Peer review file

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Reviewer A

Comment 1: The reason that 20-50% of mesotheliomas that have negative BAP1 IHC do not show mutations by Sanger or NGS is because this technique is reliable to detect nucleotide level mutations, not small or larger deletions that are common in mesothelioma. This has been clearly demonstrated and there is no reason to create confusion here suggesting mechanisms of post translational regulation account for this large number of cases- see and cite: Nasu M et al., JTO 2015; Yoshikawa Y et al., PNAS 2016; Carbone M et al., Ca Cancer 2019 and Cancer Discovery 2020. So explain this and tone down the later discussion in which you attribute the 20-50% difference to miRNA, maybe they contribute but there is a very clear cut explanation already for the discrepancy between Sanger/NGS and IHC, see references above.

Reply 1: Thank you for the suggestions. As suggested, the discrepancy has been clarified either in the introduction (page 3-4, line 63-70) or in the discussion (page 10, line 234-235). As well, the suggested references have been added.

Comment 2: Following up on the comment above, the choices of the reference list are difficult to understand, rather than citing original references and papers in high impact factor journals, the authors cite references of papers that confirmed data or were in minor journals. There are reviews on BAP1 and its clinical implications in mesothelioma Ca Cancer 2019 –the journal with the highest IF- and reviews in Cancer Discovery and Nature Review Cancer 2020. Please read these reviews, it will help you to have a more complete understanding of the topic and cite original references.

Reply 2: We agree. The suggested references have been included in the manuscript.

Comment 3: The authors should clarify the very different survival implications of

germline versus somatic BAP1 loss. BAP1 loss is associated (maybe) with a small survival gain –according to some studies when the mutation is somatic, but with a dramatic increase in survival when the meso patient carries a germline BAP1 mutation –that almost always becomes biallelic mutation in tumor cells (Baumann F et al., Carcinogenesis 2015, Pastorino S et al., JCO 2018; Panou et al., JCO 2018; Hassan R, PNAS 2019 and references cited above.

Reply 3: Thank you for the suggestions. The implication of the somatic and germinal mutations on the patient outcome has been mentioned in the discussion (Page 10, line 220-225), and the suggested references added.

Comment 4: How was asbestos exposure "documented"? This needs to be clearly stated. If is based on questions asked by the nurse or the physician to a patient for example is totally unreliable, if it is based on objective clinical or pathological evidence it is reliable. If the patient worked in a well-established asbestos trade –say asbestos miner, Ethernit, etc- is reliable.

Reply 4: We fully agree. The asbestos exposure was established either by the presence of benign asbestos-related diseases (fibrosis, pleural plaques and asbestosis) or according to the occupational tasks of the patients, who were working at the asbestos-related industry (page 4, line 83-87).

Comment 5: Since the authors discuss in detail BAP1 IHC, could they please show a nice figure of IHC? See Nasu et al., JTO 2015 and Carbone et al., Cancer Discovery 2020 for reference to correctly interpret the IHC.

Reply 5: A representative figure depicting the BAP1 has been included as Figure 1, and BAP1 revealed by IHC has been discussed at page 10, line 220-225.

Comment 6: The logic is a bit circular. If miR-31 is a post-transcriptional regulator of BAP1, its high expression could interfere with BAP1 if the latter is present.

Reply 6: Most likely, miR-31 by targeting BAP1 may regulate its expression. However,

we have to take in account that in tissue other miRNAs, as well other mechanisms, may be involved in the regulation of BAP1 expression. Unfortunately, due to the total RNA degradation, we couldn't detect BAP1 expression in FFPE samples where miR-31 was analyzed.

Comment 7: The authors argue that miR-31 was highly expressed in s-MPM compared to e-MPM the data supporting this is not shown because in Fig.3 only e-MPM is represented. The authors need to show the data.

Reply 7: The distribution of miR-31 expression among the three MM histotype was shown in the figure 2, now in figure 3.

Comment 8: The authors should have investigated BAP1 gene expression, in the same RNA extracts used to assay miR-31. If possible this should be done.

Reply 8: Due to total RNA degradation in FFPE tissues, we couldn't detect BAP1 expression in these samples.

Comment 9: Some suggestions to improve the manuscript are listed below:

(1) start with representative BAP1 IHC staining of MPM patients tumor samples; continue with Fig. 1 (but insert the y-axis definition).

Reply 9 (1) : A representative image of BAP1 IHC has been included as Figure 1. The y-axis definition has been added in the Figure 3.

(2) then switch to data on s-MPM, insert this in table 2 (OS-PFS) more data are needed to show the functional interrelation between miR-31 and BAP1.

Reply 9 (2) : As suggested, multivariate analysis in non-epithelioid MPM (s-MPM and b-MPM) has been added in Table 2.

(3) in figure 3B miR-31 is highly expressed in 9 samples, in these 9 samples analyze and correlate the BAP1 levels to OS and PFS.

Reply 9 (3): OS and PFS have been evaluated also in patients with highly miR-31 expression and the data shown at page 9, line 194-197.

