Peer Review File

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-Reviewer A-

We would like to thank Reviewer A for her/his constructive comments on our manuscript. Below are our answers to her/his comments.

The manuscript reports immunohistochemical findings about PD-1 and PD-L1 expression in archival mesothelioma tissue samples. The authors collected a nice sample size of 203 samples. Their findings showed that high expression of PD-L1 on tumor cells is a negative predictive factor for survival in MPM patients.

The manuscript is well written but lacks substantiating arguments for some of the findings. On top of that some important references in the mesothelioma research field are missing (see further comments). The authors claim three times to be "the first one to…" (lines 296, 332 and 347) while this is not the case. They should have a closer look into literature.

Comment #1: Line 80-81: in 2004 the FDA approved cisplatin + pemetrexed as first line treatment for mesothelioma. So far there is no official approval for cis + pem + bevacizumab in first line. This should be adjusted.

Reply #1: We thank Reviewer A for bringing this inaccuracy to our attention. We agree with the Reviewer, that although the addition of bevacizumab to the pemetrexed-cisplatin regimen improved both PFS and OS of newly diagnosed malignant pleural mesothelioma (MPM) patients in a large phase III trial [1], so far there is no official approval of pemetrexed-cisplatin+bevacizumab in first-line therapy. Therefore, we have modified the list of approved drugs administered in first-line setting accordingly.

Changes in the text #1: Bevacizumab was deleted from the list of drugs approved in first-line setting. The following sentence was revised in the "*Introduction*" chapter:

-page 4, lines 80-81: "Platinum-based chemotherapy (CHT) has been used in MPM treatment and still remains the backbone for current combination strategies [reference_5]"

Comment #2: Line 87 "… an unmet need to identify prognostic markers to predict outcome…": the way in which this sentence is worded is confusing because to predict outcome one would think you will look into predictive markers.

Reply #2: We thank Reviewer A for pointing out this issue. In order to improve the clarity and readability, now we clearly state that we are referring to overall survival (OS).

Changes in the text #2: The following sentence was revised in the "*Introduction*" chapter:

-page 4, line 93: The word "outcome" was replaced with "OS (overall survival)"

Comment #3: Line 96: typo "the prognostic role OF the tissue biomarkers … "

Reply #3: Thank you for pointing out this typo. The misspelling was corrected.

Changes in the text #3: The preposition "of" was inserted into the following sentence:

-page 5, line 102: "… the prognostic role of these tissue biomarkers to date remains unclear in lung cancer."

Comment #4: Line 127-129: the samples are from patients that were treated in the past but nowhere in the manuscript there is made a distinction between the different treatments neither do the authors describe if this might have an influence on the PD-1/ PD-L1 expression that they observe? One should elaborate on this in the manuscript results/discussion.

Reply #4: We do agree that different therapeutic agents might influence the PD-L1 expression in general. However, in our study, only a small fraction (11%) of the included patients received neoadjuvant platinum-based chemotherapy (CHT) prior to tissue sampling and all other samples were retrieved from CHT-naïve patients as stated in the "*Tissue samples*" subsection (page 6, lines 143-146). In addition, the dynamic changes in PD-1/PD-L1 expression caused by systemic CHT as a potential study limitation is also mentioned in the "*limitations*" paragraph of the "*Discussion*" section (page 16, lines 382-384: "although the majority of included patients were CHT-naïve at biopsy, the application of CHT prior to tissue sampling can possibly confound expression patterns").

The therapeutic agents (i.e. platinum-based CHT) and approaches (i.e. palliative radiotherapy) presented in the "*Treatment*" subsection mentioned by the Reviewer are referring to the treatment modalities after the diagnosis, and consequently after tissue sampling. Nevertheless, for a better understanding, we have revised the "*Treatment*" subchapter. Furthermore, in order to maintain the chronology, we also changed the order of the subsections, and now the "*Treatment*" subchapter is inserted after the "*Tissue samples*" and "*Immunohistochemistry*" subchapters.

