# Peer Review File

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**First round of peer review**

**Reviewer A:**

1. The major concern is the lack of adequate information in the procedures of obtaining the first biopsy (EBUS, mini video-assisted thoracotomy, other).

Answer:

Thanks to the reviewer's careful reading and pointing this important question out. We have completed the information. The procedure of obtaining the first biopsy sample is by EBUS. It is rectified at Page 4, Line 63 in red.

2. Also,the second biopsy after osimertinib therapy, it was from a metastatic site. There were biopsies from tumor tissue?

Answer:

Thanks to the reviewer's careful reading. The biopsy sample after osimertinib treatment was in the left upper lobe of the lung and the procedure of obtaining it was by EBUS. It is rectified at Page 6, Line 108-109 in red. Accurately it was not a metastatic site, the sample was obtained from the primary site of the lung.

3. Were liquid biopsies performed through the evolution after second line therapy with chemotherapy and bevacizumab?

Answer:

Thanks to the reviewer's careful reading and questions. The answer to this question is that the sample was obtained by pulmonary puncture guided by CT. It is rectified at Page 5, Line 90-91 in red. Usually, tumor tissue is the "gold criterion" for the diagnosis in pathology and molecular pathology. Compared with tumour tissues, ctDNA is easily obtained non-invasively (or minimally) and can be a specific and sensitive biomarker for the detection of *EGFR* mutations in patients with NSCLC. According to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology, a plasma ctDNA assay could be used to identify *EGFR* mutations, only if tumor tissue is limited or insufficient for molecular testing. When the tumor tissue is not enough to perform NGS,

clinicians could use liquid biopsy instead. The tumor tissue obtained after progressed with "bevacizumab plus carboplatin plus pemetrexed" by pulmonary puncture guided by CT was enough to carry out NGS, so we didn't use liquid biopsy.

4. Presumably, liquid biopsies were also carried out since at least four different targeted NGS analysis were done. The first 8-gene panel (Buring Rock), a second 56-gene panel (Burning Rock) in a second tumor biopsy? (In which site was the biopsy taken and with which method?).

Answer:

Thanks to the reviewer's careful reading, we apologize for the carelessness of this case report. We are sorry that lacking of enough explanations in each molecular test misled you. To be more clearly and in accordance with the reviewer's concerns, we have corrected the deficiencies both in the manuscript and in the table. Indeed, there were 2 different liquid biopsies during the treatment.

The biopsy sample of the first 8-gene panel was obtained in the left pulmonary hilum by EBUS. This biopsy sample was made into FFPE not in cell free DNA. It is rectified at Page 4, Line 63-67 in red.

The biopsy sample of the 56-gene panel was obtained in the tumor tissue of the left lung using pulmonary puncture guided by CT. This sample was made into FFPE, too. It is rectified at Page 5, Line 89-91 in red.

The time axis of two liquid biopsy is as follows:

The first liquid biopsy was performed after using gefitinib, and it was tested on two platforms at the same time: ddPCR and NGS（A plasma ctDNA-associated 9-gene panel (Anoroad, China)）. Because *EGFR* T790M mutation is the most common acquired resistance mechanism for the first *EGFR* TKI, the main objective of that liquid biopsy testing was to determine if there was *EGFR* T790M mutation developed after progressed with gefitinib. Usually, ddPCR is much more sensitive than NGS in testing *EGFR* T790M mutation, so we used both ddPCR and NGS to perform the testing sufficiently. However, the results were just *EGFR* exon 19 deletion and *TP53* mutation. It is rectified at Page 4, Line 74-76 in red.

The second liquid biopsy sample was taken after using bevacizumab plus sindilimab. Besides *EGFR* exon 19 deletion and *TP53* mutation we found *EGFR* T790M mutation in this 9-gene panel (Anoroad, China) NGS testing. It is rectified at Page 5, Line 92-93 and in red.

5. The metastatic sites from which tumor tissue was retrieved should be reported as well as if liquid biopsies were also performed.

Answer:

We are glad to receive reviewer's recommendations, and we are sorry for our negligence. All the tumor

tissue obtained to perform molecular testing were from the primary tumor site in the left lung. We have marked liquid biopsies and FFPE separately both in the manuscript and in the table.

