Peer Review File

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Reviewer A

The paper titled "CircRHOT1 restricts gastric cancer cell ferroptosis by epigenetically regulating GPX4" is interesting. CircRHOT1 promoted GC progression and suppressed ferroptosis by recruiting KAT5 to initiate GPX4 transcription. Our findings show that cirRHOT1 is a promising target for GC treatment. However, there are several minor issues that if addressed would significantly improve the manuscript.

Comment 1:

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

Reply: Thank you for your kind advice. We have modified the abstract: "Despite the developments on prognosis and treatment the prognosis of patients with advanced gastric cancer remains poor. Hence, identification of detailed molecular mechanisms and potential therapeutic targets is of great importance for GC study." (see Page 2, line 40-42).

Comment 2:

The description of some methods in this study is too simplistic, please describe in detail.

Reply: Thank you for your kind advice. We have added detailed description on methods: "The cells were incubated with 5 μM probe for in the dark for 30 min at 37 °C. Cells were then harvested and resuspended in serum-free medium. Samples were then analyzed by the BD FACs system", "For MDA detection, cells were homogenized using MDA lysis buffer and centrifuged at 13,000 g for 3 min. After reaction with thiobarbituric acid (TBA), the absorbance values were measured at 532 nm. For GSH detection, cells were lysed with 5% 5-sulfosalicylic acid (SSA) solution and incubated with enzyme mix. Then absorbance values were detected at 450 nm" (see Page 6, line 223-225, 232-236).

Comment 3:

There are many circRNAs that regulate the gastric cancer. Why did the author choose circRHOT1 for research? Please describe the reason.

Reply: Thank you for your kind advice. The circrRHOT1 has been identified as a potential diagnostic factor and therapeutic target for several cancers, and the circRHOT1 is capable of modulating gene expression in epigenetic and post-transcriptional level. However, its role in gastric cancer has not been determined. Hence, we wondered if circRHOT1 participate in the progression of gastric cancer. Corresponding description has been added in introduction section : "Increasing evidence have identified the participation of circRHOT1 in proliferation,

autophagy, apoptosis, and ferroptosis of various cancers such as hepatocellular carcinoma, breast cancer, pancreatic cancer (26,30,31). The diagnostic role of circRHOT1 has been identified in pancreatic cancer. Furthermore, the regulatory effects of circRHOT1 involve epigenetic regulation and post-transcriptional regulation. For example, circRHOT1 directly binds to miR-125a-3p, acting as an endogenous sponge to inhibit its activity, hence targets the E2F3 expression to promote proliferation and invasion of pancreatic cancer cells (32). In lung cancer, circRHOT1 modulate cell apoptosis and cell cycle via directly interact with acetyltransferase KAT5 to modulate the recruitment of RNA polymerase II and the histone H3 lysine 27 acetylation (H3K27ac) modification to the promoter region of c-MYC (33). Therefore, targeting circRHOT1 is a promising strategy for cancer." (see Page 4, line 115-126).

Comment 4:

There is an error in the description of circRHOT1 in the conclusion of the abstract. Please carefully check and make corrections.

Reply: Thank you for your kind advice. We have corrected the error in abstract: "CircRHOT1 promoted GC progression and suppressed ferroptosis by recruiting KAT5 to initiate GPX4 transcription. Our findings showed that cirRHOT1 is a promising target for GC treatment" (see Page 2, line 62).

Comment 5:

What are the correlations between ferroptosis-related genes and the tumor microenvironment? How valuable are circRHOT1 in predicting survival and drug sensitivity in gastric cancer patients? It is recommended to add relevant content.

Reply: Thank you for your kind advice. Our current work does not focus on the correlations between ferroptosis-related genes and the tumor microenvironment. We modified the introduction section: "Based on the reported functions of circRHOT1 in cancer cell growth and death, we speculate that circRHOT1 may be a potential diagnostic factor and therapeutic target for GC" (see Page 4, line 127-129).

