samples of baseline for Cohort A and those of progressive disease (PD) for Cohort B. We have changed the description of collecting blood samples in Cohort B and added a sentence to explain the samples used in the study.

Changes in the text:

"In Cohort B, blood samples were routinely collected after study enrollment following the treatment response evaluation schedule. And in the end, blood samples were collected when tumor progression was confirmed for Cohort B patients. Although this study was initially designed to analyze serial cfDNA samples from both cohorts A and B, due to time and cost limitations, subsequent procedures were limited to analyzing only blood samples at baseline in Cohort A and those of progressive disease (PD) in Cohort B." (Page 6, Line 120-124)

2. Section 3.3

There was no explanation as to how the 78 patients were chosen to have ctDNA sequencing analysis out of total of 129 patients, and at the same time, the identical mutational profiles with exactly the same percentage distribution also added to the possibility of inherent bias in the selection of the above 78 / 129 patients.

Reply 2:

We tried to conduct ctDNA sequencing for all collected samples and did not choose any samples to test in other ways. Unfortunately, there were some samples with too low ctDNA concentration or the total amount of ctDNA and a few samples with gDNA contamination that made fail to ctDNA sequencing and dropped out of the study. The identical percentage distribution of TP53 and EGFR between tumor tissue and ctDNA sequencing was a coincidence, and the other genes of KRAS, APC, CDKN2A, etc. had different percentage distribution. We have added the cause of unavailable ctDNA sequencing data in the limitation part of the Discussion section.

Changes in the text:

"Despite efforts to obtain appropriate samples and sequencing data, there was some loss of samples and inadequate data among Cohort A and B patients (low concentration or total amount of ctDNA and genomic DNA contamination)." (Page 13, Line 345-347)

3. Section 3.3

Please consider further elaboration or using alternative terms of 'significantly mutated'.

Reply 3:

We appreciate the reviewer's comments. We agree that the expression 'significantly mutated' was awkward so we have changed the text to 'frequently mutated' instead.

Changes in the text:

"TP53, KRAS, STK11, EGFR, NF1, ATM, APC, EPHA3, CDKN2A, and ERBB4 are frequently mutated in lung adenocarcinoma." (Page 9, Line 227-228)

4. Table 1

What were the 'others' under Pathology?

Reply 4:
Several patients diagnosed with non-small cell lung cancer (NSCLC) with neuroendocrine differentiation, pleomorphic carcinoma, or NSCLC-not otherwise specified (NOS) were included in this 'others' category. To afford more information, we have added the footnote for 'others' of pathology at the bottom of Table 1.
Changes in the text:
*"*Others: NSCLC with neuroendocrine differentiation, pleomorphic carcinoma, or NSCLC-not otherwise specified"*, (Page 18, Line 472, Table 1)

Reviewer C

Sheehyun Kim et al. conducted a prospective study assessing cfDNA/ctDNA in lung cancer patient. Two cohorts of patients were studied, each involving around 60 patients. They studied correlation between cfDNA and OS, and concordance of tissue and plasma genomic results. Earlier studies have shown that cfDNA concentration is increased in patients with lung cancer, with a high level being correlated with a poor prognosis. ctDNA only accounts for a very small proportion of the whole cfDNA, thus the correlation between cfDNA and ctDNA is awaited. I guess there are major limits in this work. Most important, definition of the population included within the two cohort is very unclear, in the whole manuscript (abstract, methods, results). Moreover, I guess conclusions need to be revise, and major papers that did similar works need to be included in the discussion part.

Introduction
L97-99: The objective is very unclear. What is clinical utility for authors?

Reply (Introduction):
We appreciate the reviewer's comments. By improving the ctDNA analysis, we suggest that cfDNA (or ctDNA) concentration can be used as a new prognostic biomarker, and ctDNA sequencing can be alternatively used to detect tumor mutational profiles for molecular diagnosis before (baseline) and after treatment (PD). To clarify the clinical utility written in the Introduction section, we have modified the manuscript as follows.
Changes in the text:
"Here, we evaluated the clinical implementation of ctDNA in patients diagnosed with advanced non-small cell lung cancer (NSCLC) and investigated its potential clinical utility (prognosis prediction and molecular tumor diagnosis) using the NGS platform." (Page 5, Line 95-97)

Met & Met
I do not understand definition of cohort A (newly diagnosed, but only patients treated with chemo?) and cohort B (relapse after a first-line therapy? Authors said cohort B including patients during targeted treatment, but they also talk about immunotherapy (L109 p3).
"recurrent or metastatic lung cancer" (abstract, text): what does it mean recurrent OR metastatic? After a first-line systemic therapy? After a first treatment at localized stage? What about a localized recurrence?