Changes in the text #4: The order of the subsections was changed in the "*Methods*" chapter: the "*Treatment*" subchapter is now placed after the "*Tissue samples*" and "*Immunohistochemistry*" subchapters. Additionally, the following sentence was revised in the "*Treatment*" subchapter":

-page 8, lines 173-175: Accordingly, after the diagnosis and tissue sampling, patients were treated with either platinum-based CHT, palliative radiotherapy (RT), combined chemoradiotherapy (CHT-RT) or best supportive care (BSC).

Comment #5: Line 141: neoadjuvant chemotherapy might have an influence on the expression of PD-1/PD-L1 (references Sheng J, Sci Rep 2016 and Marcq E, Oncoimmunology 2017). Where there also tissue samples available from treatment naïve patients? The authors did not report whether previous treatments can have an influence on the expression. This is something that should be mentioned.

Reply #5: The Reviewer's comment is well taken. The suggested references were inserted into the "*limitations*" paragraph of the Discussion section. Please also see Reviewer A answer #4.

Changes in the text #5:

-The following references were inserted to the limitations paragraph of the Discussion chapter:

[37] Marcq E, Siozopoulou V, De Waele J, et al: Prognostic and predictive aspects of the tumor immune microenvironment and immune checkpoints in malignant pleural mesothelioma. Oncoimmunology 6:e1261241-e1261241, 2016

[54] Sheng J, Fang W, Yu J, et al: Expression of programmed death ligand-1 on tumor cells varies pre- and post-chemotherapy in non-small cell lung cancer. Scientific Reports 6:20090, 2016

-please see Changes in the text #4; Reviewer A, Comment #4

Comment #6: Line 145: clone E1L3N of the PD-L1 antibody was used. It has been reported that this clone results in poor staining of tumor cells and immune cells in comparison to other clones. Are the authors aware of this? Might it not be that their findings are an underestimation of the true PD-L1 expression?

Reply #6: We are grateful for this observation. We understand that PD-L1 antibodies used today (including E1L3N) are very similar, but not interchangeable. Notably, however, there is a plethora of publication demonstrating that there are no relevant differences in staining when using this clone in comparison to other clones [2-4]. Meanwhile, others found that the antibody

clone E1L3N might indeed show poor staining in both tumor cells and stromal/immune cells [5]. Furthermore, another group reported that the staining intensity and quality might also be influenced by the tumor type and affected organ [6]. All is all, there is no clear consensus on the pathological relevance of different PD-L1 antibody clones. We have decided to use this particular antibody clone because at the time point when this study was initiated there was not enough data concerning the differences between different clones and, moreover, this antibody was already tested and used in our routine histopathology diagnostics. In addition, the PD-L1 antibody clone E1L3N was already used and validated in several other studies and clinical trials as well [2, 4, 7, 8]. Yet, we fully agree with the Reviewer, that the antibody clones might as well have an impact on our results. Therefore, we now clearly state this in the "*limitations*" section of the *Discussion* chapter.

Changes in the text #6: The following sentences were inserted into the "*study limitations*" subsection of the "*Discussion*" chapter:

-page 16, pages 378-381: In this study, we used the commercially available E1L3N antibody for PD-L1 staining. Importantly, however, not all antibody clones show a similar staining pattern and positivity [reference_52]. Therefore, our results should preferentially be considered when using the E1L3N antibody clone.

Comment #7: Line 209: why only looking at PD-1 expression on TILs while it might also be expressed in tumor cells (reference Marcq E, Oncoimmunolgy 2017). Did the authors observe any positivity on tumor cells after PD-1 staininig of the tissue samples?

