Peer Review File

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Reviewer A

The study idea is interesting. However, the manuscript falls short in describing the change in TIME in BtC.
First the number of series is too small (:5)
Second the urge is so not present the data for the changes in different subtypes
Third the cohort is too homogenous with FGfR2f, HER2a and other molecular alterations that are known to impact TIME
Reply: Thank you for your review. Regarding your comment about the sample size, the original intention was to examine IHC changes in a substantial number of patients. However, limitations in funding and the availability of adequate tissue constrained us, resulting in the generation of data sufficient for a case series. Consequently, we view the study results as merely hypothesis-generating. Despite the limited numbers, we believe our research offers guidance for future studies involving larger patient cohorts.
In reference to your comment on molecular markers such as FGFR2 and Her2, recognized for their influence on TIME (as acknowledged on page 10 line 291-296) across various malignancies, we acknowledge the established evidence supporting their capacity to modulate TIME dynamics. Again, given the small number of patients in our cohort, we are unable to study the impact of these markers in further detail in this specific cohort.
Changes in the text: We added the following lines to address Review A's comments in page 10 lines 296-298: It is imperative to underscore, however, that due to the limited size of our patient cohort, a comprehensive exploration of the nuanced impact of these markers remains unattainable within the scope of our study.

Reviewer B

In this study, Dharmapuri et al. seek the effects of neoadjuvant chemotherapy on the tumor immune microenvironment in biliary tract cancer. BTC and CCA are heterogeneous cancers, and early diagnosis is challenging. Treatment options are limited, so it is beneficial and supportive for doctors and patients to show that NACT is worth to try. This study used 5 samples, so sample numbers are low and data are still preliminary, but worth to read as a case study. I have a few suggestions for this manuscript.

Comment 1: There are previous studies showing that NACT impacts immune cell infiltration and tumor microenvironment. Those studies are in other cancers, but it would be good if the authors discuss about NACT effects on immune cells and microenvironment in the introduction as basic information.
Reply : Thank you for your review. We have discussed the impact of NACT on immune modulation of TIME on page 4-5, lines 125- 143 in length.
Changes in the text: NA

Comment 2 & 3: The authors show CD8:FOXP3 ratios in Table 2, but previous studies used various ratios, such as neutrophil to lymphocyte ratio, to predict prognosis. Why do the authors look at only CD8:FOXP3 ratios and do not see neutrophils? Please describe the rationale and discuss ratio-based prognosis prediction. The author's approach is based on IHC using liver tissues, but tumor tissues may be heterogeneous, so IHC results may not accurately identify immune cell populations. On the other hand, NLR can be obtained by blood testing, which is much easier and accurate. Why do the authors looked at CD8:FOXP3 ratio but not NLR? If the authors focus on tumor microenvironment, that is fine, but at least the authors discuss other methods or possibilities for future studies.
Reply : As you mentioned, inflammatory markers such as NLR, PLR, and others derived from peripheral blood have undergone extensive examination in the context of biliary tract cancer, as well as other malignancies. Their role as prognostic markers has already been well established (Reference: PMID: 35879385). It is crucial to note, however, that our study differs from these aforementioned

investigations. Specifically, our focus lies in exploring the influence of Neoadjuvant Chemotherapy on the tumor microenvironment in biliary tract cancers—a facet that, to the best of our knowledge, has not been previously explored in detail. In this study, our primary objective was to comprehend the impact of Neoadjuvant Chemotherapy on different cell populations by examining Immunohistochemistry markers within the TIME. The rationale for selecting each marker is elucidated on page 9 line242 - 259. Specifically, the inclusion of the CD8:FOXP3 ratio as a marker of interest was motivated by its well explored role in other tumors, as detailed on page 10 line 286 - 290.
Changes in the text: NA