Reply (Material and Methods 1):
Most of all, all patients enrolled in our study were diagnosed with metastatic non-small cell lung cancer (NSCLC) classified as stage IV. Some patients were initially diagnosed with metastatic NSCLC, and others were diagnosed with the localized stage but metastatic tumor recurrence was newly found after local treatment. The "metastatic or recurrent lung cancer" included both kinds of patients anyhow first diagnosed with metastatic stage IV lung cancer.
The newly metastatic NSCLC-diagnosed patients would be treated with first-line systemic therapy and these patients were enrolled in Cohort A to evaluate pre-treatment status at baseline. On the other hand, several metastatic NSCLC patients already being treated with targeted treatment or immunotherapy were enrolled in Cohort B to evaluate post-treatment status after disease progression. We have changed and added description about definition of Cohort A and B in Section 2.1.
Changes in the text:
"On the one hand, Cohort A included patients newly diagnosed with metastatic or recurrent NSCLC and scheduled to receive first-line systemic chemotherapy (to evaluate pre-treatment status at baseline). On the other hand, patients in Cohort B had metastatic or recurrent NSCLC, had been receiving molecular targeted therapy or immune checkpoint inhibitors, and had once experienced tumor shrinkage and regrowth during treatment (to evaluate post-treatment status after disease progression)." (Page 5, Line 104-108)

This is also very unclear the timing of blood samples collection. Cohort B: up to six times? Which one was analysed and included in the result part?
"A total of 152 patients (Cohort A: 73 and Cohort B: 79) were enrolled in the study, and data from 129 patients (Cohort A: 65 and Cohort B: 64) with matched tumor samples and clinical data were analyzed." This sentence should be in the result section.
Data end of follow-up?

Reply (Materials and Methods 2):
We are sorry for the ambiguous descriptions and confusing explanations. An attempt was made to describe an initial study plan to analyze serial cfDNA sequencing by sequentially taking blood samples, but the lack of explanation confused the meaning of the results of subsequent studies. Actually, we obtained multiple blood samples for the study patients, but we finally used the blood samples of baseline for Cohort A and those of progressive disease (PD) for Cohort B. We have changed the description of collecting blood samples in Cohort B and added a sentence to explain the samples used in the study. Also, following the reviewer's comment, we have moved the sentence about the number of analyzed patients to the Result section.
Changes in the text:
"In Cohort B, blood samples were routinely collected after study enrollment following the treatment response evaluation schedule. And in the end, blood samples were collected when tumor progression was confirmed for Cohort B patients. Although this study was initially designed to analyze serial cfDNA samples from both cohorts A and B, due to time and cost limitations, subsequent procedures were limited to analyzing only blood samples at baseline in Cohort A and those of PD in Cohort B." (Page 6, Line 120-125)
"From March 2017 to January 2019, a total of 152 patients (Cohort A: 73 and Cohort B: 79) were enrolled in the study, and data from 129 patients (Cohort A: 65 and Cohort B: 64) with matched tumor samples and clinical data were analyzed." (Page 8, Line 183-185)

Results
How long is the follow-up of each cohort?
Table 1: Treatment without MTT: would it be possible to have details (chemo, IO?)?

