Peer Review File

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Reviewer Comments

-The title is misleading. Of the 17 pages of the main body of the review, only 7 deal with liquid biopsy. Also, although the authors focus on lorlatinib, they also review the literature about other ALK TKIs. A possible alternative title would be "NGS in NSCLC patients with ALK rearrangements, a focus on liquid biopsy and lorlatinib"

Reply: we thank the reviewer for suggesting a new title, we have modified the title to: "Next Generation Sequencing using liquid biopsy in the care of patients with ALK-rearranged Non-Small Cell Lung Cancer, a focus on lorlatinib."

-Lines 96-98. Testing for ALK fusions is mandatory for treatment with ALK TKIs

Reply: the sentence we had written was referring to the fact that the development of new generation ALK inhibitors, biomarkers of resistance to guide subsequent ALK therapies was not required. We have modified the sentence (lines 96-99): "However, unlike T790M mutation status to select treatment with osimertinib in EGFR-mutant lung cancer, the selection of new generation ALK inhibitors after crizotinib did not include mandatory biomarker assessment of resistance mechanisms to guide treatment to subsequent ALK TKIs (1,2)

-Lines 116-117. Several resistance EGFR mutations have also been described in patients progressing to third generation EGFR TKIs

Reply: we have added the following sentence and citation in line 117: "*Resistance mutations to third-generation EGFR inhibitors, such as C797S have also been described using liquid biopsy (9). Moreover, characterization of C797S mutation in cis or trans with T790M, has therapeutical implications, with reports of response to the combination of first and third generation EGFR inhibitors in patients with mutations in trans (10).*"

-Lines 144-147. Virtually all ALK-positive NSCLC patients have an EML4-ALK fusion, other partners are extremely rare. This fact should appear clearly in the text

Reply: we have added the following data to the sentence (lines 149): "Echinoderm microtubule-associated protein-like 4 (EML4) gene is the most common ALK fusion partner, present in 81% of ALK-positive NSCLC (18–20)".

-Lines 166-168. Without treatment selection? Please clarify what you mean

Reply: we have removed the phrase "without treatment selection" and the sentence is as follows (line 170): "The median progression-free survival (PFS) of patients treated with upfront crizotinib was reported at 10.9 months, and the median PFS with second-generation inhibitors given sequentially ranges from 5.4 months to 15.6 months (27–29)"

-Line 176. Please specify median PFS in months for both studies.
Reply: we thank the reviewer for this suggestion we have added the PFS values in line 179: "the median PFS was also significantly superior with the second-generation ALK inhibitor (24.0 vs 11.0 months; HR 0.49, p = 0.001) (29)"

-Line 189. The frequency of G1202R mutation in patients progressing to crizotinib is lower than 8%
Reply: we have corrected this sentence in line 193: "Contrarily, the solvent front ALK G1202R mutation, present in about 2% of crizotinib samples, is the most common ALK-dependent resistance mechanism in patients treated with second-generation ALK inhibitors (~40%) (1,38)".

-In line 206 the frequency of G1202R is 44% in v3, in line 210 only 3%. Please clarify or provide an explanation
Reply: We thank the reviewer for this correction it was a typing mistake the correct number is 32%, we have changed this value in line 215: "(32% vs. 0%, p = 0.001) (22)."

-Lines 236-237. Lorlatinib is also active first line, as even the authors mention later (line 263)
Reply: We updated the data from the CROWN trial publication in NEJM that led to the approval in the first line in line 296: "Moreover, objective response was significantly higher in the lorlatinib group (79% vs 58%), with 70% of patients in response at 12 months. In addition, lorlatinib treatment resulted in higher intracranial responses (66% vs 20%) and central nervous system (CNS) time to progression, 96% of patients without CNS progression at 12 months with lorlatinib vs 60% with crizotinib (45). This subsequently led to the FDA-approval of lorlatinib as a first-line treatment option in treatment naïve patients."

-Line 290. Please specify median PFS in months for both studies.
Reply: Median PFS was added in line 295 "median PFS: Not Reached vs 9.3 months; HR: 0.28 [95% CI: 0.19-0.41])"

-Line 306. Please provide the rationale for giving the patient an HSP90 inhibitor
Reply: the inhibition of HSP90 disrupts oncogenic signaling in crizotinib resistance preclinical models (Sang J et, Cancer Discovery 2013). The patient received the

treatment in a clinical trial. We haven't added more information about this rational in order to keep the flow towards the concept that wants to be portrayed in this paragraph. We modified the sentence in line 314 "The patient continued treatment with ceritinib experiencing primary progression. an HSP90 inhibitor in a clinical trial, followed by standard chemotherapy

-Lines 338-362. A table listing compound mutations and associated sensitivities would be of great help to the readers
Reply: we thank the author for this suggestion, we have developed Table 1 with characterized compound mutations and effect on ALK inhibitors.