Comment 6:

It is suggested to increase the analysis of the relationship between clinical features, such as tumor stage, tumor grade and tumor size, and the expression of circRHOT1, which may be more meaningful.

Reply: Thank you for your kind advice. We will collect clinical samples to analyze the correlation between expression of circRHOT1 with tumor stage and grade in future study.

Comment 7:

The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "Abrogation of ARF6 in promoting erastin-induced ferroptosis and

mitigating capecitabine resistance in gastric cancer cells, J Gastrointest Oncol, PMID: 35837166". It is recommended to quote this article.

Reply: Thank you for your kind advice. We added citation in introduction section: "Studies have shown that ferroptosis plays a critical role in modulating various diseases, especially cancer (16,17). In gastric cancer, targeting genes that regulate ferroptosis possibly confer the drug resistance and repress cell growth (18,19)" (see Page 3, line 102-103).

Comment 8:

Please analyze the mechanism of ferroptosis regulation in gastric cancer based on the results of this study and in combination with relevant literature.

Reply: Thank you for your kind advice. We modified the discussion section to analyze the mechanism of ferroptosis in GC basing on our findings: "GPX4 is a well-known suppressor and pivotal regulator of ferroptosis and could remove the hydrogen peroxide products from membrane lipids and prevent intracellular oxidative stress (14). Here, we found that KAT5 could epigenetically regulate the acetylation and expression of GPX4 in GC cells, and circRHOT1 promoted the recruitment of KAT5 to GPX4, suggesting that circRHOT1 may suppress ferroptosis via GPX4" (see Page 12, line 406-413).

Reviewer B

Comment 1:

First, the title needs to indicate the research design of this study, i.e., in vitro or vivo.

Reply: Thank you for your kind advice. We modified the tile of results section: "CircRHOT1 suppression induced ferroptosis in the GC cells in vitro", "CircRHOT1 epigenetically modulated GPX4 expression in the GC cells by recruiting KAT5 in vitro", "Restoration of KAT5 impairs circRHOT1 deletion-suppressed GC tumor growth in vivo" (see Page 9, line 305, 320, 349).

Comment 2:

Second, the abstract needs some revisions. The background did not explain why there is a need to focus on CircRHOT1 and what the clinical significance of this research focus was. The methods need to briefly describe the research procedures and their underlying hypotheses to be examined. The results need to quantify the findings by reporting main statistics such as expression levels and accurate P values. The conclusion needs more detailed comments for the possible implications of the findings.

Reply: Thank you for your kind advice. We modified the abstract section: "Despite the developments on prognosis and treatment the prognosis of patients with advanced gastric cancer remains poor. Hence, identification of detailed molecular mechanisms and potential

therapeutic targets is of great importance for GC study", "The knockdown of circRHOT1 significantly suppressed cell growth ($p<0.05$) and stimulated the ferroptosis of the GC cells ($p<0.05$). CircRHOT1 recruited KAT5 (Acetyltransferase Tip60) to promote the acetylation of lysine 27 on histone H3 protein subunit (H3k27Ac) of the GPX4 gene and stimulated gene transcription. The overexpression of KAT5 and GPX4 notably reversed the anti-proliferation effect of circRHOT1 depletion ($p<0.05$)" (see Page 2, line 57, 61).

Comment 3:

Third, the introduction of the main text is not adequate. At least, the authors need to briefly review what has been known on the biological mechanisms underlying GC, have comments on their limitations, explain why the CircRHOT1 path is potentially important, and importantly, what the potential clinical contribution of this research focus is.

