

Peer Review File

Article Information: <https://dx.doi.org/10.21037/tcr-23-1292>

Reviewer A

The paper titled “The Activation of HAMP Promotes Colorectal Cancer Cell Proliferation through Zinc-Mediated Upregulation of SMAD4 Expression” is interesting. The study demonstrates that zinc metabolism-related genes HAMP may serve as an individual prognostic indicator in CRC patients. Further, the activation of HAMP promotes CRC cell proliferation through zinc-mediated upregulation of SMAD4 expression, which reveals the relation between zinc metabolism and CRC and provides new clinical indicators for CRC patients. However, there are several minor issues that if addressed would significantly improve the manuscript.

1)The abstract is not sufficient and needs further modification. The research background did not indicate the clinical needs of the research focus.

Reply 1: Thank you very much for reviewing our paper and for your valuable suggestions. Regarding your mention of the inadequacy of the abstract and the need for further revisions, we have taken the following steps to address your request: Abstract Revision: We have revisited and revised the abstract to ensure that it provides a more detailed and clearer overview of our research. We have paid particular attention to key information that highlights the focus of the study so that readers can better understand our work. Revision of the research context: We have revisited the research context in detail to reveal the research focus more fully. We emphasized the context and motivation of the study to ensure that readers have a clear understanding of the importance and practical implications of the study on first reading. While making these changes, we ensured that we followed academic norms and best practices in dissertation writing. We realize that your review comments are crucial to improving the quality of your paper and we will do our best to meet your expectations. Once again, thank you for your careful review and valuable suggestions.

Changes in the text: we have modified our text as advised (see Page1-3, line 27-84).

2)There is A-E in Figure 1, but only A-D in the figure legend. Please carefully check and make corrections.

Reply 2: Thank you very much for your valuable comments. I apologize for the problem you pointed out in Figure 1 and have double-checked the legend to correct it. After double-checking, I have confirmed that the legend does indeed contain only A-D and does not include E. To address this issue, I have added the appropriate label E to the legend to ensure that the diagram is consistent with the textual description. Thank you again for your corrections, which are crucial to improving the quality of the paper. Thank you for your time and professional input.

Changes in the text: we have modified our text as advised (see Page 17, line 501-503).

3)What are the relevant characteristics of the tumor microenvironment of CRC? What is the

correlation between HAMP and the tumor microenvironment? What are the possible goals of future drug development? It is recommended to add relevant content to the discussion.

Reply 3: Thank you very much for reviewing our paper and providing valuable comments. In response to the three questions you mentioned, we have added relevant content to the Discussion section to answer these questions more comprehensively. Regarding the relevant features of the CRC tumor microenvironment: we further discuss in depth the relevant features of the CRC tumor microenvironment, including but not limited to changes in immune cell infiltration, angiogenesis, and other aspects. This helps readers better understand the interactions between HAMP and the tumor microenvironment. Correlation between HAMP and the tumor microenvironment: we describe in detail the expression of HAMP in the CRC tumor microenvironment and its possible effects. This includes the potential mechanisms of HAMP regulation of immune response, as well as interactions with other important molecules in the tumor microenvironment. Possible targets for future drug development: we look to the future to explore the clinical landscape based on our findings, including potential drug targets, possible therapeutic strategies, and consider possible challenges in drug development. This helps to highlight the practical implications of our research and the potential for future clinical applications. These additions will better respond to the questions you have raised and make our paper more comprehensive and convincing. Once again, thank you for your valuable suggestions and we will ensure that the paper is appropriately revised to meet your requirements.

Changes in the text: we have modified our text as advised (see Page 13, line 376-378; see Page 14, line 415-419; see Page 15, line 431-432).

4)What are the significant roles of the zinc homeostasis-regulatory proteins in CRC pathophysiology? It is suggested to add relevant contents.