-Line 391. Reference 48 does not make sense here
Reply: we moved the citation (52) upwards, the sentence is now: "Acquired MET amplification has been well known to cause resistance to EGFR inhibitors, and clinical trials combining EGFR TKIs and selective MET inhibitors, like osimertinib and savolitinib in the TATTON trial, have shown encouraging clinical results (52")

-Line 397. NGS is a technique (same as FISH or IHC), not a biomarker.
Reply: we changed the title to (line 406) "The role of liquid biopsy NGS to study response and resistance to lorlatinib"

-Lines 410-422. The paragraph is confusing, particularly at the end, and should be extensively re-written. In the case of ALK, IHC can be used and only requires a few tumor cells. In consequence, it is not clear that "liquid biopsies can overcome the limitations of small biopsies", as the authors claim, since small biopsies are often enough to determine ALK by IHC. Also, the authors do not mention that the sensitivity of liquid biopsies is significantly lower for fusion detection than for mutation detection. This fact should be mentioned, and reports about the sensitivity of liquid biopsies vs. tissue should for fusion detection be included and discussed.
Reply: we thank the author for this suggestion, we have extensively re-written this paragraph from (line 419): "Liquid biopsy is an alternative tool to study ALK fusions at diagnosis when tissue is unavailable, though the sensitivity of NGS in plasma to detect ALK fusions ranges from 67% to 91% (54,55). Patients in which ALK rearrangements are detected by liquid biopsies, as expected, also benefit from treatment with ALK inhibitors. In the BFAST trial in 2219 patients screened using Foundation Liquid NGS assay, ALK-rearrangements were found in 5.4% of plasma samples. Patients with ALK-rearranged NSCLC detected by liquid biopsies achieved an ORR of 92% and a 12-month PFS rate of 78.4%."

-Lines 423-427 and 438-445 are repetitive and should be removed
Reply: we have changed the wording to improve the understanding of the concept we want to portray in this paragraph. In the first part we are referring to PFS according

to ALK variants. In the second part we support this by showing higher rates of acquired ALK mutation in variant 3. The paragraph is (line 427): "Pretreatment determination of the type of EML4-ALK rearrangements might have clinical implications in the future. As previously addressed, plasma biomarker study of the ALEX trial showed that in patients with EML4-ALK rearrangement detected in plasma the median PFS with alectinib was 34.8 months for variant 1, 24.8 months for variant 2, and 17.7 months in variant 3, though this difference was not statistically significant (26). However, in a biomarker analysis of the phase III ALTA1L study comparing frontline treatment with brigatinib to crizotinib, PFS was significantly shorter in patients with variant 3 EML4-ALK rearrangements compared to variant 1 treated with brigatinib [HR 2.38 (95% CI: 1.04-5.5)] and crizotinib [HR 2.96 (95% CI: 1.44-6.09). This could be explained by the fact that EML4-ALK variant 3 tumors have higher rates of acquired ALK resistance mutations (44.4% variant 1 vs. 75% variant 3) and ALK G1202R mutations (0% in variant 1 (0/9) vs. 50% (4/8) in variant 3) compared to variant 1 EML4-ALK fusions (22). In another study evaluating the use of plasma NGS with InVisionFirst-Lung assay from Inivata, 37% of EML4-ALK variant 3 fusions had ALK kinase domain mutations compared to 13% with variant 2 and 0% in variant 1 fusions, and all G1202R mutations were seen in variant 3 EML4-ALK rearrangements (54)."

-Lines 446-453 should not be there, they should be moved to the part of the manuscript where resistance mutations are described

Reply: we thank the reviewer for this suggestion, this sentence was removed from the manuscript as it is hypothetical for the moment.

-Lines 470-472. An 82% specificity is not acceptable in the clinical setting. The authors should mention this fact.

Reply: we have added the following phrase to the paragraph (line 460): "Using tissue biopsies from "de novo samples" as a reference, the sensitivity of plasma NGS for ALK mutations was 61% and the specificity was 82%, with an overall accuracy for plasma NGS of 73%, which needs further improvement"

-Lines 473-499. The authors only discuss Guardant (and conclude there is no role for it) but ignore that many other techniques have been used for ALK mutation detection in plasma

Reply: For the moment, this is the only published study to address the efficacy of lorlatinib according to the pre-treatment study of ALK mutations in the clinical setting. This study was done with Guardant 360 as a biomarker testing in the phase II lorlatinib clinical trial.

-Line 483. "De novo" samples is not an accepted terminology

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Reply: we have modified the sentence removing "de novo" and switching for "Using tissue biopsies from lorlatinib pre treatment samples" in line 460.

-Lines 500-507. The type of NGS used should be specified
Reply: The type of NGS is specified in line 491 "using Guardant 360"

-Lines 534-525. The previous paragraph already describes the use of NGS for "the study of lorlatinib resistance mechanisms"
Reply: we thank the author for this suggestion we have removed the sentence: "Another clinical scenario in which plasma NGS can be implemented is in the study of lorlatinib resistance mechanisms". We also moved the reference for table 3.

-Lines 593-594. Absolute MET copy number >2.1 is not a standard criterion for MET amplification, >5 or higher are actually used.
Reply: we agree with the reviewer that the cutoff to determine MET amplification in this study is lower than in other reports. We have modified the sentence in line 583: "Among 106 plasma samples, MET focal amplification (defined in this study as absolute MET copies ≥ 2.1 based on the validation study of plasma comprehensive cancer genotyping assay".

Comment to the reviewer:
We apologize as we inverted the order of Table 2 and 3, now this has been amended.

Comment to the Editor:
We have changed the order of authors according to the contribution in this review. Currently: "Juan B. Blaquier[1], Andrés F. Cardona[2,3,4], Alessandro Russo[5], Christian Rolfo[5], Gonzalo Recondo[1]"