Peer Review File

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Reviewer A

Comment 1: Recently, how about the application of FOLFOX for BTC? Please state in the introduction.

Reply 1: We appreciate your insightful suggestion. FOLFOX was reported to provide survival benefit in second line treatment of advanced BTC in the ABC-06 study (DOI:https://doi.org/10.1016/S1470-2045(21)00027-9), but has not been confirmed feasible in the first-line setting. We have stated this in the introduction.

Changes in the text: We have added the application of FOLFOX for BTC in the introduction (see page 4, line 130-132).

Comment 2: It was advised to add related reference (DOI: 10.21037/cco.2019.12.07) about the systemic therapy for BTC.

Reply 2: Thanks for your suggestion. We have added the reference (citation 15) as your advised to our paper.

Changes in the text: A reference has been added (see page 6, line 172).

Comment 3: There were several targeted agents for BTC. How to choose targeted agents in the study? Please state in the introduction.

Reply 3: Thanks for your valuable comment. As you pointed out, there were several targeted agents for BTC.over the past several years. Previous trials targeting the epidermal growth factor receptor (EGFR) pathway demonstrating limited success, and inhibition of the vascular endothelial growth factor (VEGF) receptor pathway yielding suboptimal results, but these studies did not restrict drug administration to patient populations with corresponding target mutations. Recent evidence, including clinical trials employing the isocitrate dehydrogenase (IDH)-targeted agent ivosidenib in cholangiocarcinoma patients harboring IDH1 mutations and a cohort study utilizing a BRAF inhibitor in patients with BRAF V600E-mutated cholangiocarcinoma, has demonstrated efficacy, highlighting the importance of tailoring targeted therapies to specific patient populations with relevant mutations. (DOI: 10.21037/cco.2019.12.07)
However, the financial burden associated with genetic testing renders the selection of targeted therapeutic agents based on individual patient mutations impractical in a clinical setting. Anlotinib, a small-molecule targeted agent, has shown promising antitumor activity in intrahepatic cholangiocarcinoma (CCA) through the inhibition of VEGFR-2 phosphorylation

and inactivation of the PI3K/AKT signaling pathway. Moreover, a phase II trial revealed that, following a median follow-up of 8.76 months, anlotinib in combination with sintilimab, an anti-PD-1 monoclonal antibody, resulted in a median progression-free survival of 6.50 months, an objective response rate of 40.0%, and a disease control rate of 86.67%, with a favorable safety profile in the second-line treatment of BTC.
In light of these findings, So, we tried anlotinib in combination with Anti-PD-1/PD-L1 immunotherapy in clinical practice and retrospectively assessed the efficacy in this study.

Changes in the text: We added how to choose targeted agents in the study in introduction (see page 5/ line 167- page 6/ line 179).

Comment 4: There were adverse events occurred in the study? How to prevent adverse events? Please state in the discussion.

Reply 4: The adverse events were discussed in page 12/line 387-407. The three regimens showed manageable toxicities with no additional adverse events compared to previous reports. To prevent adverse events, we implement the following measures: 1. Prior to initiating the treatment regimen, assess patients for high-risk factors of immune-related adverse events. For patients presenting with relevant risk factors, such as autoimmune diseases, pulmonary tuberculosis, or cardiac disorders, the use of ICIs should be exercised with caution; 2. During outpatient follow-up, monitor relevant laboratory parameters to identify trends indicative of adverse events or early stages of AEs, and promptly intervene as necessary, such as administering leukocyte elevation therapy or hepatoprotective treatments; 3. Depending on the patient's condition, consider adjusting the medication dosage or temporarily discontinuing treatment in a timely manner. Furthermore, gemcitabine was used once in a 2-week cycle, rather than on D1 and D8 in a 3-week cycle, to reduce the risk of adverse events.

Changes in the text: We added content about how to prevent adverse events in discussion (see page 12/line 398- line 404).

Comment 5: What were the advantages of combination therapy for BTC? Please state in the discussion.