Reply #7: Thank you for raising this point. Although it was not mentioned in the manuscript text, we did evaluate the PD-1 staining on tumor cells (TCs) as well. However, we did not observe any positive reaction in our cohort. We now clearly state this in the "*Results*" and "*Discussion*" chapters. Of note, in the study mentioned by the Reviewer (Marcq E, et al. Oncoimmunology 2017), PD-1 expression on TCs was also rarely seen (only 4 of 54 cases showed TC PD-1 expression). Additionally, it is important to mention, that they used a different antibody clone for PD-1 staining. We have revised the "*Discussion*" chapter accordingly and the aforementioned article is now mentioned in the manuscript text.

Changes in the text #7: The following sentences were inserted to the "*Results*" and "*Discussion*" chapters:

-page 10, lines 223-224: Meanwhile, PD-1 expression was analyzed solely in TILs because we did not observe any positivity on TCs.

-page 14, lines 317-322: In our study, PD-1 expression of TILs could be measured in 164 patients, whereas we did not observe any PD-1 positive TCs. Our results are in line with the findings of Marcq and colleagues who demonstrated that PD-1 is expressed to a great extent on immune cells in MPM [reference_37]. Additionally, they also showed that PD-1 positive TCs are rarely seen in these patients (only 4 of 54 patients had PD-1 positive TCs in their study) [reference_37].

Comment #8: Line 214 "PD-L1 TIL expression was rarely seen": this is a somewhat unexpected finding. Might it be that there is an underestimation of the positivity due to the PD-L1 antibody clone that was used?

Reply #8: We agree with the Reviewer that TIL PD-L1 expression is rather low in our cohort compared to others. A possible explanation for this discrepancy might be that PD-L1 expression can be mostly seen in non-epithelioid MPM, and in our study, only 19.2% of the patients were diagnosed with such histology. In addition, as suggested by the Reviewer, the antibody clone used in our study might also influence the positivity. Please also see Reviewer A reply #6.

Changes in the text #8: The following sentence was inserted to the "*Discussion*" chapter:

-page 13, lines 312-315: "A possible explanation for the relatively low number of cases with PD-L1 expressing TILs might be that TIL PD-L1 positivity is usually seen in sarcomatoid MPM, whereas the majority of patients included in our study had epithelioid type MPM [reference_37]."

-please also see Changes in the text #6; Reviewer A, Comment #6

Comment #9: Line 215: what about that one case with a high PD-L1 TIL expression? Can this be due to the treatment in the past or…?

Reply #9: We thank the Reviewer for picking this up. Although different therapeutic agents might indeed influence the PD-L1 expression both on TCs and TILs, this particular patient with high PD-L1 TIL expression was a CHT-naïve patient. Accordingly, in this case, the high TIL PD-L1 expression cannot be due to the treatment in the past. As mentioned in Reply #8 (Reviewer A), the histological subtypes might also influence the TIL PD-L1 expression. Unfortunately, however, in case of this particular patient we do not have any available information on the exact histopathological subtype.

Changes in the text #9: No changes in the text.

Comment #10: Line 223: there is no table 1B?

Reply #10: Thank you for pointing this out. Table 1.B (Patient characteristics and PD-L1 expression of TCs in human MPM) was included in the original submission, however, due to a technical reason, it seems that it was not merged into the final PDF file created by the Journals' Editorial Manager. We will upload it once again together with the revised manuscript.

Changes in the text #10: No changes in the text.

Comment #11: Line 296: please read and refer to the papers from Combaz-Lair C (Hum Pathol 2016) and Marcq E (Oncoimmunology 2017) who already evaluated PD-1 expression in MPM.

Reply #11: The Reviewers' comment is well taken. We have revised the aforementioned paragraph of the "*Discussion*" section accordingly, and the articles suggested by the Reviewer are now cited in the text.

Changes in the text #11: The following sentence was inserted to the "*Discussion*" chapter:

-page 14, lines 316-317: So far, two major studies investigated the detailed expression pattern of PD-1 in MPM [reference_37_38].

Comment #12: Line 308: the discordance between results might also be due to an underestimation of the expression based on the antibody clone that was used in this study?

