Peer Review File

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Reviewer A

The paper titled “Esophageal cancer-related gene 4 inhibits gastric cancer growth and metastasis by upregulating Krüppel-like factor 2 expression” is interesting. The results show that ECRG4 promotes GC progression via Krüppel-like factor 2 signaling and highlight ECRG4 as a potential GC biomarker and therapeutic target. However, there are several minor issues that if addressed would significantly improve the manuscript.

Comment1: What are the relations of ECRG4 promoter methylation to pathology, age, gender and lymph node metastasis? It is suggested to add relevant contents.
Reply1: In this study, I did not conduct a study on ECRG4 promoter methylation. In the reference "Downregulation and DNA methylation of ECRG4 in gastric cancer" PMID: 30034241 studied the relationship between ECRG4 methylation and gastric cancer, and his research showed that the expression level of ECRG4 is positively related to lymph node metastasis. Let's add line296-297.
Changes in the text: Line 296-297.

Comment2: The manuscript mentions "The average tumor volume in the group with ECRG4 overexpression and KLF2 knockdown was 1.43 times higher than that in the group with only ECRG4 overexpression (Figure 5G)". According to the figure, should the tumor volume be 5H? Please check carefully and correct it.
Reply2: Thank you for correcting. We have corrected it on line 369.
Changes in the text: Line 369.

Comment3: The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “Abnormal arginine metabolism is associated with prognosis in patients of gastric cancer, PMID: 35116560”. It is recommended to quote this article.
Reply3: Thank you for correcting. We have corrected it on line 87；
Changes in the text: Line 87，527-529.

Comment4: Does the ECRG4/KLF2 signaling in this study affect the radioresistance of gastric cancer? What impact might it have? It is recommended to add relevant contents.
Reply4: This part of content has not been studied in this study, which will be considered for further research in the future. At present, only the effect of ECRG4 on radioresistance of gastric cancer has been reported.
Changes in the text: No.

Comment5: There are many genes that regulate the gastric cancer cell progression. Why did the author choose ECRG4 for research? Please describe the reason.
Reply5: We observed the differential expression of ECRG4 in gastric cancer tissues and adjacent non-tumor tissues, and found that ECRG4 was associated with the prognosis of gastric cancer in clinical work, so ECRG4 was selected as the object of study.
Changes in the text: No.

Comment6: Can ECRG4 be used as a potential biomarker for patient risk stratification and local regional metastasis in gastric cancer? It is recommended to add relevant content.
Reply6: This relevant content has been added. ECRG4 may serve as a biomarker for risk stratification and local metastasis in patients with gastric cancer.
Changes in the text: Line461-462.

Comment7: It is recommended to increase the study of lncRNA or miRNA regulating ECRG4, which may make the whole study more complete.
Reply7: This study content has been added. MiR-196b was significantly up-regulated and ECRG4 was significantly down-regulated in gastric cancer cells. Down-regulation of MiR-196b can inhibit the proliferation, migration and invasion of gastric cancer cells, while down-regulation of ECRG can reverse this effect.
Changes in the text: Line413-417.

Comment8: What is the impact of this study on the further treatment and prognosis of gastric cancer? It is recommended to include relevant content in the discussion.
Reply8: We have already added this section. Our study found the relationship between ECRG4 and KLF2 in gastric cancer, which is of certain value in predicting the prognosis and treatment success of patients with gastric cancer, and this finding has practical significance for the research on the treatment and management of patients with gastric cancer.
Changes in the text: Line446-447.

Reviewer B

1. The article follows the ARRIVE checklist for reporting standards. Please revise your manuscript according to the attached checklist.
   Reply: OK

2. For any experiments involving animals, the authors must indicate the nature of the ethical review permissions, relevant licenses (e.g., Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals by which the research was conducted.

Reply：The above content has been added to lines 251-254.

Please also provide a statement that the participants gave informed consent before taking part (or a statement that it was not required and why). Authors should also state that the study conformed to the provisions of the Declaration of Helsinki (as revised in 2013), available at: https://www.wma.net/wp-content/uploads/2016/11/DoH-Oct2013-JAMA.pdf

Reply: I have revised.

3. Table 2

Please check the P value.