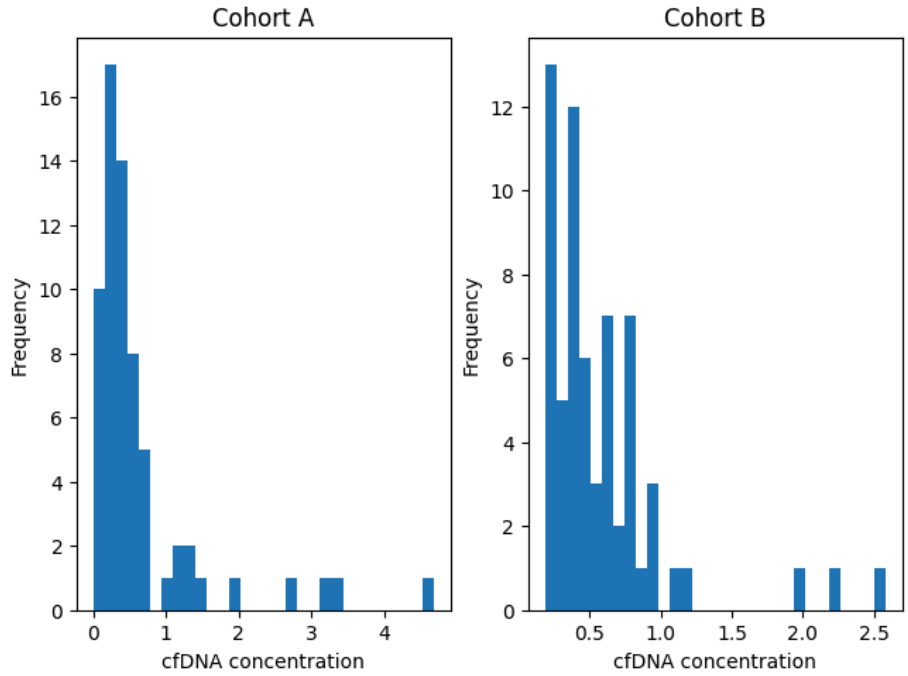
Reply (Results 1):
The median follow-up period for survival analysis was 13.1 months. And as the reviewer's comment, treatment without MTT included cytotoxic chemotherapies and immunotherapies. We have added footnote of Table 1 to describe detailed treatment of "Treatment without MTT".
Changes in the text:
**Treatment without MTT: Cytotoxic chemotherapies (gemcitabine, pemetrexed, or paclitaxel + cisplatin or carboplatin) or immunotherapies (pembrolizumab, atezolizumab, or nivolumab, etc.)* (Page 18, Line 473-474)

Part 3.2
What is the range and the distribution of cfDNA concentration in both cohort? It will be interesting to have a histogram of the distribution.
It is cfDNA concentration before any treatment?

Reply (Results 2):
We appreciate the reviewer's valuable comment. We have added description regarding the distribution of cfDNA concentration within both cohorts as well as histogram figure. The Cohort A represented pre-treatment (baseline) and the Cohort B represented post-treatment (PD).

Changes in the text:
"Most patients had cfDNA concentration below 1 ng/μL (0.226, 0.370 and 0.603 ng/μL; Cohort A and 0.330, 0.455 and 0.714; Cohort B for 25%, 50% and 75% of samples, respectively. And the minimum and maximum cfDNA concentrations were 0.010 and 4.680 ng/μL for Cohort A and 0.188 and 2.580 ng/μL for Cohort B, respectively (S1 Fig.)." (Page 8, Line 201-204)

<Figure S1>



Part 3.3
Would it be possible to pool Fig 2A and suppl fig S2, to show in parallel results in tissue and ctDNA? What is the rate of concordance/discordance? It is the aim of the study in the first sentence of the abstract.

Reply (Results 3):
We separated two oncoplots of tumor tissue (Fig S3, previous S2) and ctDNA (Fig 2A) because frequently mutated genes would be different once we pooled those two plots. As shown in Fig 2A and Fig S2, frequently mutated genes for tumor tissue and ctDNA are different. Except TP53 and EGFR, 3rd to 10th mutated gene in cfDNA were DNMT3A, KRAS, APC, BRCA2, CDH1, FGFR2, GNAQ and IDH2, while KRAS, APC, CDKN2A, CTNNB1, MET, SMARCA4, GNAQ and HDAC9 for tissue. Also, we demonstrated our tissue oncoplot into supplementary as our focus was on ctDNA results. We believe that separation of tissue and ctDNA would give clearer illustration of mutated gene landscape for both tissue and ctDNA, instead of pooling those two figures.
Regarding 'concordance/discordance', we calculated sensitivity and precision of ctDNA results to tumor tissue results, instead of looking at concordance and discordance, because concordance and discordance are implemented in the use of sensitivity and precision.
Changes in the text:
No change in the manuscript for this reply.

Part 3.4
"However, although we tested the association between PD-L1 IHC results and TP53, KRAS, and EGFR mutation status in tumor tissue and ctDNA sequencing for cohorts A and B, none were significant." Where are the results?