Reply 4: Thank you very much for your careful review and valuable comments on our paper. Regarding the question you mentioned, "What is the important role of zinc homeostatic regulatory proteins in the pathophysiology of colorectal cancer?" We have added relevant content based on your suggestion to answer this question more comprehensively. Importance of zinc in cell biology: We extend the discussion on the important role of zinc in cell biology, especially in cell differentiation, proliferation and apoptosis. This contributes to the understanding of the role of zinc in the pathophysiology of colorectal cancer. Regulatory role of zinc in cancer: We describe the regulatory mechanisms and role of zinc in cancer development. This helps to reveal the unique contribution of zinc in colorectal cancer pathogenesis. Role of zinc homeostatic regulatory proteins: We describe in detail the role of zinc homeostatic regulatory proteins in colorectal cancer. This includes their interactions with other proteins and their role in regulating cell signaling pathways. Clinical significance and potential therapeutic targets: We explore the clinical significance of zinc homeostatic regulatory proteins in colorectal cancer and discuss their potential as therapeutic targets. This will make our paper more practical and provide direction for future research. We believe that these additions will make our paper more complete, detailed, and better answer the questions you have asked. Again, we appreciate your time and guidance.

Changes in the text: we have modified our text as advised (see Page 14, line 399-402).

5)Some fonts need to be enlarged, as shown in Figures 1,2,3,6.

Reply 5: Thank you for your valuable comments on my thesis. I have read your suggestions carefully and have revised the thesis according to your suggestions. In particular, you mentioned the need to enlarge some of the fonts such as Figures 1, 2, 3 and 6. I have taken the following steps to fulfill your request: Enlargement of fonts: I will increase the font size of Figures 1, 2, 3 and 6 covered in the thesis in order to improve readability and to clearly present the relevant information. Review of Layout: I have also reviewed the layout of the entire document to ensure that the overall aesthetics and clarity of the document is maintained while the font size is enlarged. Please be assured that I will take your suggestions seriously and submit an updated version with revisions. Thank you again for your guidance and review.

Changes in the text: we have modified our figures as advised (see figure revised).

6)What is the correlation between the expression of HAMP and the prognosis of CRC patients? How to gain in-depth understanding through bioinformatics? It is recommended to add relevant content.

Reply 6: Thank you for your careful review and valuable suggestions on our paper. Regarding the issues you mentioned, we have included more content about the prognostic relevance of HAMP expression in CRC patients in the revised version and detailed how to gain insight into this relationship through bioinformatics approaches. This contributes to a more comprehensive understanding of the role of HAMP in CRC and provides additional background information for the reader. We explored the association between HAMP gene expression and prognosis of CRC patients in depth by bioinformatics methods. First, we used RNAseq data provided by TCGA and its clinical data, and performed a gene global batch survival analysis by R software to screen for genes associated with significant prognosis in CRC. Genes associated with zinc metabolism were obtained from websites specialized in the field through gene set enrichment analysis (GSEA). To find overlapping genes, we used Venn Diagram Online to draw Venn diagrams of genes related to zinc metabolism and genes with significant prognostic significance in CRC. We further applied the STRING database to predict and catalog the protein interaction networks between these overlapping genes and performed functional enrichment analysis using the DAVID database. GO contains biological processes, cellular components and molecular functions that provide biofunctional explanations for these genes. While by KEGG analysis it was analyzed and visualized how these genes interconnected in the PPI network function through specific pathways. In order to build a more personalized prediction model, we performed expression testing of five interconnected genes in CRC samples and normal control samples from the TCGA database, and compared these genes with the clinical features of CRC using univariate Cox proportional risk regression analysis. Further, we used multivariate Cox proportional risk regression analysis to assess which genes or clinical features might be promising prognostic indicators. Ultimately, we constructed a prognostic diagnostic model containing independent prognostic factors and assessed the predictive power of the model by calibration curves. Based on the TCGA database, we further analyzed HAMP

gene expression. We performed comparative analyses for different cancer stages, patient gender, age, lymph node metastasis status, and TP53 mutation status to identify potential correlations or trends between these parameters and HAMP expression levels. We believe these further analyses and additional content will more fully answer your questions regarding the prognostic relevance of HAMP expression in CRC patients. and improve the quality of our paper. Thank you again for your valuable suggestions and review.

Changes in the text: we have modified our text as advised (see Page 9-11, line 255-310).

7)The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “TRPV3 inhibits colorectal cancer cell proliferation and migration by regulating the MAPK signaling pathway, J Gastrointest Oncol, PMID: 36388700”. It is recommended to quote the articles.

