Peer Review File

Article information: https://dx.doi.org/10.21037/jgo-23-151

Reviewer Comments-Reviewer A

General comments
This study investigated the downstream target genes of enolase 1 (ENO1) by RNA-immunoprecipitation sequencing. Several possible target genes were identified. Although the effects of ENO1 and these target genes were not investigated, the work is novel and interesting. However, the quality of the manuscript needs to be improved, particularly the language quality. I have some comments.

Title:
Comment 1: The title should be revised because the effects of ENO1 on glycolysis and tumor progression were not investigated.
Reply 1: We have modified our title as advised: RNA-binding protein *ENO1* promotes the tumor progression of gastric cancer by binding to and regulating gastric cancer-related genes. (see Page 1, line 2-3)
Changes in the text: RNA-binding protein *ENO1* promotes the tumor progression of gastric cancer by binding to and regulating gastric cancer-related genes.

Abstract
Comment 2: Lines 39 to 41: The sentence should be removed. It is a conclusion rather than a result.
Reply 2: We have modified our text as advised: The sentence has been removed. (see Page 2, line 43)
Changes in the text: The sentence has been removed.

Comment 3: Lines 42 to 43: ENO1 may play a role in GC by binding to and regulating GC-related genes and affecting cell proliferation, migration, and apoptosis. This sentence should be rephrased as ENO1 may play a role in GC by binding to and regulating GC-related genes. The effects of ENO1 on proliferation, migration, and apoptosis were not investigated in this study.
Reply 3: We have modified our text as advised: *ENO1* may play a role in GC by binding to and regulating GC-related genes. (see Page 2, line 44)
Changes in the text: *ENO1* may play a role in GC by binding to and regulating GC-related genes.

Introduction
Comment 4: The 1st paragraph should be rephrased. The epidemiology and molecular mechanisms of GC have been introduced in this paragraph. The details of GC epidemiology should be enhanced, and the relationship between PD1 and GC should be removed.
Reply 4: We have modified our text as advised: At present, chemotherapy is still the main

treatment for advanced gastric cancer, but neither changing the number of chemotherapy drugs, different combinations of chemotherapy drugs, nor the currently recommended immunotherapy can solve the high mortality rate of gastric cancer. So, more studies on the mechanisms underlying tumor progression need to be conducted due to the high heterogeneity of GC.(see Page 2, line 55-60)

Changes in the text: At present, chemotherapy is still the main treatment for advanced gastric cancer, but neither changing the number of chemotherapy drugs, different combinations of chemotherapy drugs, nor the currently recommended immunotherapy can solve the high mortality rate of gastric cancer. So, more studies on the mechanisms underlying tumor progression need to be conducted due to the high heterogeneity of GC.

Comment 5: The 2nd paragraph: Further, we found that the expression of ENO1 and pyruvate kinase M2 (PKM2)~~~~. A reference should be cited here.

Reply 5: We have modified our text as advised: This reference has been added. (see Page 3, line 76, Page 14, line 438-440)

Changes in the text: This reference has been added.

Comment 6: ENO1 binds to and degrades the mRNA of the IRP1 gene, thus regulating metabolic homeostasis~~~~~. A reference should be cited here.

Reply 6: We have modified our text as advised: This reference has been cited. (see Page 3, line 93)

Changes in the text: This reference has been cited.

Comment 7: Lines 78 to line 80: Please report the relationship between IRP1 and RBP.

Reply 7: We have modified our text as advised: They found that ENO1 protein binds to (Ironregulatoryprotein 1, IRP1) mRNA, and promotes the degradation of IRP1 mRNA by recruiting RNA degradation factor CNOT6. Combined with previous findings that ENO1 degrades RNA in prokaryotes, the research results reveal the conservation of ENO1 function among species. (see Page 3, line 87-91)

Changes in the text: They found that ENO1 protein binds to (Ironregulatoryprotein 1, IRP1) mRNA, and promotes the degradation of IRP1 mRNA by recruiting RNA degradation factor CNOT6. Combined with previous findings that ENO1 degrades RNA in prokaryotes, the research results reveal the conservation of ENO1 function among species.

Methods

Comment 8: There are two subtitles of cell culture. Please combine them, as well as "Resuscitation of cells," together.

Reply 8: We have modified our text as advised: These two parts have been merged together. (see Page 4, line 120)

Changes in the text: These two parts have been merged together.

Results

Comment 9: After IP was repeated twice, the ENO1 protein could still be detected in the supernatant, which indicated that all the ENO1 protein in the lysate had been precipitated, and

the immunoprecipitation was successful. It seems that this sentence is incorrect. Please check it.

Reply 9: We have modified our text as advised: Protein signal of ENO1 in IP sample is detected, which proves that IP is successful; ENO1 protein can still be detected in the supernatant, which indicates that the ENO1 protein in the lysate has not been completely precipitated, and the immunoprecipitation was successful.(see Page 7, line 204-207)

Changes in the text: Protein signal of ENO1 in IP sample is detected, which proves that IP is successful; ENO1 protein can still be detected in the supernatant, which indicates that the ENO1 protein in the lysate has not been completely precipitated, and the immunoprecipitation was successful.