6. The evolution of metastatic sites is not included as well as if radiotherapy was delivered from metastatic lesions.

Answer:

Thanks to the reviewer for pointing this question out, and we have completed it according to your ideas. The patient had CNS metastatic when she came to our hospital the first time. Although she had a metastatic lesion in the left frontal lobe, she didn't receive radiotherapy during the treatment. The evolution of metastatic sites has been completed in the manuscript. It is rectified at Page 4, Line 61-63; 68-71.

7. No data on toxicity of the treatments, no information on the performance status trough the progress of the disease.

Answer:

We appreciate it very much for this good suggestion, and we have completed it according to your ideas. There were hepatic damage, allergy, urinary retention and vomiting. We have completed this part of information at Page 5, Line 85-88 in red.

8. Osimertinib was not better than gefitinib neither in PFS nor in OS in the FLARA trial in the subgroup of Chinese patients. Soria et al. N Engl J Med 207, Ramalingam et al. N Engl J Med 2019). Conversely and interestingly, furmonertinib (also a third-generation EGFR tyrosine kinase inhibitor) was superior compared with gefitinib as first-line therapy in Chinese patients with EGFR mutation-positive NSCLC with an acceptable toxicity profile (Shi et l. Lancet Respiratory Medicine 2022).

Answer:

Thanks to reviewer's careful reading and kind reminder. The reason why clinicians used osimeitinib was that the disease of patient progressed after taking gefitinib, and *EGFR* T790M mutation was found in the fourth NGS. Osimertinib selectively inhibits both *EGFR* sensitive mutation and *EGFR* T790M resistance mutations, with lower activity against wild-type *EGFR*. Besides due to the good blood–brain penetration of osimertinib and the fact that this patient developed CNS metastases when she came to our hospital for the first time. So osimeitinib was a good choice at that time. We have completed this part of information into the discussion at Page 9, Line 161-167.

We all agree with you that furmonertinib targets both sensitising *EGFR* and *EGFR* T790M mutations. And furmonertinib is more suitable for Chinese patients compared with osimeitinib. We have completed this part of information in discussion at Page 11, Line 218-220 and Page 12, Line221. However, the time of furmonertinib approved for marketing was not consistent with the time at which the patient suffered NSCLC. Besides, there was no relative clinical trials in our hospital at that time. This was also one of the reasons why we chose osimertinb.

9. Other observations to keep in mind immunotherapy is not effective in patients with EGFR mutant Nsclc, regardless the PD-L1 tumor expression . The patient hava 90% PD-L1 expression after osimertinib progression. In EGFR mutant lung adenocarcinomas increase B7-H4 expression, an alternative immune-checkpoint molecule associated with inhibition of CD8+ T-cell function (Lu et al. Oncogene 2021). Note that EGFR e746-A750 deletion mutation induces also anergic dendritic cells to repress antitumor immunity through exosomes (Oncogene 2020).

Answer:

Thanks to the reviewer's careful reading and useful suggesting. We have read the literatures you recommended carefully. These two literatures make the mechanism of PD-L1 inhibitors are not effective for patients with *EGFR* exon 19 deletion profound.

Lu et al. found that *EGFR* mutation can increase the level of *BH-H4* by activating MEK/ERK pathway. Increased *B7-H4* downregulates the level of granzyme B, eventually the cascade makes the expression of PD-L1 diminished. Also, there is a background that in the normal condition *EGFR* exon 19 deletion inhibits the function of $CD8^+$ T cells. However high expression of PD-L1 leads to reverse the inhibition. With the help of $CD8^+$ T cells, the tumor-growth was repressed and patients gain longer survival in NSCLC. However, there is a paradoxical point in this case that the expression of PD-L1 was more than 90% in the patient who carried *EGFR* exon 19 deletion. This phenomenon is not consistent with the signaling pathway put forward by Lu et al. We think that the reason maybe individual difference or processing problems in the assay of immunohistochemistry.

Yu et al. shed a new train thought that *EGFR* exon 19 deletion induces anergic dendritic cells to repress antitumor immunity through exosomes. The new knowledge broadens our horizon. Thanks to the reviewer again.

As suggested by reviewer, we have added the suggested content to the manuscript at Page 10, Line

190-200 in red.