Reply 7: Thank you for reviewing my thesis and providing your valuable comments. I greatly appreciate your professional advice, especially regarding the deficiencies in the introduction section. I have carefully considered your suggestions and have made changes accordingly to ensure a more comprehensive introduction. You pointed out that "TRPV3 inhibits colorectal cancer cell proliferation and migration by regulating the MAPK signaling pathway," J Gastrointest Oncol, PMID: 36388700" is indeed a very important study and closely related to my research topic. At your suggestion, I have cited this article to strengthen the link between my thesis and related studies. Not only does this make my introduction more comprehensive, but it also helps to better frame my research in the current field. Thank you again for your detailed review and suggestions. I am deeply honored to have received your expert guidance and to have ensured that my dissertation is of high quality standards, both in terms of the introduction and in other areas. Thank you again for your time and professional advice.

Changes in the text: we have modified our text as advised (see references).

8)Can zinc be used as a potential adjuvant in the treatment of CRC with chemotherapy drugs? It is suggested to add relevant contents.

Reply 8: First of all, thank you very much for your careful review of our paper and your valuable comments. We deeply appreciate your concern regarding the question of whether zinc can be used as a potential adjuvant to chemotherapeutic agents in the treatment of colorectal cancer (CRC) and would like to respond accordingly. Indeed, relatively few studies have been reported on the specific role of zinc and its potential use in the treatment of CRC, which is one of the reasons why we have been cautious in stating this in the article. However, zinc plays a very important role in living organisms and is involved in several biological processes such as cell survival, proliferation, and apoptosis. These fundamental cell biological functions make zinc of interest in cancer therapy. Despite the more limited reports so far, we believe that the study of zinc as a potential adjuvant in CRC treatment is a direction that deserves to be explored in depth. We will explicitly point this out in the paper and suggest directions for future research to more fully explore the potential application of zinc in CRC therapy. Such in-depth studies will not only help to reveal the mechanism of action of zinc, but also provide new ideas for developing more effective therapeutic strategies. Thank you again for your patient review and

valuable suggestions.

Changes in the text: we have modified our text as advised (see Page 14, line 402-403).

Reviewer B

1. Please provide the full name of “RNAseq” “FBS” “PCR” “RIPA” “BCA” “ANOVA” “mRNA” in the main text. Please also check through your article to make sure **all** the abbreviated terms have been defined when they **FIRST** appear in the Abstract and the main text.

Response: Thank you for your careful review and valuable suggestions on our paper. We have carefully considered your comments and find that our failure to provide full names when we first introduced the terminology abbreviations was an oversight on our part, for which we apologize. In response to your suggestion, we have revised the paper accordingly by adding an explanation of the full title, and we believe that this improvement will significantly enhance the quality of the paper. In addition, we have scrutinized the abstract and the text to ensure that all abbreviated terms have been clearly defined when they first appear. We hope that these changes will fulfill your requirements and make the paper clearer and more understandable. Thank you again for your time and professional advice, your guidance has been crucial to our research.

2. Please recheck the full name of “TMB” in the text.

associated with tumor mutation load (TMB).

Response: Thank you very much for your careful review of our paper and your valuable suggestions. We apologize for the inaccuracy of the full name "TMB" due to a spelling error. The correct term should be "tumor mutational burden (TMB)". We have reviewed the full text to ensure that all relevant sections have been corrected and necessary corrections have been made in the appropriate places. We value your comments and would like to thank you for pointing out our errors. We do our best to ensure the accuracy and quality of the paper. Thank you again for your patience in reviewing the paper and your valuable comments.

3. Please indicate the source of cell lines.

233 **Cell culture and treatment**

234 Both CRC cell lines (RKO, HCT-116, SW480, SW620) and immortalized normal

235 intestinal epithelial cell lines (FHC) were cultured in **Dulbecco's Modified Eagle**

Response: Thank you for reviewing our paper and raising questions about the source of the cell lines. Here, we are more than willing to provide detailed information about the source of the cell lines to ensure the transparency and scientific validity of the study. The cell lines we used

were sourced from the Culture Collection Center of the Chinese Academy of Sciences (located in Shanghai, China). The information was obtained through official means of purchase, ensuring the traceability and credibility of the cell lines. We have amended the text accordingly to clearly indicate the source of the cell lines and appreciate your suggestions, which will help improve the quality and credibility of our paper. Once again, thank you for your patience in reviewing and your valuable suggestions.

