Peer Review File

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Reviewer A

Comment 1: The abstract is somewhat confusing. I suggest re-writing the abstract with more clarity and with 1) clarification on the prospective nature of cfDNA sample collection, 2) summary or cohort overview at the beginning of results section before moving on to detailed description of results.

Reply 1: The abstract has been modified according to the reviewer's suggestions with clarification on the prospective nature of cfDNA sample collection, as well as a cohort overview at the beginning of Results

Changes in the text: We have modified the abstract as advised (mainly lines 48-52, changes have been highlighted).

Comment 2: Table 1 is crowded. To improve its readability, consider changing the format to n (%), instead of % followed by number and denominator.

Reply 2: The format of Table 1 has been changed to to n (%), instead of % followed by number and denominator in order to improve readability according to the reviewer's suggestion.

Changes in the text: We have modified Table 1 as advised (changes have been highlighted).

Comment 3: The authors may want to clarify two things: 1) Since there were 133 events of radiographic changes among 56 patients, it is likely that a patient had more than one radiographic change event. How was this handled in the analysis? 2) Was there a difference in survival or time to treatment failure among cfDNA positive patients who received subsequent TKI-based vs chemotherapy-based regimens?

Reply 3: 1) The number of progression events (and analyzed samples) per patient is now given in the first section of the results (mean value 2.46, range 1-7, page 9, lines 194-196). Since primary endpoint of our study was the relationship between ctDNA results and radiologic changes, events/samples from the same patient were analyzed as independent. The biologic rationale for this, is that there is considerable heterogeneity between different progression events in the same patient, for example successive progression events occur at different sites (intracranial vs. extracranial), develop with different rates, under treatments of different potencies (TKIs of various generations or chemotherapy), present with varying tumor loads etc. All these factors have a significant impact on the probability that the liquid biopsy drawn at progression will be positive of negative for each patient, and they affect different patients in a similar manner. According to the suggestion of the reviewer, the independent handling of all samples in this study has now been clarified in the Methods section of the revised manuscript (page 8, lines 175-180) .

2) Based on the suggestion of the reviewer, we provide these results here: there was no significant difference in time to treatment failure among ctDNA positive cases with subsequent TKI vs. chemotherapy regimens: 3.1 vs. 3.8 months, respectively p=0.39. The indifference in unselected patients is not surprising, because whether TKI or chemotherapy would help more

in this setting, depends on whether the detected ctDNA alteration is resistant or sensitive to available TKI. Regarding overall survival of ctDNA positive patients from the time of liquid biopsy, it was significantly longer for subsequent TKI vs. chemotherapy treatment: 20.1 vs. 7.0 months, respectively, p=0.0003. This is difference is probably secondary, due to the fact that chemotherapy is usually given after TKI options have been exhausted. Therefore, we have not included these results in our manuscript.
Changes in the text: The Methods section has been modified as advised (page 8, lines 174-179, highlighted). We also provide the number of samples per patient in the main text (page 9, lines 194-196, highlighted).

Reviewer B

In this manuscript, data from 139 samples from 56 ALK positive NSCLC is reported. Authors mainly found that detection of ctDNA is associated with poorer outcome and extracraneal disease. The association of ctDNA levels with prognosis has been extensively documented by many authors, even in the subset of ALK + NSCLC. Likewise, it has been extensively documented that detection of ctDNA is challenging in patients with brain mets exclusively. Therefore, these results merely confirm what it is already known. As far as I am concern, the association of ctDNA detection and variant 3 ALK-EML4 or TP53 mutations is less documented and authors could stress the novelty of these findings. NGS data is not well presented in the manuscript. In addition, I am not sure about the statistical methods. As a far as I understood, samples are considered as independent while many of them are from the same patient. I am not sure how appropriate is this in terms of calculations of HRs. In addition, there are some technical issue that are not well presented in the manuscript as listed below

Comment 1: Data from 139 samples are presented. These samples correspond to 56 patients. The number of samples analyzed per patient should be specified in the main text. On the other hand, a supplementary table specifying which samples comes from which patients should be available. Line of treatment should be also be indicated. Are the same mutations detected over the course of treatment? Did the number of mutations increased over the number of line treatments?. Did ctDNA levels increase with sequential diagnosis of disease progression?
Reply 1: According to the recommendation of the reviewer, we have now specified the number of samples analyzed per patient in the main text (first paragraph of the Results section: mean value 2.46, range 1-7, page 9, lines 194-196). The detectable alterations generally increased over the number of treatment lines and with sequential disease progressions, as indicated by the significantly higher number of previous treatment lines for liquid-biopsy-positive vs. negative samples in Table 1 (2.8 vs. 1.5, $p<0.001$, line 18 of Table 1). We have already published this phenomenon in more detail in a recent study (Figure 2 of PMID 33161228) and it is therefore not a main subject in the current work. A detailed overview of all samples, including the line of treatment during which each sample was drawn, is given in supplementary Figure 1. In this Figure, the study material has been arranged according to liquid biopsy positivity (on the left) vs. negativity (on the right), in order to convey the main results of the study, instead of an arrangement according to individual patients, because many patients had both positive and

negative samples at different progression timepoints over the course of their disease, which would disrupt the positive/negative classification and impair visual interpretability. Using *TP53* mutations as an example, as shown in the new supplementary Table 2 (which was prepared as part of the response to comment 4 of the same reviewer below), the detected mutations could be similar or differ over the course of the disease in each patient. One important factor that influenced ctDNA positivity at the time of disease progression was the site of disease of progression: when progression was restricted to the central nervous system, liquid biopsies were mostly negative (Figure 2 and suppl. Figure 1).
Changes in the text: The Results section has been modified as advised (page 9, lines 194-196, highlighted). The legend of Supplementary Figure 1 has been improved (page 2 of the Supplements).

