Peer Review File

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Reviewer Comments

Comment 1: Both in the Abstract and Discussion, it is not clear which genetic events are considered the main resistance mechanisms in this patient. They should be stated more clearly. The current formulation in both sections is vague and a bit speculative, as if the authors are not sure about these mechanisms.

Reply 1: Thank you for your critical comments. The patient progressed rapidly and we did not found a good therapy to allow prolonged responses. Longitudinal assessment of progressions by rebiopsies and/or ctDNA showed potential different mechanisms of resistance to ALK-TKIs. Ultimately, we speculate that EMT induced by the deletion of FBXW7 and MLL3 may represent the main mechanism of resistance to ALK-TKIs. But it is too late when we realized it and no more rebiopsy was available to validate. We revised the manuscript accordingly (line 40-41, 170-177).

Comment 2: Molecular methods are poorly described. For ex., it is not clear how the apparently DNA-based (or was there also RNA analysis involved?) method utilized in the study detects gene fusions such as ALK-fusions. Please explain more in detail the sequencing method for detecting SNV, CNV and fusions in tumor biopsies and in cfDNA. Any commercial kits/platform for NGS should be cited (manufacturer's name etc.), and possibly briefly described if it's not been done previously (in case cite related reference/s).

Reply 2: Thank you for the advice. We have added the information in line 68-70 of revised manuscript and Supplementary Method.

Comment 3: Figure 1: The CT-scan figures are small and not very clear. To make them more reader-friendly, please mark somehow the most significantly changes (regression or persistence of lesions, appearance of new lesions). The most significant genetic alterations related to each progression could be shown in the figure as separate drawing or included in the text under the scans.

Reply 3: Thanks for your thoughtful suggestion. We have now indicated the relevant changes by arrows in the revised figure 1 (with revised legend accordingly).

Comment 4: Table 1: The mutations detected in the patients during the time course are interesting, but they should be described more accurately. There are many mutations shown in table 1, but there is no mention at all on which are verified (in COSMIC and possibly other databases) as pathogenic mutations and which are VUS or known polymorphisms. In particular, FBXW7 and other mutations that are present from baseline throughout the time course (TP53, SETD2, MLH1, POLD1) should be described in terms of pathogenic vs VUS, as they are stably present, and occur in genes involved in major pathways of tumor

suppression and DNA repair. Related references, if available should be cited to support a role of FBXW7 and, in case, the other 3 genes in ALK-TKI resistance. The authors focus on the FBXW7-mutation, a this was potentially targetable with mTOR inhibitors, but the potential involvement of the other genes in TKI-resistance should also be discussed.
Reply 4: Thank you for pointing this out. We mainly focus on the FBXW7-mutation, because we have found no direct involvement of the other genes (SETD2, MLH1, POLD1) in ALK-TKI resistance by analysis of the current body of literature. Base on other comments, we further assessed the association between mutations with EMT. We have modified our text (line 142-147, 167-172) and table 1 as advised.

Comment 5: VUS and polymorphisms, if any, should not be shown, to avoid confusion in terms of relevance of the mutation displayed in table 1.
Reply 5: Thank you for your comments. We updated table 1 and retained some key variants.

Comment 6: The time course could be illustrated better as it is somewhat confusing and difficult to follow with so many different dates for CT scans in figure 1 and genetic aberrations in table 1. It's an additional reason for making a drawing with tumor history (baseline + progressions), focusing on the most relevant mutations.
Reply 6: We are appreciative of the reviewer's suggestion. We revised some descriptions about the time course instead labeled them with the dates that those events occurred in table 1. We also added the most relevant mutations in figure 1 accordingly.

Comment 7: The MET gene amplification detected by NGS should be validated at the protein level by IHC. This would show whether there is Met receptor protein overexpression in tumor cells, otherwise it is not sure that MET amplification translates into a biologically relevant event and therapeutic target.
Reply 7: Thank you for your comments. To our knowledge, there is growing evidence that MET IHC may not be a good screening test for MET amplification or METex14 mutation in lung cancer (J Thorac Oncol. 2018 Dec;13(12):1962-1967. doi: 10.1016/j.jtho.2018.08.008. J Thorac Oncol. 2019 Sep;14(9):1666-1671. doi: 10.1016/j.jtho.2019.06.009. J Thorac Oncol. 2020 Jan;15(1):120-124. doi: 10.1016/j.jtho.2019.09.196.), considering how frequently MET IHC is positive in lung cancers. Moreover, multiple trials have used MET IHC as a predictive marker for MET-directed therapies, but have largely been unsuccessful (J Clin Oncol. 2017 Feb;35(4):412-420. doi: 10.1200/JCO.2016.69.2160. Lancet Oncol. 2016 Dec;17(12):1661-1671. doi: 10.1016/S1470-2045(16)30561-7.). In contrast, ongoing studies with MET tyrosine kinase inhibitors have seen more success with use of high MET copy

numbers (gene copy number >5) and MET/CEP7 ratios as predictive markers (J Clin Oncol. 2018 Nov 1;36(31):3101-3109. doi: 10.1200/JCO.2018.77.7326.). So we did not validate the protein level of MET by IHC.