Reply (Results 4):

Because p-values from association test between PD-L1 IHC and mutation status were highly poor, we described as "none were significant", but we now have added p-values in the manuscript according to the reviewer's comment.

Changes in the text:

"However, although we tested the association between PD-L1 IHC results and TP53, KRAS, and EGFR mutation status in tumor tissue and ctDNA sequencing for cohorts A and B, none were significant (Cohort A, tissue: p = 0.77, 0.73 and 1.0; Cohort A, cfDNA: p = 0.89, 0.82 and 1.0; Cohort B, tissue: p = 1.0, 0.12, 0.53; Cohort B, cfDNA: p = 1.0 1.0 and 1.0 for TP53, EGFR and KRAS, respectively)." (Page 10, Line 245-247)

Part 3.5

"Among the patients with EGFR mutations in PD from tissue sequencing data, 31.6% (six out of nineteen samples) had consistent EGFR mutations in ctDNA sequencing data". How authors explained this low rate of detection? Is it concordant with other data available in literature? This should be discussed in discussion.

Reply (Results 5):

The data mentioned by the reviewer was from ctDNA sequencing data of PD samples. To our knowledge, few studies have evaluated the accuracy of ctDNA sequencing in patients with disease progression after treatment. These patients were under treatment, and ctDNA release to blood could be decreased even if the tumor was increased. And the low ctDNA concentration in blood might lead to a low detection rate in PD patients. Following the reviewer's comment, we have added the above description in the Discussion section.

Changes in the text:

"On the other hand, disease progression under treatment without increasing ctDNA release to blood could be a reason for the low rate of variant detection from ctDNA in PD." (Page 12, Line 325-327)

Discussion

Is the sensivity of 60% of detection with ctDNA concordant with litterature data? It would be interesting to have range of detection rate from other main studies with similar design. I guess authors must add and discuss other major studies that have assessed ct/cfDNA in lung cancer patients (Leighl 2019, Li Ann Oncol 2019, ….).

Reply (Discussion 1):

We appreciate the reviewer's comments. As the reviewer mentioned, the sensitivity of tumor variant detection varied between previous studies ranging to about 60~80% (including references that the reviewer recommended). Although our data showed slightly lower sensitivity compared to other studies, detection sensitivity may vary depending on the sequencing panel, data analysis method, patient disease status, and comparison method between tumor tissue and ctDNA. Following the reviewer's comments, we modified and added the description of detection sensitivity in the Discussion section.

Changes in the text:

"Although our data showed slightly lower sensitivity (~60%) compared to other studies, detection sensitivity may vary depending on the sequencing panel, data analysis method, patient disease status, and comparison method between tumor tissue and ctDNA. Nevertheless, the sensitivity of

our data for detecting lung cancer-related tumor mutations by ctDNA analysis indicated that more optimized experimental protocols and accurate data analysis algorithms are required." (Page 12, Line 314-318)

"In addition to the EGFR T790M variant, KRAS G12C, which was specifically found in the ctDNA of one patient, could influence changes in treatment with targeted therapy, demonstrating that ctDNA analysis has clinical utility in patients with PD after treatment." We do not know if patient with EGFR and KRAS G12C mutation will respond to KRAS G12C inhibitor (dominant mechanism of resistance or a subclonal phenomenon?). Thus, it is absolutely not a demonstration of clinical utility of ctDNA.
Analytical validation studies have now accumulated and clinical validation is strongly needed. Authors wrote in the title "Clinical validity of oncogenic driver …". How do they prove clinical validity? What does it mean?
"ctDNA will widely be used in diagnosing and treating lung cancer by improving ctDNA analysis." I disagree with this conclusion based on the paper.

Reply (Discussion 2):
As listed in Table 3, the patients (B-001 and B-075) who had the EGFR T790M variant and the other patient (A-051) who had the KRAS G12C variant were different. Despite it might be a subclone that the KRAS G12C variant was only detected in ctDNA but not from tumor tissue, it is worthwhile to detect the targetable variant and have an opportunity of treating with targeted therapy such as KRAS G12C inhibitor. At this point, the clinical utility of ctDNA was shown according to the KRAS G12C variant was only detected by ctDNA analysis.We demonstrated that the cfDNA concentration in blood samples can be used as a prognostic biomarker at diagnosis, and ctDNA sequencing can be additionally or alternatively used for finding the mutational profile of tumors in advanced lung cancer patients. Similarly detected mutation results between ctDNA and tumor tissue and a few ctDNA-specific variants showed the clinical validity of ctDNA in advanced lung cancer patients.
Changes in the text:
No changes in the manuscript for this reply.