Reply 5: The application of targeted immunotherapy combinations in the field of hepatocellular carcinoma has become well-established, with a wealth of evidence supporting the safety and efficacy of such regimens (e.g., IMbrave 150: N Engl J Med. 2020 May 14;382(20):1894-1905, ORIENT32 Lancet Oncol. 2021 Jul;22(7):977-990). GEMOX chemotherapy is the standard treatment for biliary tract carcinoma. By incorporating targeted and immunotherapy agents to the chemotherapy foundation, we aim to achieve improved treatment efficacy.
To minimize the risk of adverse events, we modified the gemcitabine administration schedule from a three-week to a two-week cycle in the combination regimen in this study. Indeed, retrospective results of our study showed potential efficacy and acceptable safety of such combination. A similar treatment regimen to the targeted immunotherapy combination with chemotherapy used in our study is the use of tislelizumab combined with lenvatinib and the

GEMOX regimen for first-line treatment of advanced biliary tract carcinoma. The results of this trial demonstrated an objective response rate of 80%, with median overall survival (OS), progression-free survival (PFS), and duration of response (DoR) of 22.5, 10.2, and 11.0 months, respectively, further demonstrating the advantages of this combination strategy. A total of 56.7% of patients experienced grade ≥3 adverse events, primarily neutropenia and leukopenia, with no new safety signals observed (Signal Transduct Target Ther. 2023 Mar 17;8(1):106.).

Changes in the text: We added the advantages of combination therapy in introduction (see page 6/ line 186- line 201).

Comment 6: In the study, it was showed that anti-PD-1/PD-L1 immunotherapy in combination with anlotinib and gemcitabine provides promising efficacy and a good safety profile. Whether the treatment strategy could be improved? Please state in the discussion.

Reply 6: Thank you for your insightful suggestion. Indeed, our retrospective study demonstrated promising safety and efficacy data for the combination of anti-PD-1/PD-L1 immunotherapy, anlotinib, and gemcitabine. We think the optimization of this treatment strategy could be achieved through reducing drug doses or adjusting administration schedules to reduce toxicities and the risk of adverse event. For instance, we modified the gemcitabine administration schedule from D1 and D8 in a 3-week cycle to once in a two-week cycle, and the safety profile resulted to be acceptable in this retrospective study. Additionally, anlotinib dosing could be further optimized based on patient weight or body surface area. To further enhance treatment efficacy, incorporating local therapies such as interventional procedures and radiotherapy, into the combination regimen might be considered. However, the specific improvements would need to be carefully evaluated in the context of potential toxicities. Determining the optimal approach to enhance this treatment regimen and whether such modifications would yield better clinical outcomes for patients requires further exploration in future trials.

Changes in the text: We have stated the improvement of the treatment strategy in the conclusion (seeing Page 13 Line 424-430).

Reviewer B

Comment 1: First of all, the authors need to consider whether it is appropriate to describe this study as a real-world study, which is often characterized by a large sample, but the current study not. In the title, please indicate efficacy and safety, as well as the clinical research design, i.e., a retrospective cohort study.

Reply 1: We appreciate your observation and agree with your suggestion. The title has been changed to "A retrospective cohort study on the efficacy and safety for Combination combination therapy of immunotherapy, targeted agent, and chemotherapy versus immunochemotherapy or chemotherapy alone in the first-line treatment of advanced biliary tract carcinoma".

Changes in the text: We have changed the title of our paper (seeing page 1 line 3-6).

Comment 2: Second, the abstract needs some revisions. The background did not explain why combination therapy of immunotherapy, targeted agent, and chemotherapy is potentially effective and safe for BTC and the treatments of control groups to be compared. The methods need to describe the inclusion of subjects, follow up procedures, and measurements of safety outcomes. The results need to briefly describe the clinical characteristics of the three groups and report the baseline comparability of the three groups. The current conclusion needs to be tone down since the sample size is small and the authors did not adjust for baseline clinical and pathological characteristics.

