Peer Review File

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Reviewer A:

This manuscript is interesting but a little poorly written, but more description for the case presentation and more figures are needed, otherwise, this manuscript is not appropriate for this journal. And the Discussion is not sufficient.

> **Author response/ Changes in the text:** Thank you for your time in reviewing our work and providing helpful suggestions to improve our manuscript. We have revised the manuscript to improve the language and also added more information including the duration of crizotinib response (please refer to page 2 and 5, lines 37 and 92 to 93) and discussion (please refer to page 5, line 104 to 109). Since we were not able to obtain tumor specimen of the patient harboring AMBRA1-ALK for in vitro studies, and no gall bladder cell lines stably expressing AMBRA1-ALK and WT ALK were available from our lab so we were not able to provide in vitro and in vivo experimental data and elucidate the crizotinib sensitivity of AMBRA1-ALK.

Reviewer B:

This manuscript shared with us a stage IV case of gallbladder cancer which achieved 7 months' partial response to crizotinib. Using NGS, a new AMBRA1-ALK fusion gene was identified. The authors indicated that such partial response was due to this new fusion gene. The authors concluded that the ALK gene could be considered as a biomarker for GBS and NGS should be done too.

This case report could have filled our gap of effective target therapy towards GBC if following concerns were well presented, discussed, and explained.

1. Major revisions to make

(1) The biggest innovation of this case is the finding of this new fusion gene and its potential relationship with partial response. However, the authors have to prove this point by well-organized discussion. It's not persuasive by just saying 'it's reasonable to consider'. I don't think it's reasonable.

First, please discuss the present literature on the association between ALK and gallbladder cancer. It's mentioned in the manuscript by 'unexplored' on line 122. However, I do find some related literature. See: PMID32373528, PMID31815765, PMID24508317 etc.

> **Author response/ Changes in the text:** Thank you very much for your time in thoroughly reviewing our work and providing very detailed suggestions to further improve our manuscript. We have thoroughly revised our manuscript to incorporate all your suggestions wherever possible. More related literatures were added in both the introduction and discussion of the revised manuscript. Indeed ALK mutation F1174L has been reported for GBC (PMID: 24508317) as well as ALK overexpression in biliary tract cancers (PMID: 27136744); however, reports on ALK fusions in these cancers are very limited. These information are mentioned in the introduction section in lines 79-81 of the revised manuscript with track changes.

Second, please explain how the AMBRA1 gene could be associated with gallbladder cancer. Give us your hypothesis. It's reasonable to consider this by pathways like autophagy, apoptosis, T cell regulation etc.

Author response/ Changes in the text: The general function of AMBRA1 is described in lines 162-165. The role of AMBRA1 in tumorigenesis is mentioned in lines 166-167. A report demonstrating overexpression of AMBRA1 in cholangiocarcinoma and its correlation with poor prognosis has been added in lines 69-71 and 167-168 of the revised manuscript with track changes.

Third, please explain how the AMBRA1-ALK gene could be associated with GBC. Show your hypothesis to make it reasonable.

Author response/ Changes in the text: Since AMBRA1 is a tumor suppressor gene that plays crucial role in regulating autophagy and survival and ALK plays important roles in cell development, the fusion gene could bring about deregulated cell proliferation and promoting tumor development. We have included a more detailed explanation of this hypothesis in lines 181-186 of the revised manuscript with track changes.

Please do notice that the AMBRA1-ALK fusion gene is totally different from the ALK gene. Therefore, it's not correct to conclude in the abstract that 'This case suggests that the ALK gene could be considered as a biomarker for GBC'. Do make sure your phrasing is correct in the whole manuscript.

Author response/ Changes in the text: Thank you for pointing this out. We have revised this statement in the abstract to reflect that a subset of GBC might be driven by aberrant ALK signaling and is thus can be a potential biomarker of response to ALK inhibitors. This revised statement can be found in lines 45-47 of the revised manuscript with track changes.

(2) It's mentioned in the manuscript 'strongly suggesting that the novel AMBRA1-ALK genomic rearrangement also induces ALK protein overexpression'. Unfortunately, it's not persuasive. You have to give your hypothesis that it's this fusion gene that induces overexpression. You can't just give such a conclusion. To prove this, a gene knock-out or overexpression animal model is usually used. However, as this is a single case report, the authors should explain enough at least.