Reviewer D

The study by Kim et al. investigates utility of ctDNA testing in NSCLC and provides some interesting findings. I identified some issues that the authors should address to improve their manuscript.

Issues
1. Line 78 I am not sure what the authors mean? why would targeted agents be developed to UNDERSTAND genomic alterations? Isn't it more the other way around that our increased understanding of the effects of certain genomic alterations has led to development targeted inhibitors?

Reply 1:

We appreciate the reviewer's comments and are sorry for the incorrected writing. The opinion of the reviewer is right and we actually intended to describe that understanding the genomic alterations lead to the development of targeted inhibitors. We have corrected our manuscript as follows.

Changes in the text:

"Various targeted agents have been developed based on understanding the genomic alterations in tumors, and targeted drugs have shown dramatic responses in many patients." (Page 4, Line 76-77)

2. Line 82 I am not sure liquid biopsy is used for detection of lung cancer (although there are efforts to use it for early detection) I would think lung cancer is still detected in most patients by conventional methods. Can the authors provide a reference for lung cancer detection by liquid biopsy?

Reply 2:

We are sorry for confusing description and agree with the reviewer's comments. In fact, the sentences were intended to explain that liquid biopsy can detect and identify the molecular properties of tumors instead of detecting lung cancer itself. We have modified the sentences as follows.

Changes in the text:

"Liquid biopsy is a minimally invasive method for detecting and identifying the molecular properties of lung cancer with high sensitivity rates, facilitating integrative analyses of genomic, transcriptomic, or metabolomic markers." (Page 4, Line 80-82)

3. Line 84 can the authors elaborate on what they mean with "high false-positive rates"? do they mean false positive mutation detection in ctDNA or falsely "detecting" lung cancer in healthy individuals?

Reply 3:

We are again sorry for misunderstood sentences. As with comment #2, we wanted to describe the detection of tumor mutations by liquid biopsy instead of lung cancer itself. To clarify the meaning of "high false-positive rates", we have added an explanation to it.

Changes in the text:

"However, the nontrivial false-positive rates for tumor mutation detection, prohibitive costs, and reproducibility issues associated with the procedure need to be resolved." (Page 4, Line 82-83)

4. Line 88-90 in a way this sentence contradicts the one in line 84/85.

Reply 4:

We appreciate the reviewer's incisive comments. We have corrected both of the sentences to avoid conflicting descriptions as follows.

Changes in the text:

"However, the nontrivial false-positive rates for tumor mutation detection, prohibitive costs, and reproducibility issues associated with the procedure need to be resolved." (Page 4, Line 82-83)

"The advantages of ctDNA analysis, such as the representation of tumor heterogeneity and dynamics, drug response monitoring for patients with progressive disease, and the ability to detect

genomic markers by minimally invasive procedure, have been reported in several studies." (Page 5, Line 87-90)

5. Line 94 i) replace "tumor DNA" with "ctDNA" and ii) I assume you mean "genomic DNA" not "germline DNA" in the context of your text.

Reply 5:
We appreciate the accurate review of our manuscript. We agree with the reviewer's comments and have revised the manuscript according to the reviewer's corrections.
Changes in the text:
"For example, ctDNA concentration is lower than genomic DNA concentration, strongly correlates with tumor size and staging, and occasionally increases in patients with benign or premalignant tumors." (Page 5, Line 91-93)

6. Line 123/124 can the authors comment on why "three blood samples were obtained before the second cycle of chemotherapy"? Why would they not have taken a baseline sample?

Reply 6:
We apologize for the incorrect description of the blood samples in Cohort A due to several words being omitted during the manuscript editing. In fact, we also obtained blood samples at the start of treatment, so the three blood samples were taken at baseline, before second cycle of chemotherapy, and after confirming disease progression. We are again sorry for the confusing sentences, and actually, the baseline blood samples were taken in Cohort A. We have corrected the description as follows.
Changes in the text:
"For Cohort A patients, three blood samples were obtained at starting treatment (baseline), before the second cycle of chemotherapy, and after confirming disease progression via imaging tumor evaluation." (Page 6, Line 118-120)

7. Line 134 I assume concentration was measured not only quality since the authors found correlation of cfDNA concentration and OS. Quality is quite vague unless the definition of what is considered "good" and "bad" quality is provided.