Reply 2: We appreciate your insightful suggestion. The abstract has been revised as your suggested, except the baseline comparability of the three groups as statistical testing is meaningless given the nature of small sample size in each group.

Changes in the text: We have revised the abstract of our paper (seeing page 1 line 32- page 2/ line 68).

Comment 3: Third, in the introduction of the main text, the authors need to explain why the combination of immunotherapy, targeted agent, and chemotherapy is safe. This is important and should be addressed from theoretical perspectives. The authors should also have some reviews on the efficacy and safety of immunochemotherapy or chemotherapy alone, in particular the clinical indications for these single-agent treatments, because the patients may be different from those who undergone multiple treatments.

Reply 3: Thank you for your insightful comment. The application of targeted immunotherapy combinations in the field of hepatocellular carcinoma has become well-established, with a wealth of evidence supporting the safety such regimens (Mbrave 150: N Engl J Med. 2020 May 14;382(20):1894-1905. ORIENT32: Lancet Oncol. 2021 Jul;22(7):977-990). GEMOX chemotherapy is the standard treatment for biliary tract carcinoma, and its safety is indisputable. By incorporating targeted and immunotherapy agents to the chemotherapy foundation, we aim to achieve improved treatment efficacy.To minimize the risk of adverse events, we modified the gemcitabine administration schedule from a three-week to a two-week cycle. A similar treatment regimen to the targeted immunotherapy combination with chemotherapy used in our study is the use of tislelizumab combined with lenvatinib and the GEMOX regimen for first-line treatment of advanced biliary tract carcinoma. The results of this trial demonstrated an objective response rate of 80%, with median overall survival (OS), progression-free survival (PFS), and duration of response (DoR) of 22.5, 10.2, and 11.0 months, respectively. A total of 56.7% of patients experienced grade ≥3 adverse events, primarily neutropenia and leukopenia, with no new safety signals observed, further indicating the feasibility of such combination strategy.

Changes in the text: We have added the explanation why the combination of immunotherapy, targeted agent, and chemotherapy is safe, in the introduction (see page 6/ line 186- line 201).

Comment 4: Fourth, in the methodology of the main text, please clearly describe the clinical research design, sample size estimation, assessment of baseline clinical factors, and follow up procedures. In statistics, please first test the baseline comparability across the three groups and consider to do multiple regression analysis to adjust for potential confounders. The findings from direct univariate comparisons are problematic without adjustment analyses.

Reply 4: Thank you for pointing this out. As this was a retrospective cohort study, there was no sample size calculation, and the baseline clinical factors were assessed in accordance with routine clinical treatment procedures (see page 8, line 237). Patients were followed up weekly, based on the drug administration cycle (see page 7, line 232).
We recognize the importance of adjustment analyses for retrospective cohort studies. However, due to the small sample size in each cohort, the influence of individual participants on the results was substantial, making statistical tests less meaningful. Consequently, we did not perform tests to compare baseline data across groups or conduct adjusted analyses on the results. As such, the findings in our study are descriptive and should be interpreted with caution, serving as a reference only. We have added this limitation to the discussion section (see page 13, line 407-414) and have revised the conclusion to reflect a more cautious tone (see line 419-420).

Changes in the text: We have added assessment of baseline clinical factors (see page 8, line 239), and follow up procedures (see page 7, line 234) in methods. We have added this limitation to the discussion section (see page 13, line 409-416) and have revised the conclusion to reflect a more cautious tone (see line 421-422).

Reviewer C

Editorial Comments (Please provide your point-to-point response)

1. Please define GEMOX in Abstract.

37 carcinoma. GEMOX chemotherapy is the standard treatment for BTC. This study
38 aimed to evaluate the efficacy and safety of anti-programmed cell death protein-1 (PD-

Reply: GEMOX is defined in Abstract.

