Peer Review File

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Reviewer A

This study demonstrates the function of hsa_cir_0006646 in gastric cancer.
The meaning of underlines in the sequence of oligonucleotides in Table 2 may be explained in detail.
Reply: The shRNA consists of two short reverse repeats. shRNA cloned into shRNA expression vector consisted of two short reverse repeats separated by a stem loop sequence, which formed a hairpin structure and was controlled by polIII promoter. Then 5-6 T are attached as transcriptional terminators of RNA polymerase III. So the sequence of underlines represents the stem loop sequence.

Reviewer B

The paper titled "Function of hsa_circ_0006646 as a competing endogenous RNA to promote progression in gastric cancer by regulating the miR-665-HMGB1 axis" is interesting. The hsa_circ_0006646-miR-HMGB1 axis exerts its tumor- promoting effects in GC by activating the Wnt/β-catenin pathway. 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: We have modified our text as advised (see Page 2, line 41-44).

2) The description of some methods in this study is too simplistic, please describe in detail.
Reply: We have added some methodological details as advised (see Page 6-12, line 164-337).

3) What are the correlations between hsa_circ_0006646 and gastric cancer staging, degree of differentiation, lymphatic metastasis and survival prognosis? It is recommended to add relevant content.
Reply: hsa_circ_0006646 expression was positively correlated with TNM stage, lymph node invasion and poor survival prognosis, we added some data (see Page 13, line 360-366; Page 20, line 578-580; Page 31, line 871-874; Page 32, line 881-886).

4) There are many circRNA that regulate the progression of gastric cancer. Why did the author choose hsa_circ_0006646 for research? Please describe the reason.
Reply: We first screened the upregulated circRNAs in GC tissues by analyzing a public GEO data set GSE163416. Among all candidate circRNAs, hsa_circ_0006646 exhibited the most significant upregulation, so we choose hsa_circ_0006646 for research (see Page 13, line 354-357).

5) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "Construction of a novel ceRNA network and identification of lncRNA ADAMTS9-AS2 and PVT1 as hub regulators of miRNA and coding gene expression in gastric cancer, Transl Cancer Res, PMID: 35116422". It is recommended to quote the articles.

Reply: We added the relevant content as advised (see Page 5, line 119-124).

6) What are the changes in T cell infiltration and tumor microenvironment during EMT in gastric cancer? What are the correlations? It is recommended to add relevant content.

Reply: We are very sorry that due to the epidemic, the laboratory is still closed at present and can't do experiments. The content and experiments of this part can't be made up for the time being, and we will make up for them when we have the opportunity later. Thank you for your understanding.

7) It is suggested that the research progress of circRNA in gastric cancer should be added to the discussion.

Reply: We added the relevant content as advised (see Page 19, line 547-550).

Reviewer C

1) First, the abstract needs some revisions. The background should clearly indicate the knowledge gaps and limitations of prior studies on molecular mechanisms in GC and explain why the research focus of circRNAs is of research priority. The sentence "have not been fully elucidated" is vague and unclear. The methods need to describe the clinical sample and how the controls were obtained and matched. The results need to quantify the findings by reporting statistics such as the expression levels and accurate P values. The conclusion needs more detailed comments for the clinical implications of the findings.

Reply: We have modified our text as advised (see Page 2-3, line 41-76).

2) Second, in the introduction of the main text, the authors need to have a brief review on what has been known on molecular mechanisms in GC and have comments on the limitations and knowledge gaps of prior studies. The authors need to explain why the circRNA is more important or unique compared to other known molecular mechanisms and deserved to be studied. Please also describe the potential clinical significance of this research focus.

Reply: CircRNAs exhibit multiple functions in cellular processes including cell cycle regulation, proliferation, migration, invasion, apoptosis, and autophagy. Since circRNAs are stable, conserved, and abundant in cancers, some specific circRNAs have been deemed to be of great value for treating cancer patients. We have modified our text as advised (see Page 4, line 101-106,110-114; Page 5, line 125-126, 138-141).

3) Third, in the methodology of the main text, the authors need to have an overview of the research procedures of this study, the questions to be answered by an individual procedure,

and details of the selection of the patient sample and controls. In statistics, please describe the test of the normality of the variables and ensure P<0.05 is two-sided.

Reply: All hypothesis tests were conducted by two-sided test, and the test level was 0.05. A value of P<0.05 was considered statistically significant. We have modified our text as advised (see Page 6, line 164-165; Page 12, 339-349).

Reviewer D

Comment 1: Please unify the full name of "HMGB1".

Reply: We have modified our text as advised.

Changes in the text: Page 3, line 78-79

Comment 2: Please check all abbreviations in the main text, such as below abbreviations. All abbreviated terms should be full when they first appear.

Reply: We have modified our text as advised.

Changes in the text: Page 7, line 193; Page 12, line 336-337.

Comment 3: Please check if any more references need to be added in the below 2 sentences since you mentioned "Studies", but only one reference was cited.

1138 and trans-acting factors (17). Previous studies considered circRNAs as transcriptional

1139 noise and aberrant splicing by-products (28). However, due to the development of high-

1216 tumors (44). Accumulating studies have demonstrated that *HMGB1* is expressed at high

1217 level in multiple malignant tumors (45). *HMGB1* is a key regulator in EMT, promoting

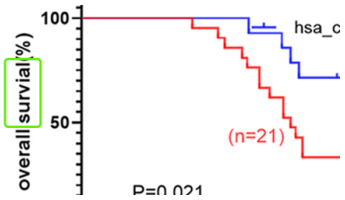
Reply: We have added relevant references.