Reply 7:
We appreciate the reviewer's comments. As the reviewer mentioned, we measured the concentration and the total amount of cfDNA and analyzed the data with OS. But in fact, we were not trying to evaluate the quality of our samples as "good" or "bad". We agree that the phrase "quality check" was confusing, so we have modified it as follows.
Changes in the text:
"and the concentration and the total amount for eluted cfDNA were measured using a Qubit dsDNA HS Assay Kit, Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA), and 2100 Bioanalyzer with High-Sensitivity DNA Chips (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions." (Page 6, Line 129-132)

8. Section 2.4: I would prefer a list of genes tested in the targeted sequencing being provided rather than referred to and the reader has to look it up in other publications.

Reply 8:
We thank the reviewer's kind comment. We now added the gene list of Axen Cancer Panel 2 in the Supplementary Table and fixed the number in the manuscript from 170 to 171.

Changes in the text:
"Targeted sequencing on both ctDNA and tumor tissue DNA was performed using Axen Cancer Panel 2 (Macrogen Inc., Seoul, Korea), targeting approximately 1.1 Mb of the genomic region containing 171 genes and 25 fusion gene rearrangements (S1 Table);" (Page 7, Line 151-153)

<Supplementary Table 1>
S1 Table. List of 171 genes covered by Axen Cancer Panel 2

ABL1	ABL2	AKT1	AKT2	AKT3	ALK	APC	AR
ARAF	ASXL1	ATM	ATR	AURKA	AURKB	AURKC	AXL
BAP1	BCL2	BRAF	BRCA1	BRCA2	BRD2	BRD3	BRD4
CBFB	CCND1	CCND2	CCND3	CCNE1	CDH1	CDK12	CDK4
CDK6	CDKN1A	CDKN1B	CDKN2A	CDKN2B	CDKN2C	CEBPA	CHEK2
CREBBP	CRKL	CSF1R	CTNNB1	DDR1	DDR2	DNMT3A	DOT1L

9. Line 157 I assume sequencing was performed on ctDNA and on tissue DNA. Please state this as part of your methods, clarify if the same method was used for both.

Reply 9:
We appreciate the reviewer's comments. As the reviewer mentioned, we conducted targeted gene sequencing both on ctDNA and tumor tissue DNA using the same method. We have added more descriptions about tissue DNA sequencing as follows.
Changes in the text:
"Targeted sequencing on both ctDNA and tumor tissue DNA was performed using Axen Cancer Panel 2 (Macrogen Inc., Seoul, Korea), targeting approximately 1.1 Mb of the genomic region containing 170 genes and 25 fusion gene rearrangements; the average sequencing depth for ctDNA was approximately 4,000x." (Page 7, Line 151-153)

10. Line 161 again I question the use of the word "germline" in the context. This should be "genomic".

Reply 10:
We appreciate and agree with the reviewer's comments. We have revised the text as follows.
Changes in the text:
"Raw DNA sequencing reads were aligned to the human reference genome (hg19) using Burrow-Wheeler Aligner, and somatic variants were called from aligned reads using GATK v4.1.2 and MuTect2 based on genomic information from whole-genome sequencing resources of 1,950 Koreans." (Page 7, Line 153-156)

11. Line 198 Replace “surface of tumor tissue” with “cell surface of tumor tissue cells”.

Reply 11:
We appreciate the detailed review and comments. We agree that the “cell surface of tumor tissue cells” is a more accurate expression, and we have changed the text accordingly.
Changes in the text:
“A total of 27.9% of patients had high PD-L1 expression (> 50%) on the cell surface of tumor tissue cells and were candidates for treatment with immune checkpoint inhibitors, including nivolumab and pembrolizumab.” (Page 8, Line 191-193)

12. Line 202 “sensitivity” to what?

Reply 12:
We now have added description of “sensitivity” to improve clarity.

