Peer Review File

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Reviewer Comments

Comment 1:
First, in the title I suggest the authors to indicate the dataset used.
Reply 1: Thank you for your valuable suggestion regarding the title of our manuscript. We have revised the title as per your recommendation to:

"Causal relationship between circulating immune cells and gastric cancer: a bidirectional Mendelian randomization analysis **using UK Biobank and FinnGen datasets**"
Changes in the text: Title in the Page 1.

Comment 2:
Second, the abstract needs some revisions. The background needs to specify the controversy regarding the causal relationship between circulating immune cells and gastric cancer and explain why MR studies are helpful for addressing the controversy. The methods need to specify the clinical sample and outcome assessment in the datasets used, as well as the selection of instrumental variables. The results need to report the statistics and P values to support the main findings. The current conclusion is vague and I suggest the authors to have mode detailed comments for the clinical implications of the findings.
Reply 2: Thanks for your insightful comments and suggestions. We have carefully addressed each point and made the following revisions:

(1) Background: We have expanded the abstract's background section to highlight the controversy surrounding the causal relationship between circulating immune cells and gastric cancer. We've also explained the value of Mendelian randomization (MR) studies in addressing this issue. The revised background now reads:

"The role of immune cells in cancer pathogenesis remains controversial due to conflicting reports, potentially arising from various confounding factors. Emerging evidence suggests that cancer can also influence immune cell populations and functions, making it challenging to investigate their causal relationship. Traditional observational studies often fail to eliminate all confounding factors and are prone to reverse causality. Therefore, we employ Mendelian randomization (MR) to determine the causal relationship between immune cells and cancer, as this method can identify causal relationships independent of confounding factors and avoid reverse causality." **(see Page 2, lines 2-7)**

(2) Methods: We've provided more detailed information about the clinical samples, outcome assessment, and instrumental variable selection. The methods section now includes:

"Genome-wide association study (GWAS) summary statistics on immune traits, encompassing 310 immune cell phenotypes, were obtained from 3,757 European individuals, with peripheral blood immune cells tested using flow cytometry. GWAS summary statistics for gastric cancer were derived from 476,116 European individuals across two large-scale

biobanks: the UK Biobank and FinnGen. Gastric cancer was identified by the International Classification of Diseases, 9th Revision (ICD-9), and 10th Revision (ICD-10) codes. Significant single nucleotide polymorphisms (SNPs) for immune traits were extracted at a threshold of $P < 1 \times 10^{-5}$, while a threshold of $P < 5 \times 10^{-8}$ was used for gastric cancer GWAS data. Linkage imbalance-based clumping was performed to obtain independent SNPs, and those with $F < 10$ were excluded to mitigate weak instrument bias. Phenoscanner V2 was used to exclude SNPs directly associated with potential confounders or outcomes." **(see Page 2, lines 8-16)**

(3) Results: We have incorporated the relevant statistics and P values to support our main findings. **(see Page 2, lines 20-23)**

(4) Conclusions: We've revised the conclusion to be more specific and to emphasize the clinical implications of our findings. The new conclusion reads:

"Circulating CD4-CD8- T cells and IgD-CD27- B cells are positively correlated with the development of gastric cancer, while the percentage of IgD+ CD24- B cells in lymphocytes are negatively correlated. These findings provide insight into the relationship between immune cells and gastric cancer pathogenesis and may serve as a basis for the development of immunotherapies for gastric cancer." **(see Page 2, lines 26-29)**

Thank you again for your constructive comments. We believe these revisions have significantly improved the clarity and comprehensiveness of our abstract.

Changes in the text: Page 2, lines 2-29.

Comment 3:

Third, in the introduction of the main text, the authors need to present examples of the controversies regarding the causal relationship between circulating immune cells and gastric cancer, analyze the potential reasons for the controversy, and explain why MR studies are useful for addressing the controversy. The authors need to further explain why they focused on bidirectional relationship since the studies reviewed above did not indicate the reverse relationship between circulating immune cells and gastric cancer.

Reply 3: Thank you for your thoughtful feedback. We have made the following revisions to address your concerns:

(1) We have provided more examples of controversies regarding the causal relationship between circulating immune cells and gastric cancer, listed potential reasons for the controversy, and explained the limitations of traditional observational studies and why MR studies are useful for addressing these controversies. We added the following content:

"In gastric cancer, tumor-infiltrating dendritic cells and natural killer cells are generally associated with improved prognosis and enhanced anti-tumor immunity (16). In contrast, effector regulatory T cells and regulatory B cells can promote immune escape, leading to poorer outcomes (7). The prognostic implications of cytotoxic T cells (CD8+) are particularly complex (17-19); while some studies link high CD8+ T cell infiltration to better survival rates, others associate it with worse prognoses. These discrepancies likely arise from the intricate interplay between different immune cell subpopulations, the tumor microenvironment, and the stage of cancer progression. Thus, elucidating the causal relationships between immune cells

and gastric cancer is crucial. However, traditional observational studies are often confounded by factors such as reverse causality, making it challenging to establish definitive causal links." (**Page 3, lines 10-18)**

(2) We have also explained that cancer itself can influence immune cell populations and function, and clarified why we focused on the bidirectional relationship between immune cells and gastric cancer. We added the following content:

"While previous studies have primarily focused on the impact of immune cells on cancer development, emerging evidence suggests that cancer itself can influence immune cell populations and function. This bidirectional relationship between cancer and the immune system is known as cancer immunoediting (31). During this process, the tumor can shape the immune response, potentially leading to changes in circulating immune cell populations (31,32). Therefore, investigating the bidirectional relationship between the immune cells and gastric cancer is crucial for understanding the complex interplay between them." (**Page 3, lines 25-30)**

Changes in the text: Page 3, lines 10-18 & lines 25-30.