Reply: Thank you for your kind advice. We have modified the introduction section: "Genetic and epigenetic regulations, such as the transcriptional factors and non-coding RNAs, have been reported to control the growth, death, and metabolism of GC (5,6). Surgical operation is the primary choice for GC patients, and the chemical therapy and radiotherapy are commonly adopted for adjuvant treatment before and after surgery (7). Besides, immunotherapy such as immune checkpoint inhibitors for GC has emerged (8)", "Increasing evidence have identified the participation of circRHOT1 in proliferation, autophagy, apoptosis, and ferroptosis of various cancers such as hepatocellular carcinoma, breast cancer, pancreatic cancer (26,30,31). The diagnostic role of circRHOT1 has been identified in pancreatic cancer. Furthermore, the regulatory effects of circRHOT1 involve epigenetic regulation and post-transcriptional regulation. For example, circRHOT1 directly binds to miR-125a-3p, acting as an endogenous sponge to inhibit its activity, hence targets the E2F3 expression to promote proliferation and invasion of pancreatic cancer cells (32). In lung cancer, circRHOT1 modulate cell apoptosis and cell cycle via directly interact with acetyltransferase KAT5 to modulate the recruitment of RNA polymerase II and the histone H3 lysine 27 acetylation (H3K27ac) modification to the promoter region of c-MYC (33). Therefore, targeting circRHOT1 is a promising strategy for cancer." (see Page 3, line 80-85, 115-126).

Comment 4:

Fourth, in the methodology of the main text, please first have an overview of the research procedures and the questions to be examined by them. In statistics, please ensure $P<0.05$ is two-sided and statistical tests for the pair-wise comparisons after the ANOVA analysis.

Reply: Thank you for your kind advice. We have modified the methods section: "For analyses of datasets with parametric distribution, the two-tailed Student's t-test and a one-way analysis of variance followed by pair-wise comparisons were used for the comparisons between 2 or multiple groups. For the analysis of datasets with non-parametric distribution, the differences between two or more groups were compared using Mann–Whitney U test and Kruskal–Wallis

test with Dunn's multiple comparisons post-test, respectively. P values <0.05 were considered statistically significant" (see Page 8, line 280-288).

Comment 5:

Finally, please consider to cite several potentially relevant papers to enrich the background of this study: 1. Jiang M, Hu R, Yu R, Tang Y, Li J. A narrative review of mechanisms of ferroptosis in cancer: new challenges and opportunities. Ann Transl Med 2021;9(20):1599. doi: 10.21037/atm-21-4863. 2. Geng D, Wu H. Abrogation of ARF6 in promoting erastin-induced ferroptosis and mitigating capecitabine resistance in gastric cancer cells. J Gastrointest Oncol 2022;13(3):958-967. doi: 10.21037/jgo-22-341. 3. Weng W, Zhang M, Ni S, Tan C, Xu M, Wang X, Sun H, Wang L, Huang D, Sheng W. Decreased expression of claudin-18.2 in alpha-fetoprotein-producing gastric cancer compared to conventional gastric cancer. J Gastrointest Oncol 2022;13(3):1035-1045. doi: 10.21037/jgo-22-462.

Reply**:** Thank you for your kind advice. We have modified the introduction section: "Studies have shown that ferroptosis plays a critical role in modulating various diseases, especially cancer (16,17). In gastric cancer, targeting genes that regulate ferroptosis possibly confer the drug resistance and repress cell growth (18,19)." (see Page 3, line 100-103).

Reviewer C

Comment 1:

There are two reference lists in the manuscript. Please check which one is correct, and delete the unnecessary one.

Reply: Thank you for your kind advice. We have deleted the unnecessary reference list in main text (see Page 14, line 464).

Comment 2:

The authors mentioned "studies...", while only one reference was cited. Change "Studies" to "A study" or add more citations. Please revise. Please number references consecutively in the order in which they are first mentioned in the text.

A number of studies have shown that circRNAs participate in multiple cellular processes, including cell growth, metastasis, and autophagy, by sponging microRNAs to modulate downstream mRNA expression or directly interacting with proteins [25]. In recent years, studies examining the functions of non-coding RNAs, especially circRNAs, have drawn great attention [25].

Reply: Thank you for your kind advice. We have corrected these two sentences (see Page 4, line 109 and Page11, line 259).