Changes in the text:
“3.2 Poor survival rates and improved sensitivity rates to mutations detected from tissue in patients with high cfDNA concentration at diagnosis” (Page 8, Line 197-198)

13. Line 208 how do these concentrations relate to the volume of plasma or blood used for extraction? They are currently only meaningful if the elution volume is consistent (was it?) and the input blood/plasma volume the same (was it? this information is not stated). I would prefer the authors calculate and provide a value of ctDNA / mL plasma (or blood). This is even more important since they did some tests regarding correlation of amount of cfDNA tested for mutation detection (Fig 1b). This would really be more useful for the reader (especially clinician reader) if your data would help deciding how much blood is necessary/optimal to get reliable biomarker information.

Reply 13:
We appreciate the reviewer’s insightful comments. Unfortunately, we did not calculate the accurate value of ctDNA / mL plasma (or blood) in this study, but as we described in Method section, we consistently tried to obtain 10 mL of whole blood and 5 mL plasma for all the studied samples. We could regard the amount of input samples were consistent and hope our results will help the reader determine how much blood sample to collect.
Changes in the text:
No change in the manuscript for this reply.

14. Line 215 why were ctDNA sequencing data not available for all ctDNAs? Had the other samples been tested but the test failed or was inconclusive? Or was some patient's cfDNA simply not tested? How were the patients chosen to have ctDNA sequencing?

Reply 14:
We tried to conduct ctDNA sequencing for all collected samples and did not choose any samples to test in other ways. Unfortunately, there were some samples with too low ctDNA concentration or the total amount of ctDNA and a few samples with gDNA contamination that made fail to

ctDNA sequencing and dropped out of the study. We have added the cause of unavailable ctDNA sequencing data in the limitation part of the Discussion section.

Changes in the text:

"Despite efforts to obtain appropriate samples and sequencing data, there was some loss of samples and inadequate data among Cohort A and B patients (low concentration or total amount of ctDNA and genomic DNA contamination)." (Page 13, Line 345-347)

15. Line 220 what do you mean with "true mutation sets"? Why would mutations not detectable in an isolated, small tissue sample but in ctDNA not be considered as potentially "true"? Please clarify.

Reply 15:

We agree with the reviewer's comment that mutations detected in ctDNA can be 'true mutation' as we discussed. We measured sensitivity and precision of ctDNA results to the tumor tissue results, so "true mutation sets" term is highly inappropriate. We now removed 'true mutation sets' to avoid such confusion and improve clarity.

Changes in the text:

"We extensively compared ctDNA and tumor tissue NGS data by calculating the average sensitivity and precision of ctDNA compared with the results derived from tumor tissue samples." (Page 9, Line 215-217)

16. Line 236 I notice DNMT3A was frequently found mutated in cfDNA but not tissue DNA. Is that result acquired using the same testing (ie was DNMT3A among the genes tested for both cfDNA and tissue?)

Reply 16:

We performed targeted sequencing on both cfDNA and tissue using the same cancer panel, called Axen Cancer Panel 2 (containing DNMT3A) as described in the Method section. A few mutations from several genes were detected only in cfDNA. And the DNMT3A is one of the genes for which cfDNA-only mutations are most frequently found. DNMT3A is well-known as a gene related to clonal hematopoiesis, and these cfDNA-only mutations of DNMT3A were possibly clonal hematopoiesis of indeterminate potential (CHIP) mutations. But we did not segregate the CHIP mutations in this study.

Changes in the text:

No change in the manuscript for this reply.

17. Line 256 & 277 "PD" acronym was never introduced

Reply 17:

We appreciate the meticulous review. We have added full text where the acronym "PD" was first used.

Changes in the text:

"The advantages of ctDNA analysis, such as the representation of tumor heterogeneity and dynamics, drug response monitoring for patients with progressive disease (PD), and the ability to detect genomic markers by minimally invasive procedure, have been reported in several studies." (Page 5, Line 87-90)

18. Figure 1 b label of Y-axis should be μg/μL not ug/ul

Reply 18:
We appreciate the reviewer's comments. We agree that it was an incorrect unit notation. In fact, it was ng/μL so that we have changed the Figure 1b label of the Y-axis.
Changes in the text:

