Peer Review File

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Reviewer A

Comment 1: We reviewed this article with great enthusiasm. Historically. PDAC patients do not respond to immunotherapy as they lack of target (PD1 or PDL1). There is a grade need for understanding the biology of this unresponsiveness to immunotherapies of PDAC.

Here the authors, identified increased expression of the PD-L1 in a subset of pancreatic cancers, with squamous cell differentiation (PC-ASC). The authors asked a simple question about increased PDL1 expression in PC-ASC patient specimens compared to PC-AC patients. Pathology and IHC methods are straightforward in this paper. The E1L3N method is not routinely used in clinical practice. Most immunotherapies, such as Nivolumab, and pembrolizumab, approval were based on compendium tests other than E1L3N. But, for this paper, it may not make any difference, as E1L3N is a valid method.

it would be interesting to see, if high PD-L1 expression correlated with other biomarkers of immunotherapy such as TMB, MMR status or other mutations, such as KRAS mutations. We wonder if the authors could look into this data, if available.

it is a very good paper. I recommend accepting this paper

Reply 1: We thank the reviewer for your thoughtful comment. You have raised an important point here. When we performed this study at the former institution, comprehensive genomic profiling was not performed routinely on pancreatic or biliary carcinoma cases. Hence, there is no reportable data regarding the correlation between PD-L1 expression status and other biomarkers such as TMB, MMR status or other mutations for the cases studied. However, based on the interesting results of this study, we are currently preparing for a genetic and transcriptional profiling of pancreaticobiliary adenosquamous carcinomas at our current institution. We believe that this study and our future analysis will contribute to creating a foundation for developing/refining biomarkers that can optimize and personalize therapeutic strategies in pancreaticobiliary adenosquamous carcinoma patients.

Reviewer B

This is a very well written paper and albeit low-powered yet provides quintessential insight into this interesting topic of immunogenic TME in pancreaticobiliary subtypes including squamous and adenosquamous carcinoma.

Minor comments:

Comment 1. Statement “Pancreatic ASC is associated with more aggressive” line 47-49 needs a refernce.

Reply 1: To address your point, the following reference articles are newly added as refs 3 and 4 in the revised paper. These new reference articles are highlighted red in the Reference section.

3. Huang Z, Wang J, Zhang R, et al. Pancreatic adenosquamous carcinoma: A population level analysis of epidemiological trends and prognosis. Cancer Med 2023;12:9926-36.

4. Xiong Q, Zhang Z, Xu Y, et al. Pancreatic Adenosquamous Carcinoma: A Rare Pathological Subtype of Pancreatic Cancer. J Clin Med 2022;11:7401.

Comment 2. Line 67, T and B- what? I would mention lymphocytes, can be confusing.

Reply 2: We think this is an excellent suggestion. We have addressed your point below:
The phrase "T, B, and antigen-presenting cells" was changed to "T-lymphocytes (T-cells), B-lymphocytes, and antigen-presenting cells". This revised phrase is highlighted red in the revised manuscript.

This change would also help explaining what "T-cell/T-cells" in the following sections mean.

Comment 3. Chart study was done from 2000-2019, why not until 2024? Can we see if more data can be added? Line 101. This is just a suggestion.

Reply 3: This study was conducted at the University of North Carolina (UNC) at Chapel Hill following Institutional Review Board approval, which was granted at the end of 2019 (IRB Number: 19-1862). Since the author (principal investigator) left UNC at Chapel Hill later, modification of the protocol (testing for additional cases, if present) was difficult. Therefore, we needed to adhere strictly to IRB approved protocols including case numbers.

Comment 4. Line 102, "No cases of intrahepatic or extrahepatic bile-duct ASC were found in our record. So were all the biliary patients gallbladder cancer? Please clarify, you can just gallbladder and panc.

Reply 4 : Yes. All the biliary patients in this study are gallbladder cancer cases. To clarify this, the sentence on Line 102 was changed from "No cases of intrahepatic or extrahepatic bile-duct ASC were found in our record." to "No cases of intrahepatic or extrahepatic bile-duct ASC were found in our record and thus, biliary tract carcinomas in this study are virtually limited to gallbladder lesions." This sentence is highlighted red in the revised manuscript.