Comment 8: Results, line 61-62, "The result revealed EML4-ALK fusion (AF=14.5%) with loss of the amplification of MET (Table 1).": CNV loss in cfDNA may be due to non-shedding of the amplified gene, real elimination of the amplified clone, or too little ctDNA in the plasma sample. A negative result for a relevant mutation should ideally be confirmed on a tumor re-biopsy, to see whether it is a real "loss of target". The mechanisms implicated in this case should in any case be discussed.
Reply 8: Thank you for your constructive comments. We revised the manuscript accordingly (see Page 6, line 128-134).

Comment 9: The rationale for treating the patient with cabozantinib and anlotinib should be briefly specified.
Reply 9: Thank you for the advice. We have added the information in line 93 and line 100-101 of revised manuscript.

Comment 10: Line 70-71, "The overall tumor molecular load increased": explain in a more articulated way what this means with regards to the mutations shown in Table 1.
Reply 10: Thank you for your comments. We revised the manuscript accordingly (line 97-99).

Comment 11: Line 93-95, "Surprisingly, in addition to the EML4-ALK fusion …, MET amplification was detected": Perhaps "surprising" should be avoided , as MET-amplification is a well-described off-target mechanism of acquired resistance to EGFR- or ALK-TKIs in NSCLC given that it activates a parallel pathway bypassing the EGFR or ALK blockade by TKIs. The initial response to Crizotinib is not surprising either, as this is indeed a well-known dual MET-ALK inhibitor.
Reply 11: Thank you for the valuable comments. We revised the manuscript accordingly (line 123).

Comment 12: Line 101-104, "According to the result of tissue and ctDNA sequencing, secondary mutations of the ALK gene were not detected during the disease progression, neither phenotypic changes such as small-cell lung cancer transformation nor epithelial-to-mesenchymal transition in pathology, partly known mechanisms of ALK-TKI resistance": Transformation to SCLC or EMT are not detectable by ctDNA sequencing, thus it is not clear how the authors reach this conclusion w/o showing/mentioning any data in this regard. Have they investigated these possible changes in tumor re-biopsies taken from the patient?

Reply 12: The third tumor rebiopsy taken from hepatic metastases was obtained for immunohistochemical examination. Pathologic findings showed adenocarcinoma with positive immunohistochemical staining for CK8/18 and negative staining for CK5/6, P40, synaptophysin and CD56. Transformation from NSCLC to SCLC was excluded. But we also found that the tumor cells had almost completely lost the expression of the adenocarcinoma-marker CK7 and TTF1. And we did not perform IHC analysis by using the epithelial marker E-Cadherin and the mesenchymal marker Vimentin. We should not exclude the possible presence of EMT. So this conclusion is not religious and we deleted this paragraph (line 135-141).

Comment 13: In this respect, FBXW7 inactivation is known to partly induce TKI-resistance by promoting EMT (Mol Oncol. 12:883-95, 2018. doi: 10.1002/1878-0261.12200). Moreover, EMT has recently been implicated in resistance to lorlatinib in patient-derived cell lines (Clin Cancer Res 26:242-55, 2020. doi: 10.1158/1078-0432.CCR-19-1104) and to alectinib and lorlatinib in patients receiving these drugs (Int J Mol Sci. 21:2847, 2020. doi: 10.3390/ijms21082847). This should be discussed by the authors in relation to their case. The possible presence of EMT should be investigated not only by histology, but also confirmed using markers such a positive immunostaining for vimentin and loss of E-cadherin expression or analyzing the expression of transcription factors and other genes involved in EMT.
Reply 13: Thank you for raising this interesting issue. We agree with the reviewer entirely and we added some data in line 164-175. Unfortunately, rebiopsy was not enough for further IHC analysis. This suggestion reminded us to examine features of EMT after TKI resistance.

Comment 14: Line 113-114, “However, a combination of ALK-TKI and mTOR inhibitor in our case did not seem to overcome ALK-TKI resistance”: The negative result in the patient should be interpreted with caution as Liza et al.'s case was EGFR-wt and w/o ALK-fusions. In addition, the combination lorlatinib and everolimus was attempted in the current case as 6th line of therapy not 1st/2nd line. Finally, Liza et al. used another mTOR inhibitor, which might also be relevant for the clinical response.
Reply 14: We strongly agree with this comment. We have added the information in line 154-157 of revised manuscript.

Comment 15: The non-reciprocal ALK-translocation should be shown and its clinical relevance explained more accurately.
Reply 15: Thank you for the advice. We have added the information in line 178-184 of revised manuscript.

Comment 16: Line 124-5 should be “In conclusion, we report the case of an EML4-ALK fusion-positive NSCLC patient, who progressed rapidly”.

Reply 16: Thank you for your comments. We revised the manuscript accordingly (line 185-186).

Comment 17: Check thoroughly the reference list: reference 4, 8, 9, 10, 11, 14, 15, and 17 are w/o journal name … Then use the journal names as indicated in the instructions to authors. Right now, some journals are spelled out, others are abbreviated.
Reply 17: Thank you for your comments. We revised the reference accordingly.

Comment 18: Correct to proper English on: Line 30, “progression may be have occurred”; line 76, “he was given with lorlatinib”; line 86, “improves prognosis treatment-naive ALK-positive”; line 116, “reduced Mcl-1 expression which independent of restoring PI3K/Akt”.
Reply 18: Thank you for your comments. We revised the manuscript accordingly (line 54, 115, 158-162).