Comment 2: If I have understood well, this samples are considered as individual samples (in the statistical analysis) while they are paired samples as they come from the same patient.
Reply 2: Indeed, all samples are considered individually in the statistical analysis. This is because primary endpoint of our study was not the paired comparison between samples of the same patient across different treatment lines (which could, for example, have served to analyze the increase in detectable mutations over the course of the disease, as discussed in the answer to comment 1 above), but the relationship between ctDNA findings and the anatomic pattern of disease progression. The biologic rationale for the independent handling relies on the considerable heterogeneity between different progression events in the same patient: for example, successive progression events occur at different sites (intracranial vs. extracranial), develop with different rates under treatments of different potencies (TKIs of various generations or chemotherapy), present with varying tumor loads etc. All these factors have a significant impact on the positivity or negativity of the corresponding liquid biopsy and change between progression instances in the same patient, while affecting different patients in a similar manner. Based on the comment of the reviewer, we have now clearly explained that in the Methods section of the revised manuscript (page 8, lines 175-180).
Changes in the text: The corresponding passage in the Methods has been modified accordingly (page 8, lines 174-179, highlighted).

Comment 3: Cutoff in terms of MAF for a positive call is not specified.
Reply 3 According to the recommendation of the reviewer, we have now added the cut-off in terms of MAF for a positive SNV call in the reviewed manuscript, which was 0.01%. The CAPP-Seq technology with integrated digital error suppression, on which the method used in this work was based, has *per se* a lowest limit of detection at 0.004% AF (PMID 27018799, Ref. 27 of the manuscript).
Changes in the text: The corresponding passage in the Methods has been modified as advised (page 7-8, lines 162-164, highlighted).

Comment 4: TP53 mutations detected in tumor tissue samples showed a trend for association with ctDNA positive calls. Please specified the mutations detected in a table. Please also include in which samples were the TP53 mutations detected. Were the same mutations detected in the matched plasma sample?

Reply 4: Based on the suggestion of the reviewer, we have now added a new supplementary Table 2 that shows the *TP53* mutations detected in tumor samples for each of the study patients. This table also shows the *TP53* mutations detected subsequently in ctDNA samples for each patient. In addition, the ctDNA results in conjunction with *TP53* results from tumor tissue samples and all NGS results from ctDNA samples are shown in the suppl. Figure 1 (the tissue *TP53* results at baseline (BL) are the 4th from the top variable in the annotation). As shown in the new suppl. Table 2, for some patients the *TP53* results in baseline tissue samples and subsequent liquid biopsies at progression were concordant, while for others they were discordant. This is not surprising, as it is known that both liquid biopsies and tissue NGS can miss some mutations (for tissue NGS this can for example happen due to the spatial heterogeneity of *TP53*, e.g. PMID 27646734), and also that novel *TP53* and other mutations can emerge during the course of the disease.
Changes in the text: A new suppl. Table 2 has been added as advised (page 5 of the Supplements). The Results section has been modified accordingly (page 10, lines 230-233), and a new paragraph has been added in the Discussion regarding these results (page 14 lines 306-311). The legend of suppl. Figure 1 has been improved (page 2 of the Supplements).

Comment 5: Two different panels were used. Only genomic regions covered by both panels were considered for the analysis. Please specify the regions and the genes included in these regions.
Reply 5: According to the suggestion of the reviewer we have now specified the genes and regions covered by both panels and considered in analysis precisely in the Methods section of the revised manuscript
Changes in the text: The Methods section has been modified as advised (page 7, lines 150-157, highlighted).

Comment 6: Line 105: " the outcome……" please specified that is the outcome in terms of survival.
Reply 6: According to the suggestion of the reviewer, we have now specified the outcome as survival, i.e. progression-free survival, time-to-next treatment, and overall survival.
Changes in the text: The corresponding text has been modified as advised (page 5, lines 101-102, highlighted).

Comment 7: Line 174: Please specified which variables were included in the cox model. HR were adjusted by which variables?
Reply 7: According to the suggestion of the reviewer, we have now specified that the Cox model included the liquid biopsy result (positive or negative), type of *EML4-ALK* variant, baseline *TP53* status, baseline ECOG performance status, the number of treatment lines before liquid biopsy, and whether treatment was switched or continued beyond progression after the liquid biopsy.
Changes in the text: The corresponding text has been modified as advised (page 8, lines 171-174, highlighted).

Comment 8: Lines 201-203: regarding infrequent cases with CNS-only and positive liquid

biopsies: Were these patients diagnosed as having meningeal carcinomatosis? Was the blood brain barrier clearly damage in these patients??

Reply 8: Based on the suggestion of the reviewer, we have now provided more details in the Results: apart from the higher intracranial tumor load, there was no other distinguishing radiologic characteristic of the rare cases with isolated CNS progression and positive liquid biopsies; for example, radiologic evidence of meningeal carcinomatosis was present in 2/8 cases with isolated CNS progression and a positive liquid biopsy *vs.* 2/31 cases with negative liquid biopsies. As brain metastases are known to disrupt the blood brain barrier (which also causes the contrast-enhancement in CT/MRI studies), 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. This has also been added in the Discussion of the revised manuscript.

Changes in the text: The Results (page 10, lines 213-217) and the Discussion (page 15, lines 336-339) have been modified as advised (highlighted).