2. Two references are included in your paper, please keep the final version and remove the unnecessary one.

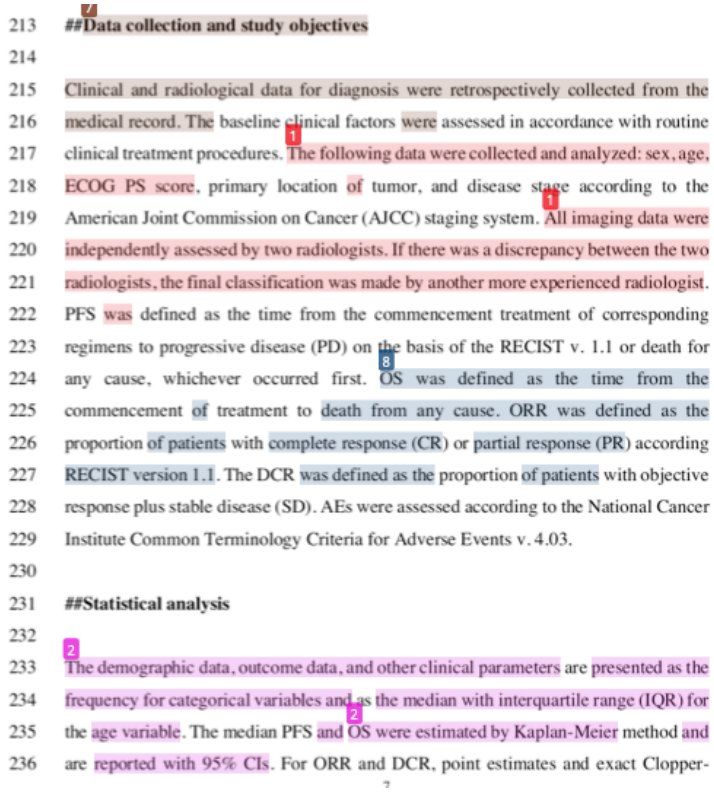
And in the second reference list, Ref.13 and Ref.22, Ref.18 and Ref.25 are the same, please check.

13. Zhou Jian, Jia Fan, Guo-Ming Shi, et al. Gemox chemotherapy in combination with anti-PD1 antibody toripalimab and lenvatinib as first-line treatment for advanced intrahepatic cholangiocarcinoma: A phase 2 clinical trial. Journal of Clinical Oncology 2021;39:4094.

22. Jian Z, Fan J, Shi G-M, et al. Gemox chemotherapy in combination with anti-PD1 antibody toripalimab and lenvatinib as first-line treatment for advanced intrahepatic cholangiocarcinoma: A phase 2 clinical trial. Journal of Clinical Oncology 2021;39:4094-.

Reply: The Ref.22, and Ref.25 are removed.

3. Originality checking of below part shows high duplication. Please revise your paper to lower the duplication rate. Attached is a report for your reference.



##Data collection and study objectives

Clinical and radiological data for diagnosis were retrospectively collected from the medical record. The baseline clinical factors were assessed in accordance with routine clinical treatment procedures. The following data were collected and analyzed: sex, age, ECOG PS score, primary location of tumor, and disease stage according to the American Joint Commission on Cancer (AJCC) staging system. All imaging data were independently assessed by two radiologists. If there was a discrepancy between the two radiologists, the final classification was made by another more experienced radiologist. PFS was defined as the time from the commencement treatment of corresponding regimens to progressive disease (PD) on the basis of the RECIST v. 1.1 or death for any cause, whichever occurred first. OS was defined as the time from the commencement of treatment to death from any cause. ORR was defined as the proportion of patients with complete response (CR) or partial response (PR) according RECIST version 1.1. The DCR was defined as the proportion of patients with objective response plus stable disease (SD). AEs were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events v. 4.03.

##Statistical analysis

The demographic data, outcome data, and other clinical parameters are presented as the frequency for categorical variables and as the median with interquartile range (IQR) for the age variable. The median PFS and OS were estimated by Kaplan-Meier method and are reported with 95% CIs. For ORR and DCR, point estimates and exact Clopper-

Reply: The duplication rate is down.