To further clarify this, the first sentence in the Methods section of the Abstract (line 28-29) "We evaluated 15 PB-ASCs (10 pancreatic, 5 biliary) and 34 control PB-ACs (22 pancreatic ductal, and 12 biliary) was changed to "We evaluated 15 PB-ASCs (10 pancreatic, 5 gallbladder) and 34 control PB-ACs (22 pancreatic ductal, and 12 gallbladder)"
Similarly, "PB-ASCs of biliary origin" in Line 160 and "ACs of biliary origin" in line 165-166 in the Results section have also been changed to "PB-ASCs of gallbladder origin" and "ACs of gallbladder origin", respectively.

Comment 5. For the IHC section page 112, I would recommend references.

Reply 5 : Thank you for pointing this out. We added the phrase "following the manufacturer's instructions." in the end of the first sentence of the Immunohistochemistry section as well as one reference article for PD-L1 IHC, which appears as ref 24 in the revised manuscript (below).

24. Song L, Zeng L, Yan H, et al. Validation of E1L3N antibody for PD-L1 detection and prediction of pembrolizumab response in non-small-cell lung cancer. Commun Med (Lond) 2022;2:137.

Comment 6. Microscopic evaluation: need references for defining TPS levels.

Reply 6 : Thank you for this suggestion. For defining TPS levels, the following 2 references (ref 25 and 26 in the revised manuscript) are added.

25. Mok TSK, Wu YL, Kudaba I, et al. Pembrolizumab versus chemotherapy for previously untreated, PD-L1-expressing, locally advanced or metastatic non-small-cell lung cancer (KEYNOTE-042): a randomised, open-label, controlled, phase 3 trial. Lancet 2019;393:1819-30.

26. Akhtar M, Rashid S, Al-Bozom IA. PD-L1 immunostaining: what pathologists need to know. Diagn Pathol 2021;16:94.

Comment 7. Page 147, was demographic collected? Did you use descriptive analysis?...looks like it.

Reply 7: Based on the reviewers suggestion, We would like to add the following sentence at the end of the first paragraph of the Results section. "Demographic characteristics (age, sex) were not statistically different in patients with PB-ASC and those with PB-AC."

Since nonparametric Mann-Whitney U test is required to analyze age differences between groups, the first sentence in the Statistical Analysis section "Fisher's exact test was performed" was changed to "Fisher's exact test and nonparametric Mann-Whitney U test were performed"

We also found that demographic characteristics were not statistically different between pancreatic ASC and pancreatic AC cases nor between gallbladder ASC and gallbladder AC cases. However, these sentences are not added since it might be somewhat redundant. Nevertheless, we believe that these facts can be easily seen/confirmed since all demographic information of each case is available in Table 2.

Comment 8. Line 164, what do mean by tumor compartment, I ould explain.

Reply 8 : We agree that the term "tumor compartment" may be misleading and therefore, has been changed to "tumor component (squamous vs. glandular)" in the revised manuscript.

Comment 9. Line 175"All of them were positive" did that inculde samples in the control PB AC arm?

Reply 9: Yes it did. All carcinomas with the II phenotype (3 cases) including 2 PB-ASCs 1 PB-AC were positive for PD-L1. To clarify this, the sentence "all of them were positive for PD-L1" was changed to "all of them (2 PB-ASCs and 1 PB-AC) were positive for PD-L1".

Comment 10. Use the PB abbreviation consistently throught text. B is mainly gallbaldder?

Reply 10 : The PB abbreviation was used consistently in the revised manuscript. As mentioned in Reply 4, B (biliary tract) in this case series is virtually limited to gallbladder. The absence of intrahepatic or extrahepatic bile-duct ASCs in our series most likely reflects their rarity. In this manuscript, we still would like to use the "PB" abbreviation (not "pancreatico-cholecystic or PC" for example), because we feel that future studies of PB-ASC with larger samples should include intrahepatic or extrahepatic bile-duct ASCs, if cases are present, rather than excluding them.