10. The patient still preserves EGFR T790M muttion after osimertinib progression (Figure 1B). Notice that T790M is preserved in 50% of patients following osimertinib treatment. Also, co-occuring mutations could be different between the sub-group with T790M preserved and those progressing with T790M loss. See Le et al. Landscape of EGFR-dependent and-independent resistance mechanisms to osimertinib and continuation therapy beyond progression in EGFR-mutant NSCLC. Clin Cancer Res 2018.

Answer:

Thanks to the reviewer's careful reading and we are grateful for the suggestion. We are all admire you for your profound knowledge in this field. We have read the literature carefully. There are two different opinions between the case report and the literature you recommended.

Firstly, the majority concurrent mutations happened in this patient were different with those in the literature except for *TP53*. In this patient, the new gene mutations happened after *EGFR* T790M mutation were *BTN2A1-BRAF* fusion, *TP53* p.K132R, *TGFBR1* p.D104y, *MYC* amplification and *FANCG* amplification in addition to *EGFR* exon 19 deletion in the finally 1021 panel by NGS.

Second, according to this literature, the resistant mechanisms to osimertinib in patients who progressed with *EGFR* T790M preserved are *EGFR* reactivation or bypass pathway. However, in this case *BTN2A1-BRAF* fusion, the off-target acquired mutation, was the only one worthful mutation identified. So we think *BTN2A1-BRAF* fusion maybe the mechanism that leads to resistance to osimertinib.

Thank you for your comments, the discussion regarding this question is presented at Page 7, Line 124-131 in red.

11. See also Schoenfeld et al. Tumor analysis reveals squamous transformation and off target alterations as early resistance mechanisms to first line osimertinib in EGFR-mutant lung cancer. Clin Cancer res 2020. See the oncoprints in Gigures 1 and 2. The authors found a case with a BRAF fusion, BRAF-TRIM24

Answer:

Thanks to the reviewer's careful reading and recommended literatures. We have read this literature carefully. We agree with you that besides *BTN2A1-BRAF* fusion, there are a few other *BRAF* fusions found to be a resistant mechanism to osimertinib.

In the literature above, *TRIM24-BRAF* fusion is developed after progression with the first-line osimertinib. While genomic alterations about *BRAF* fusion developed after later-line osimertinib are *AGK-BRAF* and *MRPS33 – BRAF*. Both *AGK-BRAF* and *MRPS33 – BRAF* are happened in patients

with *EGFR* T790M lost. The difference between this literature and our case report is that *BTN2A1-BRAF* fusion is acquired in the patient who still harbors *EGFR* T790M mutation after progressed with osimertinib.

The number and kinds of reported *BRAF* fusions are relatively rare. In this case report, it is the first time to report a new discovered *BTN2A1-BRAF* fusion which other researchers don not notice before. We think that the opinion that *BTN2A1-BRAF* fusion maybe the reason why the patient is resistant to osimertinib is the innovative discovery for clinicians in the field of NSCLC.

According to the reviewer's comment, we have provided more details about this part of information at Page 7, Line 132-138 in red.

12. In lung cancer other BRAF fusions have been reported in KRAS mutant lung cancer patients following therapy with adagrasib such as AKAP-BRAF and NRF1-BRAF (Awad et al. N Engl J Med 2021). Other studies reported fusions in the EGFR-RAS signaling pathway, such as TMEM87A-RASGRF1 (Cooper et al. Clin Cancer Res 2020) or OCLN-RASGRF1 (Hunihan et al. Clin Cancer Res 2022.

Answer:

Thanks to the reviewer's careful reading and recommended literatures. We have read this literature carefully. We are sorry to find that in the first recommended literature (Awad et al. N Engl J Med 2021) *AKAP9−BRAF* and *NRF1−BRAF* developed in a patient suffered colorectal cancer not lung cancer.