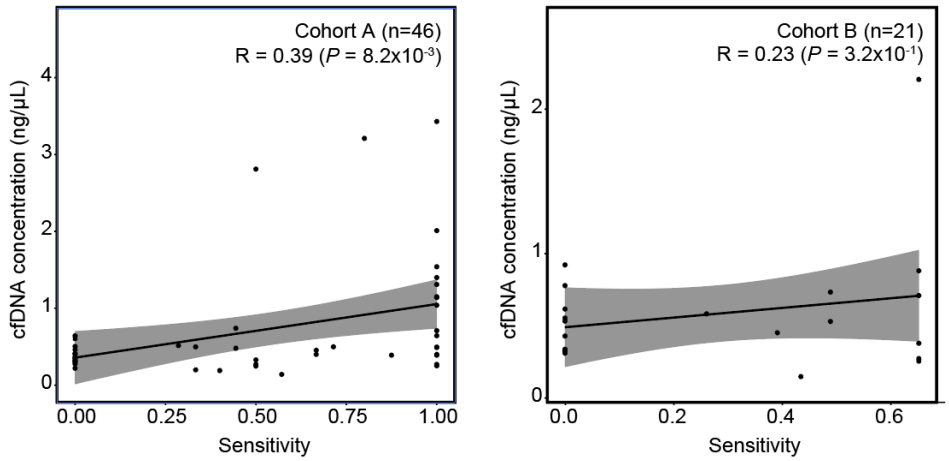


Figure 1b label of the Y-axis is changed. (Figure 1B)

19. I feel the authors should discuss clinical relevance of whether they/their data suggest ctDNA as better (clinically more practical) source of biomarker information at least as fast, economic first line approach (and if not informative tissue should still be tested if available)?

Reply 19:
As we describe in the manuscript, the cfDNA/ctDNA analysis has the potential for a prognostic biomarker and the possibility of an alternative use for molecular tumor diagnosis, but insufficient sensitivity for variant detection is still a limitation. Hence, we suggest ctDNA analysis as a supplementary approach for tumor tissue biopsy at present, but want to emphasize its potential to be more useful and better source of tumor information in the future. We have changed and added the description of clinical relevance in the Conclusions section.
Changes in the text:
"In conclusion, we demonstrated the potential of cfDNA in blood samples as a biomarker for the prognosis of advanced lung cancer at diagnosis and the possibility of alternative or additional use of ctDNA sequencing as molecular tumor diagnosis before and after treatment; however, insufficient concordance in variant detection between tumor tissue and ctDNA sequencing results remains a limitation. We suggest ctDNA analysis as a supplementary approach for tumor tissue biopsy at present, but improvement of the ctDNA analysis will make more clinical utility and will replace the tumor biopsy in the future." (Page 14, Line 362-368)

minor English language editing necessary

Reply (minor):
Thanks for the reviewer's comments. We have tried to revise and improve the manuscript with English proofreading.

Reviewer E

The authors in this study investigated the clinical utility of ctDNA in patients with NSCLC. They divided the patients into 2 cohorts: newly diagnosed, treatment naive and recurrent disease with driver mutations. The prognostic value of ctDNA has been described extensively in the literature. The new/ interesting aspects of this study: they prospectively enrolled patients and looked at correlation between ctDNA concentration and sensitivity. They also described 2 patients where EGFR T790M was detected on ctDNA and not in tissue at relapse.
I agree with the authors, the sequencing data they have is insufficient to calculate, hence I wonder what the value of is adding this analysis to the manuscript, and would recommend removing this section.

Reply 1:
We appreciate the reviewer's comments. But unfortunately, we cannot figure out which part of the analysis the reviewer recommended that we remove. We cannot be sure about the analysis that the reviewer mentioned, but it was probably about TMB calculation. In that case, we would like to carefully maintain that it is worthwhile to report the results of TMB calculation from ctDNA sequencing and the correlation of TMB to other clinical variables though the technical limitations.
Changes in the text:
No changes in the manuscript for this reply.

The figures and tables are well done and organized. In table 1, the authors need to merge some of the rows to avoid confusion. For example, in smoking the "not available" row is not separated. This needs to be applied to PD-L1, EGFR, ALK and MTT (I hope I was able to convey my suggestion).

Reply 2:
We appreciate the detailed review and comment. We have merged some of the rows that the reviewer pointed out (the table cells of PD-L1, EGFR, ALK and MTT).
Changes in the text:
The table cells of PD-L1, EGFR, ALK and MTT in Table 1 have been changed.

As I explained in the box above, the study describes the prognostic value of ctDNA which is well established in the literature. They have a few interesting/ new aspects to their study which I mentioned above. I think it's a well-done study that is worth publishing, if the authors were able to address the reviewers' comments/ suggestions. Thank you for inviting me to review this manuscript.