Reply #12: We thank Reviewer A for raising this point. Previous studies found that high PD-L1 expression (both on TCs and TILs) might be associated with non-epithelioid (i.e. sarcomatoid) histology [9, 10]. In contrast, however, we did not find any significant association between PD-L1/PD-1 expression (high vs. low) and histological subtype (epithelioid vs. non-epithelioid). A possible explanation of this discordance might be that in our study only a small portion (19.2%) of the included patients was diagnosed with non-epithelioid MPM. In addition, the different threshold values used to determine the PD-L1 high vs. low expressing subgroups might also have an impact on the outcomes. In the current study, we used a relatively strict cut-off value, and high PD-L1 expression was defined as PD-L1 >10%. Meanwhile, others used alternative threshold values or grouped the patients solely based on positivity irrespective of the expression percentage. Lastly, we agree with the Reviewer that the antibody clones we used might as well influence the staining intensity and the positivity. The "*Discussion*" chapter was revised according to the aforementioned statements and to the Reviewer's suggestion.

Changes in the text #12: The following sentences were inserted to the "*Discussion*" chapter:

-pages 14-15, lines 336-342: A possible explanation for this discordance might be related to different cut-off values. In our study, "PD-L1/PD-1 high" patients were defined as those with PD-L1/PD-1 expression >10%. Meanwhile, others used alternative threshold values or grouped the patients solely based on positivity irrespective of the expression percentage. Additionally, the relatively low ratio of patients with non-epithelioid MPM in our study might also explain these divergent results.

-please also see Changes in the text #6; Reviewer A, Comment #6

Comment #13: Line 332: same comment as for line 296. Both papers previously investigated PD-1 expression in TILs…

Reply #13: We thank Reviewer A for bringing this inaccuracy to our attention. We understand that authors of both papers [9, 10] have already investigated PD-1 expression in MPM. Of note, Combaz-Lair et al. [10] investigated the prognostic relevance of PD-L1 expression only by tumor cells. Marcq et al. [9] have indeed reported PD-1 and PD-L1 expressions by both TILs and tumor cells. Notably, however, their study included only 54 MPM patients. Nevertheless, the respective paragraph of the "*Discussion*" chapter was rewritten according to the Reviewers' suggestion and the aforementioned statements.

Changes in the text #13: The following sentences were inserted to the "*Discussion*" chapter:

-page 15-16, lines 362-370: To the best of our knowledge, ours is so far the largest study to investigate the prognostic relevance of PD-1 expression by TILs in MPM patients. Although Marcq et al. also examined the prognostic importance of PD-1 on immune cells, their study included only 54 patients [reference_37].

Comment #14: Line 347 "this is the first report that…": this should be adjusted. There already exist other reports on that in MPM. Please refer to the correct corresponding papers.

Reply #14: We thank the Reviewer for picking this up. The "Conclusion" section of the "Discussion" chapter was revised. Please also see Reviewer A reply #13.

Changes in the text #14: The following sentence was revised in the "Conclusion" section:

-page 16, lines 388-390: Furthermore, this is the largest study that comprehensively evaluates the prognostic value of PD-1 by TILs in a multicenter cohort of MPM patients.

-Reviewer B-

We are pleased that Reviewer B is positive about our paper and we thank her/him for providing the below suggestions.

A well written retrospective analysis of PD-(L)1 of 203 MPM patients in TC and TIL.
Clinical relevant given the recent introduction of immunotherapy (FDA approval for nivo/ipi).

There are only a couple of small remarks which need to be addressed:

Comment #1: There is no mention of the use of PD-(L)1 inhibitors in mesothelioma, while there is a very recent pivotal phase III study presented which led to FDA approval!.

See https://www.sciencedirect.com/science/article/pii/S1556086420306328
It would be relevant with regard to translational research to address these recent study results

Reply #1: We thank the Reviewer for picking this up. The study suggested by the Reviewer is now mentioned and cited in the "*Introduction*" chapter.