We have read the literature you recommended (Cooper et al. Clin Cancer Res 2020). We agree with you that *BRAF* fusion not only happens in lung cancer, but also in many other cancers, such as colorectal cancer, thyroid cancer, primary brain tumors, melanoma and so on. Indeed, the rate of BRAF fusions discovered in lung cancer is relatively few. As we all know that there is a signaling pathway about *RAS-RAF-MEK-ERK*. *RASGRF1* belongs to *RAS* gene. *RAS* is the upper gene of *RAF* in the *RAS-RAF-MEK-ERK* signaling pathway. According to Cooper et al. truncated *RASGRF1* loses the regulatory domain in the N terminal, and *TMEM87A-RASGRF1* is proved to be resistant to osimertinib. The difference between *TMEM87A-RASGRF1* and *BTN2A1-BRAF* is that the fusion of *RAS* conduces to activate *RAS*-GTP, which is the active status of *RAS*. There are three conserved regions (CR) in *BRAF*. CR1 has the *RAS*-binding domain and cysteine-rich domain, CR3 has the protein kinase domain, and CR2 bridges CR1 and CR3. When *BTN2A1* fuses with *BRAF*, *BRAF* gene loses CR1 and CR2. In summary, *RAS* and *RAF* are two different genes, the ways of fusion and the fusion partners varies much.

Cooper et al.think that *TMEM87A-RASGRF1* is like *AKAP9-BRAF* which happened in thyroid cancer.

Actually, the structure and the ways to form a fusion between *AKAP9-BRAF* and *BTN2A1-BRAF* are alike. Firstly, they both lose the CR1 and CR2 in the N terminal. Second, they both retain the protein kinase in the C terminal. There are 3 different points between *BTN2A1-BRAF* and *AKAP9-BRAF*. First is the fusion partner, second is the fusion site and the third is the classification of tumors in which they are found. In our opinion the discovery of *BTN2A1-BRAF* fusion enlarged the field of research of resistant mechanisms to osimertinib, and this fusion highlights a new train of thought for clinicians.

We have also read the literature of *RASGRF1* Fusions Activate Oncogenic *RAS* Signaling and Confer Sensitivity to *MEK* Inhibition (Hunihan et al. Clin Cancer Res 2022). We agree with the authors that their findings nominate the *RAF-MEK-ERK* pathway as a potential therapeutic target in *RASGRF1*-rearranged tumors. We appreciate the reviewers' insightful suggestions and agree that it would be useful to demonstrate the difference between *RAS* and *RAF*. However, *RAF* and *RAS* are two different genes, they are both significant in the oncogenic process in variable tumors. Although there is something alike between them, the distinctions still occupy much.

13. Tracing oncogene rearrangements it is of great interest as in this case: An oncoprint showing all the co-mutations and alterations found
with the use of the targeted 1021 gene panel (Geneplus) could also enrich the quality of the manuscript.

Answer:

Thank you for your grateful suggestions. We have listed all the gene mutations tested in the 1021 panel in Table1 and in the manuscript at Page 6, Line 103-106 in red and also in figure 1B.

14. One limitation of the study is the lack of in vitro evidence (a cell line model) to demonstrate the oncogenic potential of the BTN2A1-BRAF.

Answer:

Thanks to the reviewer's careful reading. We agree with you that more study or more data would be useful to explain this potential mechanism. In the future we could design an experiment about *BRAF* fusion and test its sensitivity to *EGFR* TKIs, we may explore the mechanism which lead to resistance to osimertinib and make an effort to find out the suitable drugs for *BRAF* fusion.

We have completed this part of information into the discussion at Page 12, Line 221-224.

Thank you for your careful review. We really appreciate your efforts in reviewing our manuscript during this unprecedented and challenging time. We wish good health to you, your family, and community. Your careful review has helped to make our study clearer and more comprehensive.

**Reviewer B:**

1. Second line: E for exon should be smaller.

Answer:

Thanks to the reviewer's careful reading and suggestions. We are very grateful that the reviewer pointed the typos out. We have modified this expression throughout the text according to your comment.

2.Also why did authors use bevacizumab with osimertinib, can they reference a study on which this regimen is based on?

Answer:

Thanks to the reviewer's careful reading. Tyrosine kinase inhibitor (TKI) increases the level of VEGF by inhibiting *EGFR*, so if *EGFR* and VEGF can be inhibited at the same time, the growth of tumor will be controlled. (Herbst et al. J Clin Oncol 2005) According to the literatures bevacuzumab plus *EGFR* TKI can improve the PFS of patients in NSCLC than *EGFR* TKI monotherapy. (Saito H, et al., Lancet Oncol. 2019 May;20(5):625-635. Qin Zhou et al. ESMO 2019 1480O.) Therefore, clinicians used bevacuzumab with osimertinib for this patient.

3.Last line in abstract does not make any sense, please rephrase or delete.