Changes in the text: Page 19, line 549-550; Page 21, line 612; Page 26, line 747-753; Page 27, line 798-803.

Comment 4: Figure 1:

1) Figure 1A and Figure 1E are too vague. Please resubmit Figure 1 in higher resolution to us.

2) Figure 1C: There is a spelling mistake.



3) The below cited Figure should be "Figure 1D". Please revise.

507 investigated its effects on the malignant behaviors of GC cells. As AGS and HGC27

508 cell lines exhibited a relatively higher expression of *hsa_circ_0006646* as compared to

509 the other GC cell lines (*Figure 1C*), we performed the following experiments using

Reply: We have revised our text and Figure 1. When we zoom out Figure 1A and Figure 1E, the pictures can't be seen very well and we have sent you the original pictures.

Changes in the text: Page 14, line 395.

Comment 5: Figure 2:

1) Please check whether the magnification and staining method are correct in Figure 2D and E.

1012 crystal violet staining). ***P<0.001 compared with sh-NC. (D) The effect of
1013 *hsa_circ_0006646* knockdown on invasion in AGS and HGC27 cells as detected with
1014 transwell assay (magnification 200×, crystal violet staining). ***P<0.001 compared
1015 with sh-NC. (E) The effect of *hsa_circ_0006646* knockdown on migration in AGS and
1016 HGC27 cells as detected with a wound healing assay (magnification 200×, crystal violet
1017 staining). **P<0.01 and ***P<0.001 compared with sh-NC. (F) The effect of

2) Please indicate the full name of "OD", "EMT" in the legend.

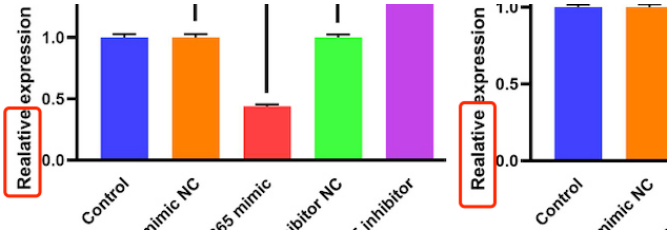
Reply: We have confirmed the magnification and staining method are correct in Figure 2D and E, we have revised other contents of our text.

Changes in the text: Page 34, line 908-909.

Comment 6: Figure 3:

1) Please indicate the meaning of *** in Figure 3D legend.

2) There is a spelling mistake in Figure 3D, F and G.



3) Please check Figure 3F legend. Is the below group name correct?

1034 The potential *miR-665* target sites in *hsa_circ_0006646* transcript. (F) The luciferase
1035 activities of the *hsa_circ_0006646* luciferase reporter vector (WT or MUT) measured
1036 after transfection with *miR-665* mimics or mimic NC into AGS and HGC27 cells.
1037 **P<0.01 and ***P<0.001 compared with *has_circ_0006646*-WT. (G) Ago2-RIP

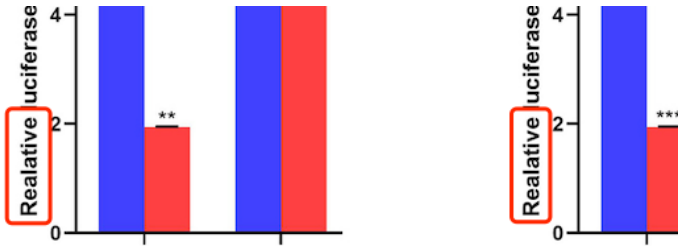
4) Please indicate the full name of "DAPI", "WT", "MUT" in the legend.

Reply: We have revised our text and Figure 3.

Changes in the text: Page 36, line 921-922; line 925; line 930-931.

Comment 7: Figure 4:

1) There is a spelling mistake in Figure 4C.



2) Please check Figure 4C legend. Is the below group name correct?

1049 binding 3'-UTR of *HMGB1* mRNA and *miR-665*. (C) The luciferase activities of the
1050 *HMGB1* luciferase reporter vector (WT or MUT) measured after transfection with *miR-*
1051 *665* mimics or mimic NC into AGS and HGC27 cells. **P<0.01 and ***P<0.001
1052 compared with *hsa_circ_0006646*-WT. (D) The effect of *miR-665* mimic on the

3) Please check Figure 4G legend. Is the staining method correct?

1056 and *miR-665* in GC specimens (n = 35). (G) The presentative IHC staining of *HMGB1*
1057 in GC tissues (magnification 200×, hematoxylin). (H) The expressions of

4) Please indicate the full name of "WT", "MUT" in the legend.

Reply: We have revised our text and Figure 4.

Changes in the text: Page 37-38, line 941-949.

Comment 8: Figure 5:

1) Please check whether the magnification and staining method are correct in Figure 5D and E

2) Please indicate the full name of "OD", "EMT" in the legend.

Reply: We have revised our text.

Changes in the text: Page 40, line 965.

Comment 9: Figure 6:

Please check Figure 6E, G legends. Is the staining method correct?

1086 Western blotting. (E) Representative IHC staining of *HMGB1* in xenograft tumors
1087 (magnification 200×, hematoxylin). (F) The expression of Wnt/β-catenin pathway and
1088 EMT-related proteins in xenograft tumors. (G) Representative IHC staining of β-
1089 catenin in xenograft tumors (magnification 200×, hematoxylin). ***P<0.001. sh, short

Reply: We have revised our text.

Changes in the text: Page 41, line 975-980.

Comment 10: References 49 and 53 are the same. Please check.

Reply: We have modified our text as advised.

Changes in the text: Page 27, line 802-804.