Author response/ Changes in the text: Thank you for pointing this out. Since no other genetic alteration was detected from the archived tumor specimen except for AMBRA1-ALK, we associated the detection of AMBRA1-ALK to the ALK overexpression status observed by IHC. The hypothesis on how the fusion induces ALK overexpression is indicated in lines 179-181 of the revised manuscript. The need for in vitro and in vivo functional studies to confirm the oncogenicity and ALK inhibitor response of AMBRA1-ALK is indicated in lines 186-188 and as limitation in lines 220-222 of the revised manuscript with track changes.

(3) Explain how you chose the dose of crizotinib and discuss how this might have affected the partial response and imply future treatment.

Author response/ Changes in the text: The dose was chosen based on the standard dose of crizotinib approved for NSCLC. This detail has been included in lines 191-194 of the revised manuscript.

(4) Provide evidence of progression after the first-line chemotherapy.

Author response/ Changes in the text: We do not have the baseline radiology scans since the diagnosis was made at a different hospital. Upon referral to our center after progression from first-line chemotherapy, MRI was performed prior to administering crizotinib (Figure 1B).

(5) Among the 520 tested genes, please clarify the status of those proved most common genes of GBC like EGFR, HER2, KRAS, FGFR1-3, SRC etc.

Author response/ Changes in the text: These genes were wild-type for this patient. This information that no other genetic alterations were detected using the targeted gene panel was included in lines 119-120 of the revised manuscript with track changes.

(6) Give your rationale of why not using surgery and radiation, while instead, having decided to do the NGS. As we could see suggested interventions in the GBC guideline.

Author response: Localized therapy, including surgery and radiotherapy, was not performed due to the presence of extensive metastatic lesions in the liver, omentum, and hilar lymph nodes of the patient. This has been included in lines 111-115. Lines 95-98 also mentioned that only palliative biopsy was performed due to the discovery of a lesion in the hepatic hilar region.

(7) Finally, in terms of the recommendation and a thought-provoking suggestion. I suggest the authors be more objective.

a. It's mentioned on line 142 that 'NGS should be considered for treatment decisions in patients with GBC'. As we know, at present target therapy is not favourable in GBC. I do think 'could' would be more appropriate than 'should'.

b. Similarly, considering previous poor results regarding target therapy and this partial response case, I would suggest the authors think and discuss how such finding implies in the whole bigger picture of GBC. How GB would be totally different from other cancers like lung cancer because of this finding? What may be our next direction from the inspiration we gain from this case?

Author response/ Changes in the text: The recommendations have been toned down to mentioning that our report provides a clinical evidence of the utility of NGS in exploring actionable mutations for therapeutic options in rare tumors including GBC. Similar to lung cancer, GBC also have complex and vast genetic heterogeneity based on numerous studies which reported the various aberrant genes in GBC and biliary tract related cancers. These changes are indicated in the abstract in lines 47-51 and the conclusion in lines 229-233 of the revised manuscript with track changes.

2. Revisions regarding the CARE GUIDELINE CHECKLIST

(1) Checklist 1: please add 'case report' int the title.

(2) Checklist 2: please add 'case report' as one of the keywords too.

(3) Checklist 3a: WHAT is unique is not well highlighted in the abstract.

(4) Checklist 4: WHY it's unique should be well introduced with enough literature in the introduction.
(5) Checklist 5c: family history and the history of this patient needs revision in detail. For instance, a family history of lung cancer (as ALK is one of the key gene mutations of lung cancer) and gallbladder cancer, and a history of lung cancer of this patient should be clarified.
(6) Checklist 7: a timeline with accurate time is needed. Make sure the figure stands alone.
(7) CheckList 10b: prognosis (latest information) of the patient after the second progression is needed.
(8) Checklist 11a: I suggest authors use one separate paragraph to LOGICALLY LIST both strengths and limitations of this case.

Author response/ Changes in the text: Thank you for pointing these out. These details have been incorporated in the revised manuscript. Case report in title and keywords indicated in lines 4 and 53 of the revised manuscript. What and why the case is unique were mentioned in the abstract in lines 42-44 and introduction in lines 79-81. Her lack of family history of cancer is indicated in lines 110-111. A timeline summarizing her treatment history is illustrated in Figure 1A. Details of her treatment after progression from crizotinib is included in lines 127-131. And a paragraph each listing the limitation and conclusion of our case report are in lines 217-233 of the revised manuscript with track changes.

3. Other minor revisions to make
This manuscript needs English polish to be more readable.

Author response: The manuscript has been thoroughly polished. We hope you find the revised manuscript more readable and more informative.