Answer:

Thanks to the reviewer's careful reading and useful suggestion. We have rephrased it into "This case demonstrates that BTN2A1-BRAF fusion potentially serves as a mechanism of acquired resistance to osimertinib in non-small-cell lung cancer and could offer a new train of thought for treatments to patients alike." It is rectified at Page 2, Line 34-36.

4.What PD1Li has to do with osimertinib?

Answer:

Thanks to the reviewer's careful reading. Indeed, PD-L1 inhibitor has limited help or little help with patients harboring *EGFR* mutation in NSCLC. Clinicians found the expression of PD-L1 was high. And at that time the patient was treated multiline of drugs, but the efficacy was still not obvious. So, they thought to try PD-L1 inhibitor due to its high expression. However, the outcome was not satisfied.

5. Case description: There is type “it should be exon 19 deletion”.

Answer:

Thanks to the reviewer’s careful reading. We are very sorry for the mistakes in this manuscript. Errors have been corrected in the manuscript throughout the manuscript.

6. Last line in case description does not make sense, please rephrase this line.

Answer:

Thanks to the reviewer’s careful reading. We are sorry that “All procedures performed in studies were in accordance with the ethical standards of the institutional and national research committee(s) and with the Helsinki Declaration (as revised in 2013).”is the format of this journal, and we could not rephrase it.

7. Introductions: It should be “deaths” not death in line 1.

Answer:

Thanks to the reviewer’s careful reading and useful suggestions. We are very sorry for this mistake and the error has been corrected at Page 3, Line 42.

8. Line 67: T790 mutation was identified

Answer:

Thanks to the reviewer’s careful reading. We are very sorry for the mistakes in this manuscript. The error has been corrected at Page 5, Line 92.

9. Line 70: absorbed is wrong here, please rephrase the sentence.

Answer:

Thanks to the reviewer’s careful reading and useful suggestions. We have changed “absorb” into “clear up”. It is rectified at Page 5, Line 97 in red.

Thank you for your precious comments and advice. Those comments are all valuable and very helpful for revising and improving our paper. We have revised the manuscript accordingly, and our point-by-point responses are presented above.

**Second round of peer review**

1. Discussion is too long, and some points are misleading, especially with regards to TMB.

Among a total number of 4017 NSCLC patients there was no correlation between tumor mutational

burden (TMB) and outcomes for immunotherapy. See Figure 3 in the article (Negrao et al. Oncogene-specific differences in tumor mutational burden, PD-L1 expression, and outcomes from immunotherapy in non-small cell lung cancer. J Immunother Cancer 2021).

Answer:

Thanks to the reviewer's careful reading and pointing this important question out. We are grateful for your recommended literature. The information of TMB has been deleted, and we are sorry for our negligence. Besides we have simplified the discussion about the expression of PD-L1, "Whereas there is a suspicious point that the expression of PD-L1 of this patient is as high as 90%. In the normal condition, with the help of CD8+ T cells, the tumor-growth was repressed and patients gain longer survival in NSCLC. EGFR exon 19 deletion inhibits the function of CD8+ T cells. High expression of PD-L1 can reverse the inhibition and leads to progression of disease. Lu et al. found that EGFR mutation can increase the level of B7-H4 by activating MEK/ERK pathway. Increased B7-H4 downregulates the level of granzyme B, eventually the makes the expression of PD-L1 diminished." has been changed to "Most data report that patients with EGFR driver mutation have a low expression of PD-L1." Please see the content at Page 11, Line 201 in underline.

2. The last statement on furmonertinib is arguable since there is not a difference in the mechanism of action with respect to Osimertinib or other 3_G EGFR TKIs.

Answer:

Thanks to the reviewer's helpful suggestion. We have deleted this part of information in the manuscript.

3. BRAF inhibitors and/or MEK inhibitors or triple combinations with ERK inhibitors should be considered to overcome resistance to BTN2A1-BRAF.

Answer:

Thanks to the reviewer, we appreciate it very much for this good suggestion. We have completed it according to your ideas. It is rectified at Page 11, Line 212-214 in underline.

4. The authors should recognize the limitations of the study as they did not carry out preclinical experiments.

Answer:

Thanks to reviewer's careful reading and kind reminder. We have completed this part of information in the manuscript, it is rectified at Page 11, Line 215-216 in underline.