ECRG4 expression (low vs. high)	0.474	0.331–0.680	0**	0.442	0.291–0.672	0**

307 metastasis, and ECRG4 expression were all linked to prognosis (P<0.01, *Table 2*). Of
308 note, ECRG4 was established as an independent prognostic factor in patients with GC
309 [hazard ratio (HR) =0.442, 95% confidence interval (CI): 0.291–0.672, P<0.01]. In

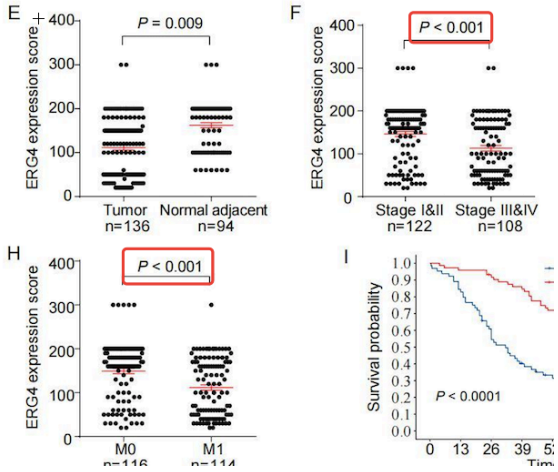
Reply: I've already confirmed that p=0.

Line309 have revised.

4. Figure 1

1) Please check the P value of figure 1F and 1H.

304 node metastasis (P=0.011), and distant metastasis (P=0.002, *Table 1*). The ECRG4
305 expression levels of patients with clinical stage III/IV (P≤0.001), lymph node metastasis
306 (P=0.002), and distant metastasis (P≤0.001) were remarkably lower than those of
307 patients in the control group (*Figure 1F-1H*). Deng P et al. 's research shows that the



Reply: I have revised the figure.

2) Please check if "(C), (N)" are correct in figure 1C legend.

Figure 1 Low expression of ECRG4 is associated with a poor prognosis of gastric cancer. (A) Relative expression of ECRG4 mRNA was significantly lower in GC tissues (T) (n=45) than in adjacent paracancer tissues (ANT) (n=45). (B) Kaplan-Meier overall survival curves of 90 patients with GC with high/low ECRG4 expression. (C) Western blot was performed to measure the protein expression levels of ECRG4 in GC tissues (C) and normal paracancerous tissues (N) from 24 patients with GC. (D)

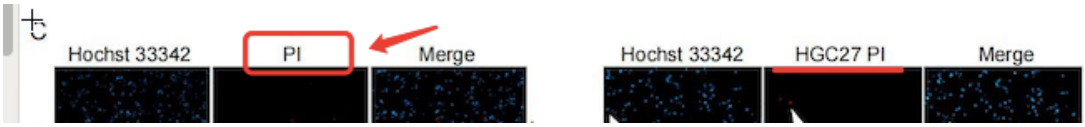
Reply: I have revised.

5. Figure 2

1) Please indicate the full term of "AGS" in figure legend.

Reply: I have revised.

2) Please check if "PI" is correct here.



Reply: I have revised the figure.

3) Please revise the format.

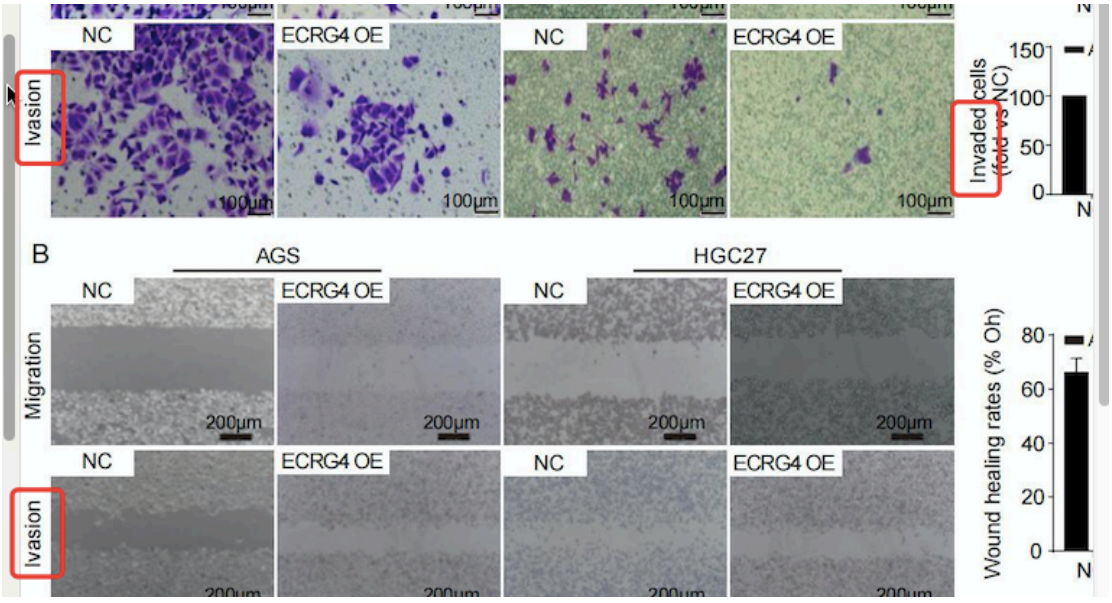
was evaluated using Hoechst-PI staining (magnification, 800×). Hoechst 33342 Stained apoptotic cells ; propidium iodide (PI)Stained apoptotic cells; Hoechst 33342 and PI