Changes in the text #1: The following sentences were inserted to the "*Introduction*" chapter:

-page 3, lines 88-91: Nevertheless, a recent phase III study investigating the efficacy of first-line nivolumab plus ipilimumab (vs. platinum doublet CHT) showed promising results with regards to OS [reference_13]. Of note, however, the progression-free survival (PFS) was similar between the treatment arms even in case of combination immunotherapy [reference_13].

Comment #2: In table 3, the CI for PD-L1 expression of TC seems reversed

Reply #2: Thank you for pointing this out. The CI was not reversed; however, the HR was indeed misspelled. Table 3 was revised accordingly.

Changes in the text #2: The following changes were made in Table 3.:

-PD-L1 expression of TCs (PD-L1 >10% vs. ≤10%): HR: 0.405 (instead of 2.486)

References

[1] Zalcman G, Mazieres J, Margery J, et al. Bevacizumab for newly diagnosed pleural mesothelioma in the Mesothelioma Avastin Cisplatin Pemetrexed Study (MAPS): a randomised, controlled, open-label, phase 3 trial [published correction appears in Lancet. 2016 Apr 2;387(10026):e24]. Lancet. 2016;387(10026):1405-1414. doi:10.1016/S0140-6736(15)01238-6

[2] Parra ER, Villalobos P, Mino B, Rodriguez-Canales J. Comparison of Different Antibody Clones for Immunohistochemistry Detection of Programmed Cell Death Ligand 1 (PD-L1) on Non-Small Cell Lung Carcinoma. *Appl Immunohistochem Mol Morphol*. 2018;26(2):83-93. doi:10.1097/PAI.0000000000000531

[3] Martinez-Morilla S, McGuire J, Gaule P, et al. Quantitative assessment of PD-L1 as an analyte in immunohistochemistry diagnostic assays using a standardized cell line tissue microarray. Lab Invest. 2020;100(1):4-15. doi:10.1038/s41374-019-0295-9

[4] Schats KA, Van Vré EA, De Schepper S, et al. Validated programmed cell death ligand 1 immunohistochemistry assays (E1L3N and SP142) reveal similar immune cell staining patterns in melanoma when using the same sensitive detection system. Histopathology. 2017;70(2):253-263. doi:10.1111/his.13056

[5] Ma, J., Li, J., Qian, M. et al. PD-L1 expression and the prognostic significance in gastric cancer: a retrospective comparison of three PD-L1 antibody clones (SP142, 28–8 and E1L3N). Diagn Pathol 13, 91 (2018). https://doi.org/10.1186/s13000-018-0766-0

[6] Kintsler S, Cassataro MA, Drosch M, Holenya P, Knuechel R, Braunschweig T. Expression of programmed death ligand (PD-L1) in different tumors. Comparison of several current available antibody clones and antibody profiling. Ann Diagn Pathol. 2019;41:24-37. doi:10.1016/j.anndiagpath.2019.05.005

[7] Fankhauser CD, Schüffler PJ, Gillessen S, et al. Comprehensive immunohistochemical analysis of PD-L1 shows scarce expression in castration-resistant prostate cancer. Oncotarget. 2017;9(12):10284-10293. Published 2017 Dec 4. doi:10.18632/oncotarget.22888

[8] Cedrés S, Ponce-Aix S, Zugazagoitia J, et al. Analysis of expression of programmed cell death 1 ligand 1 (PD-L1) in malignant pleural mesothelioma (MPM). *PLoS One*. 2015;10(3):e0121071. Published 2015 Mar 16. doi:10.1371/journal.pone.0121071

[9] Marcq E, Siozopoulou V, De Waele J, et al: Prognostic and predictive aspects of the tumor immune microenvironment and immune checkpoints in malignant pleural mesothelioma. Oncoimmunology 6:e1261241-e1261241, 2016 37.

[10] Combaz-Lair C, Galateau-Salle F, McLeer-Florin A, et al: Immune biomarkers PD-1/PD-L1 and TLR3 in malignant pleural mesotheliomas. Hum Pathol 52:9-18, 2016