4. Figures

- (1) **All abbreviations** in figures and legends should be explained. “CRC” “TCGA” “PPI” “GO” “KEGG” “CI” “BP” “CC” “MF” in Figure 1 for example. Please check all abbreviations and provide the full names in the corresponding figure legend.

Response: Thank you for reviewing our paper and providing your valuable comments. We have carefully read your suggestions and will take appropriate measures in our revisions. Our explanations of the abbreviations you mentioned, as well as others that appeared in the legend that needed to be added, have been added to their corresponding legends, and these explanations have been added to the revised version of the legend to ensure that readers will be able to accurately understand the abbreviations used in the figures. We believe this revision will further improve the clarity and readability of the paper. Thank you again for your guidance and suggestions, and we remain committed to continually improving our research to enhance the quality of our papers.

- (2) It is suggested to recheck Figure 1A legend and the main text.

“(A) Forest plot of the top 30 prognostic genes in CRC identified by genome-wide batch survival analysis of RNA data from TCGA-CRC.”

“Figure 1A shows the top 30 of these identified genes.”

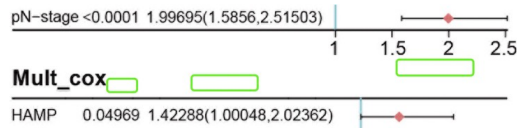
Response: Thank you very much for your valuable comments. At your suggestion, we have carefully reviewed the figure legend and text of Figure 1A and found that there is indeed an inconsistency. We described "top 30 prognostic genes" in Figure 1A, while "top 30 of these identified genes" was mentioned in the text. In order to correct this problem, we have revised the Figure 1A legend and the text to read "top 20 prognostic genes" to ensure that they are consistent. We sincerely apologize for this error and thank you for catching it in your review. We will ensure that this issue is fixed in the final version and thank you again for your time and expertise.

- (3) The words are covered in Figure 1C.



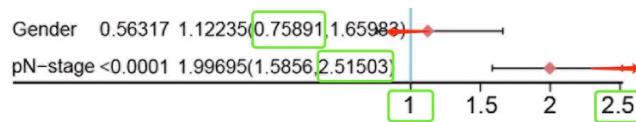
Response: Thank you for your valuable comments on my thesis. I have made a change to Figure 1C based on your suggestion to ensure that the textual content is not covered by the diagram. This change aims to improve the readability of the diagrams and make it easier for readers to understand and analyze the information contained therein to ensure the quality and accuracy of the thesis. Thank you for your careful review of my work. Thank you again for your valuable time and professional advice.

(4) Please add description in Figure 2B.



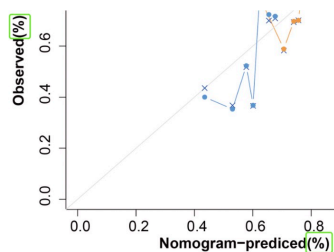
Response: Thank you for your valuable comments. We have made the changes you suggested and have now added the relevant descriptions in Figure 2B. Figure 2B shows a forest plot of the survival curves, which includes the hazard ratio (HR) and its 95% confidence interval (CI) for each group. We believe this addition will better help readers understand the results of our study. Thank you again for your valuable time and professional advice.

(5) Figure 2B: To standardize the results, the part that exceeds the horizontal coordinates should be indicated by **arrows** as below. Please check and revise.



Response: Thank you for reviewing our paper and providing valuable comments. We value your suggestions, especially the part about standardizing the results in Figure 2B. We understand your suggestions, and ensuring the standardization of results is important for clear presentation of the data. We have immediately revised Figure 2B to ensure that sections beyond the horizontal coordinates are clearly indicated by arrows. We will double-check and ensure that the graph is readable and consistent. We are committed to improving the quality of our paper and appreciate your interest and assistance in our research. Thank you again for your review and valuable suggestion.

(6) Please check if the unit “%” is correct or not in the axes of Figure 2D.