Comment 4: The authors indicate that high CD8:FOXP3 ratio may be associated with longer time to recurrence. Did the authors perform correlation analysis, such as Pearson's correlation to see if there is any correlation between immune cells (e.g., scores of CD8 or CD8:FOXP3 ratio) with better prognosis? I know the sample numbers are small so it may not be statistically significant, but at least the authors should show that and discuss probabilities in future studies.
Reply: The study team underwent extensive deliberations regarding the optimal approach for data presentation. Various statistical tests, including Pearson's correlation and paired sample T test, were considered. However, due to the modest sample size of 5, consensus emerged among us that conducting these analyses would yield results lacking statistical significance and interpretive value. Consequently, it was unanimously agreed upon that the most judicious approach for data presentation would be through descriptive statistics, allowing for a comprehensive portrayal of the observed trends without attempting statistical inferences.
We believe the adoption of descriptive statistics is a prudent strategy to mitigate the risk of deriving erroneous conclusions through scientifically invalid statistical methods. Additional line added to limitations section in the manuscript to explain the same, see below.
Changes in the text: Page 11, Lines added 318-323: 'In the presentation of the case series data, we contemplated various statistical tests, such as Pearson's correlation and paired sample T test. However, acknowledging the limited sample size of 5, a collective decision was made to opt for the presentation of descriptive statistics only. This strategic choice aims to mitigate the potential for drawing incorrect conclusions by refraining from statistical analyses that could be compromised by the constraints of the small sample size.'

Reviewer C

The authors performed multiplexed immunohistochemical consecutive staining on single slide analysis with a series of immune markers on pre-& post-chemotherapy tumor tissue of patients with biliary tract cancer. Here are the comments for the manuscript:

Comment 1: The patient sample size is too small, it is better to do some statistical analyses.
Reply: The study team underwent extensive deliberations regarding the optimal approach for data presentation. Various statistical tests, including Pearson's correlation and paired sample T test, were considered. However, due to the modest sample size of 5, consensus emerged among us that conducting these analyses would yield results lacking statistical significance and interpretive value. Consequently, it was unanimously agreed upon that the most judicious approach for data presentation would be through descriptive statistics, allowing for a comprehensive portrayal of the observed trends without attempting statistical inferences.
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Comment 2: The result description is too short, and the conclusion is not clear or solid.
Reply : Acknowledging the inherent limitations of a case series characterized by a modest sample size of 5, we concur with your assessment that the results and conclusions derived from the study lack definitiveness. It is crucial to recognize that the case series' outcomes serve a hypothesis-generating purpose, offering valuable insights that pave the way for future research endeavors. We hope that our study's results inspire researchers to conduct a larger study with more patients, allowing for conclusive findings to be drawn.
Changes in the text: NA

Comment 3: Can the authors show and analyze co-expression and co-localization of different immune markers and immune cells?
Reply : We sought guidance from our laboratory experts concerning the co-expression and co-localization analysis of distinct immune markers and immune cells. Their reply suggested that while it is feasible to carry out these analyses, it would require additional financial resources and an extended timeframe, spanning several months. Furthermore, the utility of undertaking such an analysis within the confines of this case series is uncertain due to the limited sample size. Consequently, considering the financial and temporal constraints, we deem this analysis to be beyond the scope of the current project. However, we affirm our commitment to incorporating such analyses in forthcoming research endeavors.
Changes in the text: NA

Comment 4: Figure 2 doesn't show the identification of stromal (sTILs) & tumor (tTILs) areas? And what is the difference between 'Tumor' and 'TIL' areas?
Reply: Thank you for this observation, we have corrected the caption to say 'Identification of tumor infiltrating lymphocyte(TIL)enriched tumor and non-tumor areas in pt #3" and have added an additional image showing 'Figure 3 Identification of stromal (sTILs) & tumor (tTILs) infiltrating lymphocytes in pt #3'. To your second point, definitions added to text on page 6, lines 180-184 "Intratumoral Tumor-Infiltrating Lymphocytes (TILs) were defined as lymphocytes residing within tumor nests, exhibiting direct cell-to-cell contact without intervening stroma, and engaging in interactions with carcinoma cells. Conversely, stromal TILs are situated in a dispersed manner within the stromal spaces amidst carcinoma cells, lacking direct contact with carcinoma cells."

C**hanges in the text:** ‘Identification of tumor infiltrating lymphocyte (TIL)enriched tumor and non-tumor areas in pt #3” Additional image added, Figure 3. Additional lines added to page 6, lines 180-184 “Intratumoral Tumor-Infiltrating Lymphocytes (TILs) were defined as lymphocytes residing within tumor nests, exhibiting direct cell-to-cell contact without intervening stroma, and engaging in interactions with carcinoma cells. Conversely, stromal TILs are situated in a dispersed manner within the stromal spaces amidst carcinoma cells, lacking direct contact with carcinoma cells.”