Reply: I have revised.

6. Figure 3

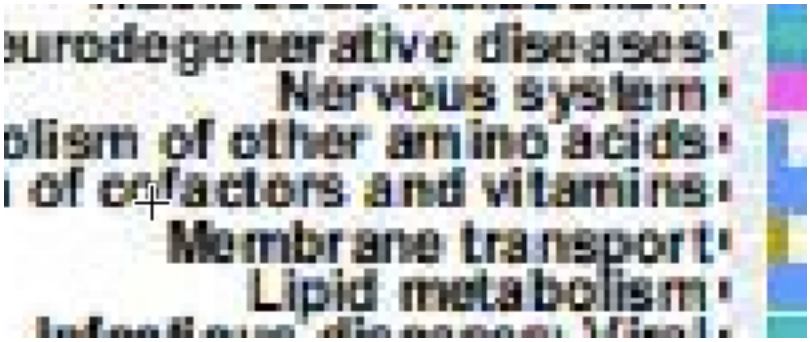
1) Please indicate the full term of "AGS" in figure legend.

2) Please revise this spelling mistake.



Reply: I have revised.

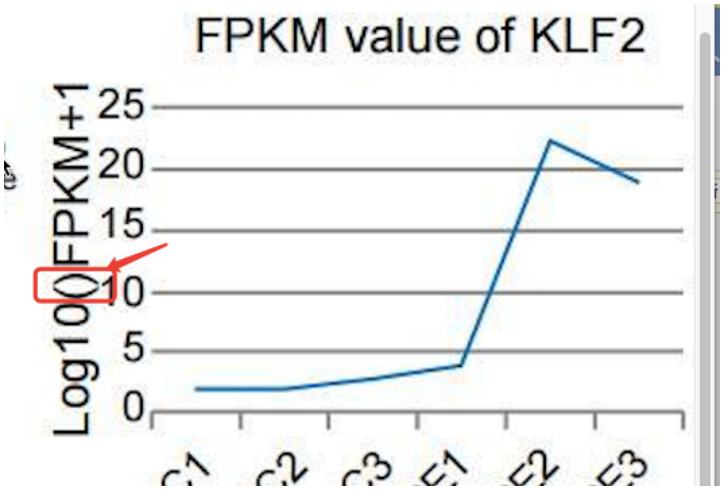
7. Figure 4A is not clear enough, please provide a clear version (higher resolution) in jpg or tiff format.



Reply: We have re-provided.

8. Figure 4

3) Please check if here is correct in figure 4D.



Reply: I have revised.

4) Please indicate the full term of “AGS, PCR” in figure 4 legend.

Reply: I have revised.

9. Figure 5

1) Please indicate the full term of “AGS” in figure legend.

Reply: I have revised.