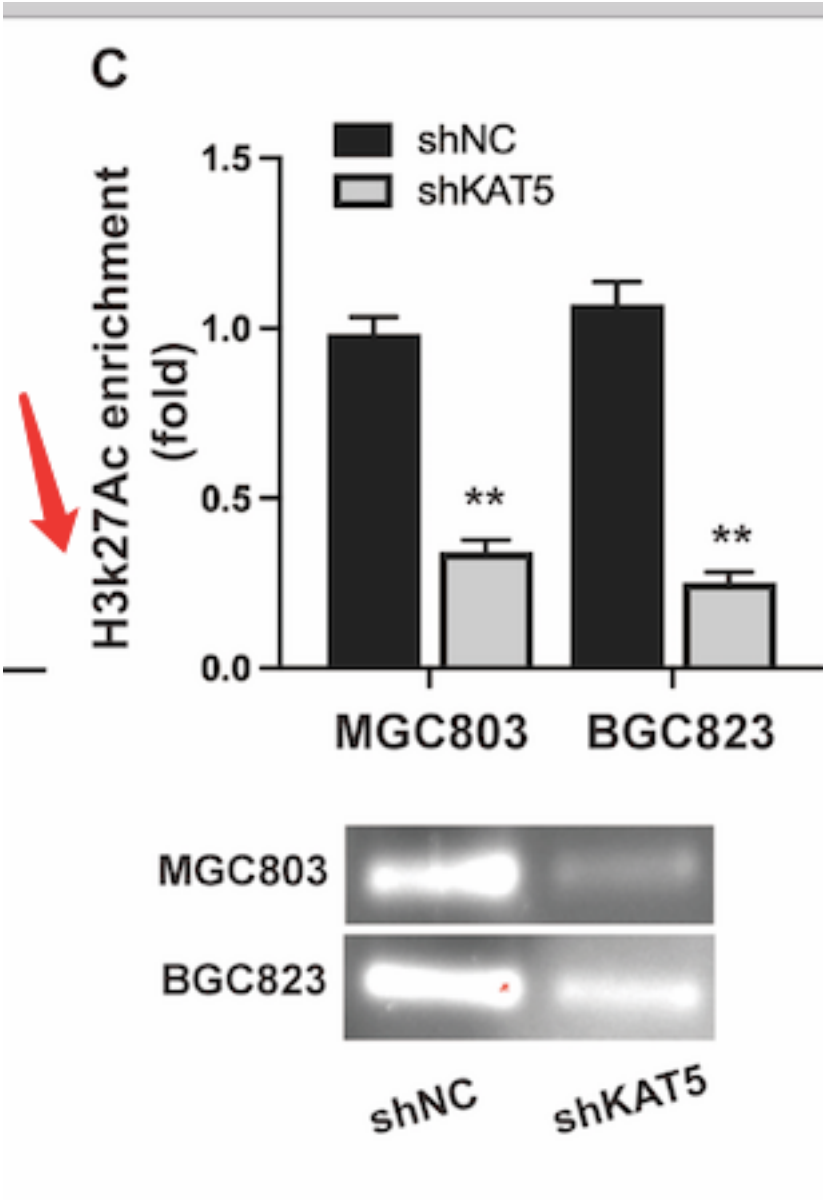
Comment 3: Figure 2

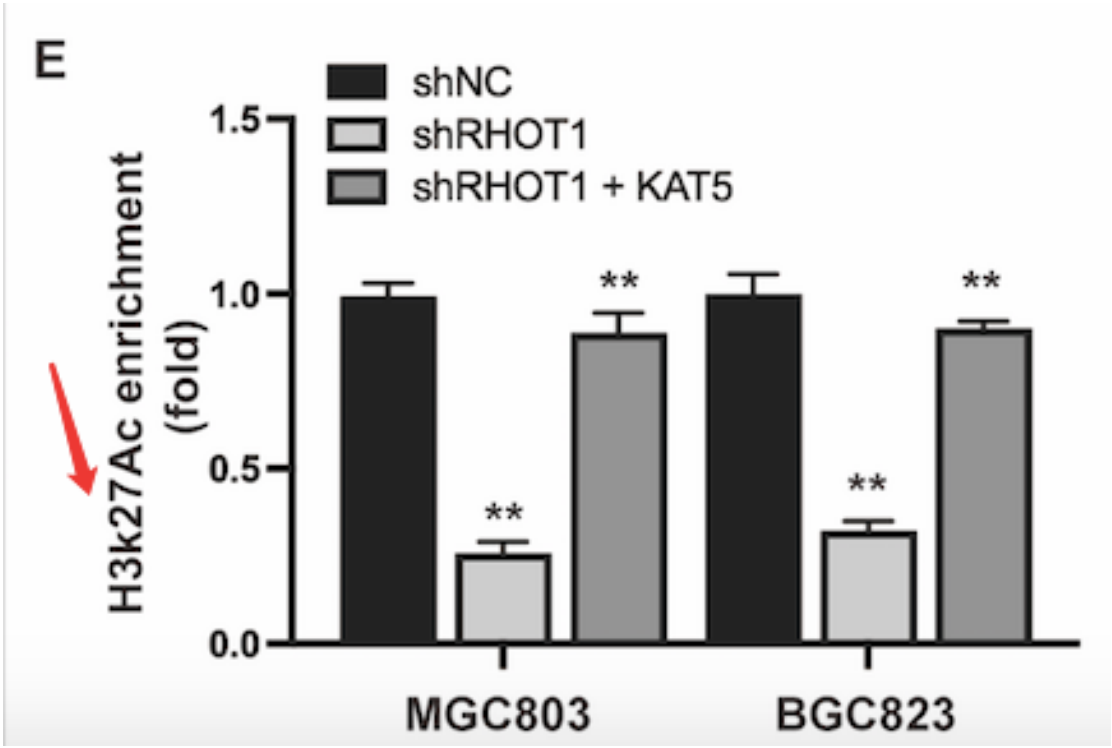
"IgG" was not in figure 2 while it was explained. Please check and revise.

Reply: Thank you for your kind advice. We have deleted the explanation of IgG in figure 2 legend (see Page 17, line 774).

Comment 4: Figure 3C, 3E

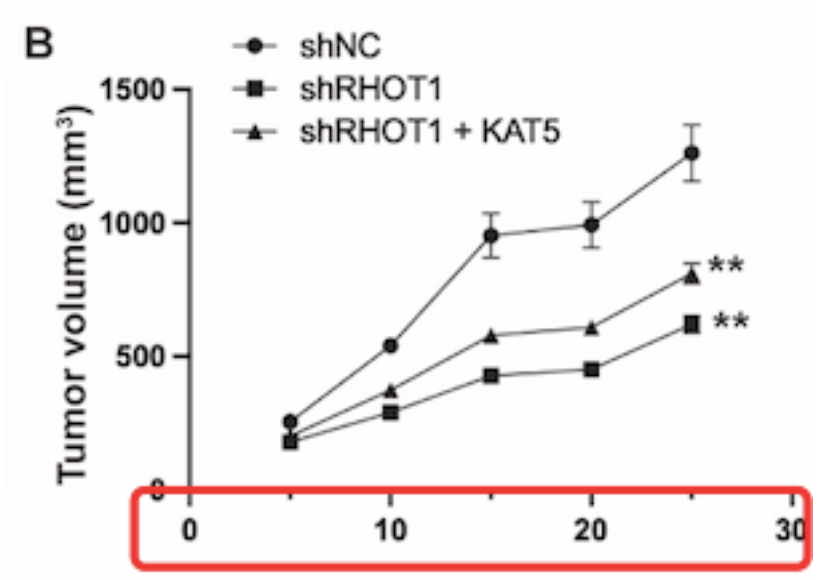
H3K27Ac **or** H3k27Ac? Which one is correct? Please check the whole text and all figure and revise accordingly.





Reply: Thank you for your kind advice. We have uniformed the format as "H3k27Ac" in the text.

Comment 5: Figure 5B
Please provide the descriptions of the x-axis (also provide unit if applicable).



Reply: Thank you for your kind advice. We have revised the manuscript and provided new figure for revision.