Comment 10:
Minor comments:
• Figure Labels: All figures need to be uniform in size, font
• punctuation errors in references

Reply 10: The figure size and punctuation in references have been corrected.

Reviewer B

MAJOR COMMENTS

Comment 1: This study comprises a small number of patients only and should likely be considered as preliminary in nature or a 'first look' at this combination of factors. The results from this study would require validation in another cohort of cases. The preliminary nature of this study should be mentioned in the abstract and conclusion; accordingly, the findings of this study should be softened.

Reply 1: We fully agree. MPM is a rare tumor; therefore, the cohort included small number of patients. As suggested, the preliminary character has been added in the abstract (page 2, line 43), discussion (page 10, line 243), and conclusion (page 12, line 279).

Comment 2: The authors should stipulate that it was only on the univariate analysis that BAP1 loss was associated with an improved OS a finding that did not extend to the

multivariate analysis.

Reply 2: This notion has been mentioned at page 8, line 171-173.

Comment 3: The authors have included analysis of PFS, however have not specified how this was measured – in particular, the frequency of follow up.

Reply 3: The progression-free survival (PFS), were evaluated by HRCT analysis during the annual follow-up period, page 4, line 88-89.

Comment 4: Also, the follow up period of 14.2 months is short if the median survival is 18 months.

Reply 4: As reported at page 7, line 151, the sentence 'At a median follow-up of 14.9 months [95% CI: 7.7-22.1 months]…' , has been deleted. In our study population the censors cases were few (3 over 55 patients), thus the reverse Kaplan Meier method to estimate the median follow up is not suitable. The median OS estimate also the median of the follow up.

Comment 5: Several prognostic factors have not been factored into the analysis – most importantly, performance status. Other prognostic factors such as anaemia have not been included or discussed.

Reply 5: Thank you for the suggestion. The anaemia, as well, the systemic immune-inflammation index (SII) have been proposed as prognostic factor for MPM (Ming et al., Cancer Manag Res. 2019;11:3973-3979). By evaluating the anaemia and the SII, in univariate and multivariate model, we found that both biomarkers were not significant prognostic factors for the MPM at epithelioid histotype. These results have been discussed at page 11, line 258-260.

Comment 6: How was the asbestos exposure measured? Was this a reliable measure?

Reply 6: The asbestos exposure was documented by clinical and pathological evidences such as the presence of benign asbestos-related diseases (ARDs), and also by interview. The patients with ARDs and subjects who were working in the asbestos-related industry were considered exposed (page 4, line 83-87).

Comment 7: In the table – please check the numbers, at times they do not add up to 55 (for example, in the histological subtype breakdown, these add up to 53).

Reply 7: The table has been corrected according to the suggestion.

Comment 8: What is the practical value of this algorithm? By the authors own discussion there is a variability in the miR-31 findings between studies. Have the authors also assessed the miR-31 results between biopsy samples from the same patient (but different sites or timepoints) to ensure the miR-31 findings are consistent?

Reply 8: The variability in the miR-31 level is probably due to different factors, which include the individual variability and the intra- inter-assay variability. The intra- inter-assay variability was tested in circulating miRNA in a previous study (Tomasetti et al. Clin Biochem. 2012;45:575-81). Here, we did not perform the intra- inter-assay variability in tissue; it is supposed that the miRNAs are more stable in biopsies.

Comment 9: In line with the practicality – there is no discussion as to the fact that the miR-31 is adjacent to CDKN2A which is deleted in approximately 60% of all mesothelioma cases (and in other publications there is evidence that miR-31 is also deleted when this occurs). Would this be a more practical test as this is now routinely performed on mesothelioma cases?

Reply 9: We agree, miR-31 expression may be affected by CDKN2A deletion, but other mechanisms may regulate the level of miR-31 in tissues. Therefore, we can postulate that detecting CDKN2A deletion by FISH or by ddPCR in MPM tissues does not reflect the level of miR-31.

Comment 10: The authors refer to ‘non-epithelioid’ behaviour and worse clinical outcome. The concern here is that (as you have suggested) there is always a spectrum of outcome in patients within the same histological subtype. Suggesting that this more aggressive behaviour can be measured by a combination of BAP1/miR-31 is premature for reasons discussed above.

Reply 10: We agree. The statement ‘non-epithelioid behavior and worse clinical outcome’ has been deleted and the preliminary character of the study has been reported.

Comment 11:
MINOR COMMENTS
Spelling and grammar issues should be addressed as in places they make the manuscript difficult to understand.
For example:
Page 3, line 77/78 – the sentence beginning ‘Data on age, gender…’ is incomplete.
Reply: Corrected.

Page 5, line 142 – ‘The 10%..’ should be altered to ‘Ten percent …’ or something similar.
Reply: Changed.

Page 7, line 220 to 222 – The sentence beginning with ‘Most probably…’ is difficult to understand the point that is being made in this sentence is lost.
Reply: The ‘Most probably’ has been replaced with ‘probably’.