Response: Thank you for scrutinizing my paper and for your valuable comments. In response to your query about the correctness of the percentage symbol “%” on the axes of Fig. 2D, I have double-checked. In Figure 2D, the use of the percentage symbol “%” is accurate. The symbol is usually used to indicate relative proportions or percentage changes, and I used percentages on the axes in the figure to show more clearly the percentage relationship of the data in question. In addition, I have confirmed the consistency and accuracy of the use of the percentage symbol to ensure the readability and accuracy of the graph as well as to ensure the quality and accuracy of the paper. Thank you again for your review and valuable comments.

(7) Please check Figure 3, whether it should be “CRC”.

Expression of HAMP in COAD based on Sample types

Response: Thank you for your review and valuable comments. Regarding your reference to Figure 3, I have double-checked and found that there is indeed an error in the original text, it should be "CRC" instead of "COAD". CRC: This is an abbreviation for "Colorectal Cancer", a widely used term covering malignant tumors of the colon and rectum. COAD: This is an abbreviation for "Colon Adenocarcinoma", which more specifically refers to one of the most common types of cancer of the colon, namely cancer originating in glandular tissue. I deeply apologize for this oversight and have made an immediate correction. I have corrected this error in the revised manuscript and made the necessary changes in the text to ensure accuracy and clarity. Thank you again for your correction, your valuable suggestions are very important to improve the quality of the paper. Thank you for your time and professional review.

(8) Please unify to use “HCT116” and “HCT-116” in Figure 4 and Figure 5.

HCT116 HCT-116

Response: Thank you for carefully reviewing our paper and providing valuable comments. Based on your suggestions, we have standardized "HCT-116" to "HCT-116" in Figure 4 and Figure 5. We appreciate your suggestion and believe that this revision will help improve the consistency and professionalism of the paper. Thank you again for your patience and guidance.

(9) Please check whether it should be “si-HAMP^{#1}” in the main text.

knockdown efficiency was estimated by qRT-PCR and WB, which indicated a better efficiency for si-HAMP^{#1} (Figure 4B). Subsequently, Transwell assays demonstrated

Response: Thank you very much for your guidance. I have double-checked the section on "si-HAMP^{#1}" in the main text of the paper and have made changes to ensure accuracy. In the revised version, I have made sure to use the correct term "si-HAMP^{#1}". Thank you for your time and professional guidance.

(10) Please indicate the staining method and magnification in Figure 4C, 4E legend.

Response: Thank you for reviewing our paper and providing valuable comments. Regarding the staining method and magnification in Figure 4C and 4E that you pointed out, we deeply apologize for not making it clear in the figure legends. In Figures 4C and 4E, we used transwell experiments with DAPI staining. The specific staining method was to use DAPI staining solution and follow the standard procedure. Magnification was 100x to ensure that clear and representative cell images were obtained. In order to enhance the clarity and readability of the paper, we have revised the figure legend to clearly indicate

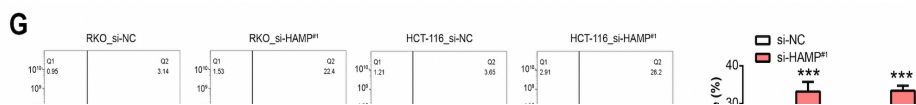
the staining method used and the magnification: (DAPI staining, original magnification $\times 100$). We hope that this revision will fulfill your requirements and make our study more accessible to readers. Thank you again for your patience in reviewing and your valuable suggestions.

(11) Please check whether the legend of Figure 4C-4F should be modified.

“(C-F) Transwell detection of HAMP knockdown regulation of RKO and HCT-116 cell invasion. On the left is a map of microscope fields, and on the right is a histogram of fine invasion counts.”

Response: Thank you very much for your valuable suggestions. The fact that the revised image does not match the description of the legend is an oversight and an error on our part, for which we apologize. Based on your comments, I have modified the legend of Figure 4C-4F. The modified legend reads, "Visualization of the microscope field of view plot, corresponding to a histogram of the number of cell invasions." This modification is intended to provide a clearer description of the plot to ensure that the reader can accurately understand the information in the graph. Thank you again for your review and guidance.

(12) The legend for Figure 4G is missing.