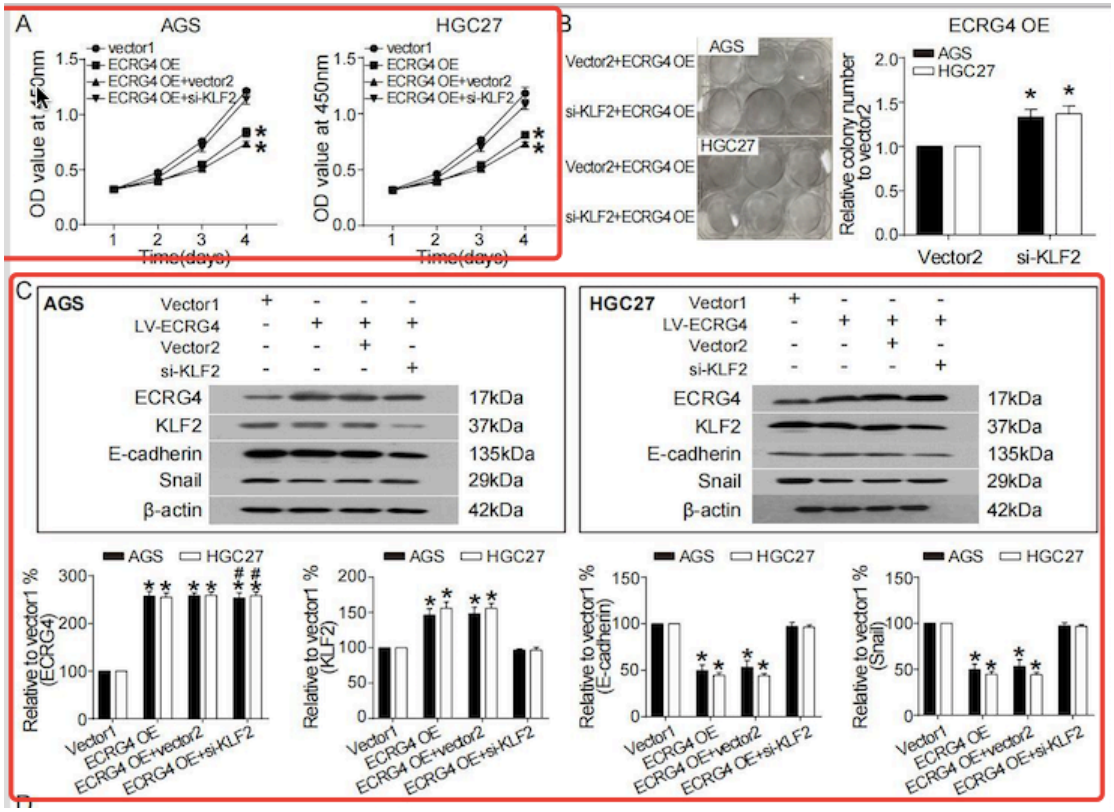
2) Figure 5F and 5H are not cited in the main text. Please check and revise.

375 ECRG4 overexpression to restrain cell migration and invasion (*Figure 5E*). Also, KLF
376 knockdown enhanced the wound healing rate, which had been inhibited in cells with
377 ECRG4 overexpression.
378 *In vivo* tumor growth assays showed that KLF2 knockdown did not reduce the body
379 weight of nude mice (*Figure 5G*). The average tumor volume in the group with ECRG4
380 overexpression and KLF2 knockdown was higher than that in the group with only
381 ECRG4 overexpression (*Figure 5G*). *In vitro* and *in vivo* analyses showed that KLF2
382 knockdown also reversed the changes to EMT markers induced by ECRG4
383 overexpression, decreasing E-cadherin and increasing Snail expression (*Figure 5C,5I*).
384 Overexpression of ECRG4 dampened the proliferation and migration of GC cells and
385 promoted apoptosis, while KLF2 counteracted these effects (P≤0.05, *Figure 5*).

Reply: I have revised it.
Line368-376 have revised.

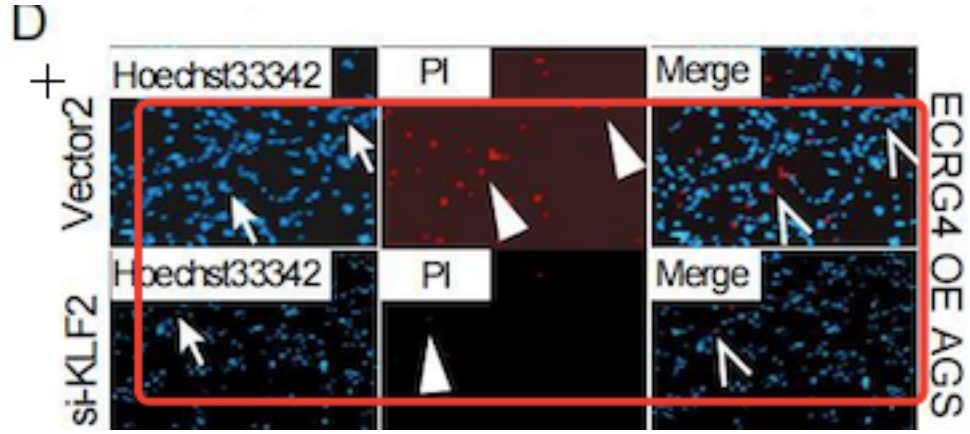
3) Please check if figure legends match the figure 5A and 5C.