Comment 4:

Fourth, in the methodology of the main text, the authors need to describe the datasets used in detail, including the clinical samples, how the diagnosis of gastric cancer was ascertained, and how the circulating immune cells were tested, as well as the details of selecting IVs. In statistics, please briefly describe the differences between the five analyses and explain why all the analyses were performed.

Reply 4: Thank you for your detailed feedback. We have made the following revisions:

(1) We have provided a more comprehensive description of the datasets used, including details about the clinical samples and how circulating immune cells were tested:

"Peripheral blood samples were collected from the donors in heparin tubes. Circulating immune cells were tested using flow cytometry, where the blood samples were stained with specific antibodies and analyzed with BD FACSCanto II flow cytometers (34). The data were processed using BD FACSDiva software, and cell populations were manually gated to ensure consistency (34)." (**Page 4, lines 17-20)**

(2) We have elaborated on how the diagnosis of gastric cancer was ascertained:

"The statistics included 24,188,662 SNPs from 476,116 European individuals across two large-scale biobanks: the UK Biobank and FinnGen. In the UK Biobank cohort, gastric cancer cases were identified through linkage with national cancer registries (37). In the FinnGen cohort, gastric cancer cases were ascertained using a combination of nationwide health registries, including the Finnish Cancer Registry, the Care Register for Health Care, and the Cause of Death Register (38). Identification of gastric cancer was facilitated by corresponding International Classification of Diseases, 9th Revision (ICD-9), and 10th Revision (ICD-10) codes." (**Page 4, lines 22-28)**

(3) We have provided a more detailed and structured description of the instrumental variable (IV) selection process:

"We employed a systematic approach to select IVs for each immune trait and gastric cancer. For immune traits, we extracted significant SNPs at a threshold of $P < 1 \times 10^{-5}$ (39,40), while for gastric cancer GWAS data, we used a more stringent threshold of $P < 5 \times 10^{-8}$. To ensure independence among selected SNPs, we performed linkage imbalance (LD)-based clumping using PLINK. For immune traits, we applied an r^2 threshold of 0.1 within a 500 kb window (39), whereas for gastric cancer, we used a stricter r^2 threshold of 0.0001 within a 1000 kb window. LD calculations were based on the 1000 Genomes Project reference panel (41). To assess instrument strength, we calculated the F statistic for each SNP and included only those with $F > 10$ to mitigate weak instrument bias (42). Furthermore, we utilized Phenoscanner V2 to exclude SNPs directly associated with potential confounders or outcomes (43,44), ensuring the validity of our selected IVs. This comprehensive selection process aimed to identify robust and independent IVs for our MR analysis." (**Page 4, lines 32-33 & Page 5, lines 1-8)**

(4) We have described the differences between the five analyses and added relevant references:

"These methods make different assumptions about pleiotropy and instrument validity: IVW assumes all instruments are valid or that pleiotropic effects are balanced (46); MR Egger allows for directional pleiotropy and provides a test for its presence (47); weighted median is robust when up to 50% of the weight comes from invalid instruments (48); simple mode assumes that the most common estimate is the true causal effect (49); weighted mode provides a more robust estimate when there is heterogeneity among the SNPs (50)." (**Page 5, lines 13-17)**

(5) We have explained why all the analyses were performed:

"We used multiple methods to assess the robustness of our findings across different assumptions." (**Page 5, line 18)**

Changes in the text: Page 4, lines 17-20 & lines 22-28 & lines 32-33; Page 5, lines 1-8 & lines 13-18.

Comment 5:

Finally, please consider to cite some related papers:

1. Ran S, Yao J, Liu B. The association between sarcopenia and cirrhosis: a Mendelian randomization analysis. Hepatobiliary Surg Nutr 2023;12(2):291-293. doi: 10.21037/hbsn-22-632.

2.Gao L, Di X, Gao L, Liu Z, Hu F. The Frailty Index and colon cancer: a 2-sample Mendelian-randomization study. J Gastrointest Oncol 2023;14(2):798-805. doi: 10.21037/jgo-23-134.

3.Yin Y, Wang B, Yang M, Chen J, Li T. Gastric cancer prognosis: unveiling autophagy-related signatures and immune infiltrates. Transl Cancer Res 2024;13(3):1479-1492. doi: 10.21037/tcr-23-1755.

4. Chen S, Ben X, Guo L, Li X. Identification of lncRNAs based on different patterns of immune infiltration in gastric cancer. J Gastrointest Oncol 2022;13(1):102-116. doi: 10.21037/jgo-21-833.

Reply 5: Thank you for your valuable suggestion. We have added the recommended references as advised. (**Reference No. 23, 24, 14, 15 in the revised manuscript**)

Changes in the text: Reference No. 23, 24, 14, 15 in the revised manuscript.