Response: Thank you for your corrections and valuable comments. We have carefully considered and incorporated your suggestions to ensure the completeness and clarity of the paper. We apologize for the omission of the description of Figure 4G. In the issue you mentioned, we have added the legend of Figure 4G and corrected it at the appropriate place. This change not only helps to improve the readability of the paper, but also helps readers to better understand our research. We appreciate your professional advice and believe that these changes will further enhance the quality and accuracy of our study. Thank you again for your time and careful review.

(13) Please indicate the meaning of “****” in Figure 4G legend.

Response: Thank you for your valuable suggestions. Regarding your reference to the meaning of “****” in the Figure 4G legend, I apologize for not being able to make this clear in the initial draft. In the revised version, I have made the appropriate changes and added an explanation in the legend that “****” is $P < 0.001$. to ensure that readers can clearly understand what “****” stands for. I have paid particular attention to your comments, which are essential to improving the quality and readability of the paper. Thank you again for your review and valuable comments.

(14) The citation of Figure 4G is missing in the text.

Response: Thank you for your review and valuable comments. Regarding the missing citation of Figure 4G that you mentioned, I have double-checked and corrected it. This one is an oversight and mistake on our part and we are very sorry for it. In the paper, I have ensured that Figure 4G is correctly cited and revised in the relevant paragraphs to ensure consistency with the citation of the figure. I have ensured that this issue has been properly addressed and hope that you are satisfied with my revisions. Thank you again for your valuable comments, which are crucial for me to improve the quality of my thesis.

- (15) Figures should be cited **consecutively** in the text and numbered in the order in which they are discussed. Therefore, Figure 5B should be cited before Figure 5C, unless Figure 5 is cited as a whole before. Please check through and revise.

379 of HAMP was reduced (Figures 5A and 5C), further confirmed by WB (Figures 5B and
380 5D). These results suggest that zinc ions can positively regulate HAMP expression, and

Response: Thank you for your careful review and valuable comments on our paper. We have carefully considered the issues you raised regarding the citation of the figures and have made appropriate changes in response to your request. Specifically, we have rechecked the chart citations in the paper to ensure that they are numbered in the same order as they are discussed in the text. We believe this adjustment will improve the logical consistency and readability of the paper. Thank you again for your guidance and advice, we are honored by your expertise. Thank you again for your guidance.

- (16) Please revise “ZnSO4” to “ZnSO₄” in Figure 5, 6.

Response: Thank you for your careful review of our paper and your valuable comments. In response to the problems you have pointed out, we have made the corresponding corrections. In Figures 5 and 6, we have corrected "ZnSO4" to "ZnSO₄". We apologize for the inadvertent errors and thank you for your corrections, which help to improve the accuracy and quality of our paper. Thank you again for your review and valuable comments.

- (17) Please indicate the meaning of “*” “**” in Figure 6 legend.

Response: Thank you for your careful review of our paper and your valuable comments. Regarding your question about the meaning of "*" and "**" in the legend of Fig. 6, we have revised it and added the corresponding explanations in the legend to make the meaning of these symbols clearer. In the new legend, *P<0.05, **P<0.01. We hope that such changes will better help readers understand the diagrams and improve the readability and accuracy of the paper. Your professional input is greatly appreciated and is vital to improving the quality of our papers. Thank you again for your valuable time and suggestions.

- (18) Please unify “CCK-8” and “CCK8” in Figures 5-6.

Response: Thank you very much for your valuable comments. Based on your suggestion,

we have harmonized the terminology in Figures 5 and 6 to "CCK-8". We have taken your feedback seriously and made corresponding changes in the paper to ensure the consistency and accuracy of the terminology. Thank you for your interest and guidance in our research. Thank you again for your time and professional input.

5. Please check whether it should be "HCT-116" in the Abstract.

"In vitro analysis showed HAMP was over-expressed in CRC, and its knockdown inhibited RKO and HCT-112 cell invasion and migration significantly."

Response: Thank you very much for your review and valuable comments. Regarding the key points you mentioned, we have carefully examined the experimental information covered in the abstract. At your suggestion, we have confirmed that "HCT-116" should be the correct expression in the abstract. We have made the necessary corrections to ensure the accuracy of the text. In the revision, we have also checked the whole paper to ensure that there are no other similar errors. Once again, thank you for your patient review and valuable comments, which are crucial to the improvement of our paper. Thank you for your support and professional advice.