705 **Figure 5** KLF2 is essential for ECRG4-mediated proliferation, migration, and invasion
706 of gastric cancer cells. (A) Representative Western blotting results of ECRG4, KLF2,
707 E-cadherin, and Snail (*, P<0.05 compared with the Vector1 group; #, P<0.05 compared
708 with the ECRG4 OE + Vector2 group). (B) Growth curves of AGS and HGC27 cells.
709 Cell viability was measured by Cell Counting Kit-8 assay (*, P<0.05 compared with
710 the Vector1 group). (C) Colony formation assay results (*, P<0.05 compared with the
711 Vector2 group). (D) Hoechst-PI staining was used to determine cell apoptosis



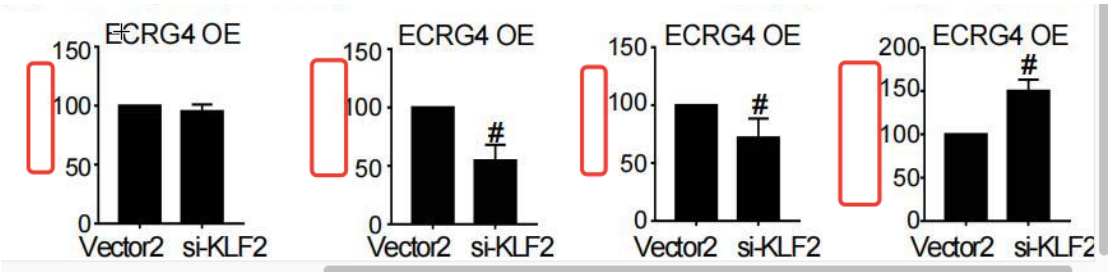
Reply: I have revised it.
Line 664-669 have revised.

4) please indicate the meaning if these symbols in figure 5D legend.



Reply: I have revised.

5) Please provide the description for the Y-axis in figure 5I.



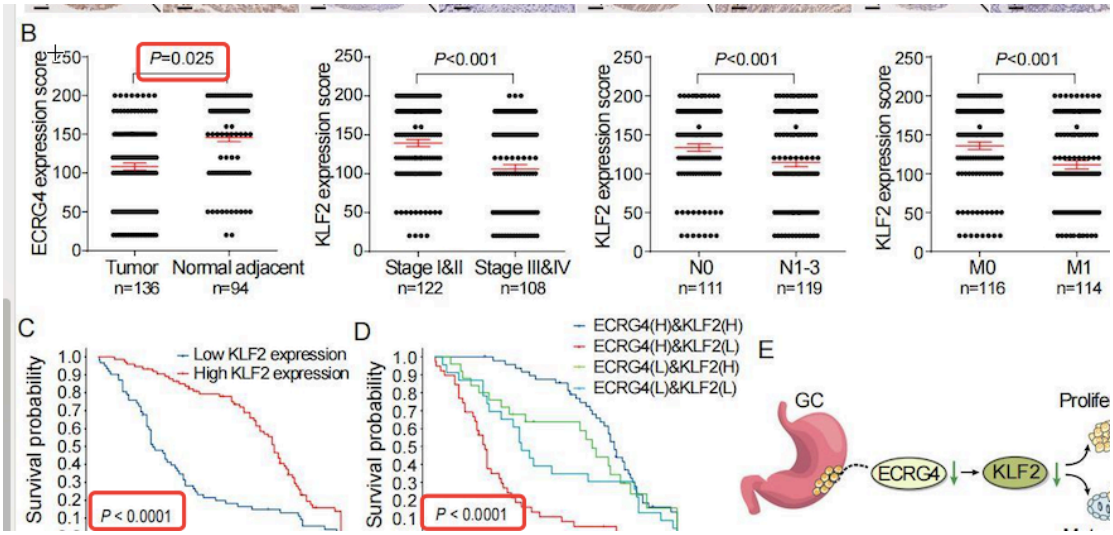
Reply: I have revised.

10. Figure 6

Please check if these P value match the main text.

396 The rates of lymph node and distant metastases were remarkably lower among patients
397 with clinical stage III/IV GC than among the controls (P=0.00059<0.001, *Figure 6B*).
398 Kaplan-Meier survival curve analysis showed that patients with GC with low KLF2
399 expression had poor overall survival (P=<0.0001, *Figure 6C*). Multivariate Cox

409 ECRG4/high KLF2 group (P=0.329). The low ECRG4/low KLF2 group had a poorer
410 prognosis than the low ECRG4/high KLF2 group (P=0.0001, *Figure 6D*). These data



Reply: I have revised.