Comment 10: Figure 1 (A/B/C/D/E/F/G/H/I) should be correctly cited in the results section.

Reply 10: We have modified our text as advised: (see Page 7, line 207-215)

Changes in the text: After IP was repeated twice, Protein signal of ENO1 in IP sample is detected, which proves that IP is successful; ENO1 protein can still be detected in the supernatant, which indicates that the ENO1 protein in the lysate has not been completely precipitated, and the immunoprecipitation was successful(Figure 1A, Figure 1B, Figure 1D). For the input sample, the IP sample reads were significantly enriched in the 5'UTR, 3'UTR, CDS, and intron regions, which showed that ENO1 has a wide range of RNA-binding activities (Figure 1E, Figure 1G). The binding motif of ENO1 obtained by the 2 experiments was consistent, and ENO1 was shown to bind to the GACGAGGA motif and CCAAG on the RNA (Figure 1F). The overlap of the ENO1 binding peaks obtained by the repeated experiments is shown in the following figure (Figure 1H). The peak-related genes were repeated in 2 experiments using the Ablife method, clustered (Figure 1C), and underwent a GO function analysis(Figure 1I).

All figures should be correctly cited in the results section. For example, when introducing the ENO1-binding peak genes of NEAT1, Figure 2A should be cited and put into a parenthesis.

Comment 11: All figures should be correctly cited in the results section. For example, when introducing the ENO1-binding peak genes of NEAT1, Figure 2A should be cited and put into a parenthesis.

Reply 11: We have modified our text as advised: (see Page 7, line 224-225)

Changes in the text: ENO1 can combine with lncRNA NEAT1(Figure 2A), PKM (Figure 2B), CD44(Figure 2C), and LINC00511(Figure 3),which are important long-chain non-coding RNAs involved in the development of GC.

Comment 12: Figures 2 and 3 should be incorporated. Figures 4 and 5 should be incorporated.

Reply 12: Because of the quality, size and layout of the picture, Figure 2 and Figure 3 are separated, and Figure 4 and Figure 5 are separated.

Changes in the text: Because of the quality, size and layout of the picture, Figure 2 and Figure 3 are separated, and Figure 4 and Figure 5 are separated.

Line 209, In the early stage, we knocked down ENO1 in the GC cell line MKN~~~. A reference

should be cited here.

Comment 13: Line 209, In the early stage, we knocked down ENO1 in the GC cell line MKN~~~. A reference should be cited here.

Reply 13: We have modified our text as advised: This reference has been added. (see Page 8, line 243)

Changes in the text: This reference has been added.

Comment 14: Figures 4B and 4C. What method was used to measure these target genes? Please report the method(s) in the methods section.

Reply 14: We have modified our text as advised: The differentially expressed genes were output by using the data of previous gene chip sequencing. (see Page 21-22, line 556-557)

Changes in the text: The differentially expressed genes were output by using the data of previous gene chip sequencing. (see Page 21-22, line 556-557)

Comment 15: Figure 6. This figure should be carefully edited. The title of the x-axis should be the figure legend.

Reply 15: We have modified our text as advised: Figure 6 has been revised and the illustration of figure 6 has been modified. (see Page 24)

Changes in the text: Figure 6 has been revised and the illustration of figure 6 has been modified. (see Page 24) .

Discussion

Comment 16: Line 292, PK should be revised as PKM.

Reply 16: We have modified our text as advised:PKM.(see Page 10, line 333)

Changes in the text: PKM is an important rate-limiting enzyme in the glycolytic pathway,

Comment 17: Generally, the clinical implications of the findings were interpreted in the discussion section. However, I have two suggestions: (1) the limitation of shit study should be discussed; (2) the introduction to the genes should be shortened to make the discussion section more focused.

Reply 17: We have modified our text as advised:(1)We have deleted this sentence: "In recent years, lncRNAs have been a popular area of research."(see Page 10, line 307), and this sentence: "Alternatively, the knockdown of lncRNA NEAT1 has been shown to significantly inhibit the migration and invasion of GC cells in vitro and regulate the expression of EMT-related proteins."(see Page 10, line 315). (2) We added the limitation of shit study: "There are some limitations in this study. Our team found that ENO1 and PKM2 are directly related, and PKM also appeared in the data of two IP repeats of ENO1 in immunoprecipitation. However, this study has not yet reached a clear conclusion on how ENO1 differentially regulates alternative splicing of PKM2 and the subsequent differences in biological effects." (see Page 11, line 350-354).

Changes in the text: (1)We have deleted this sentence: "In recent years, lncRNAs have been a popular area of research."(see Page 10, line 307), and this sentence: "Alternatively, the knockdown of lncRNA NEAT1 has been shown to significantly inhibit the migration and

invasion of GC cells in vitro and regulate the expression of EMT-related proteins."(see Page 10, line 315). (2) We added the limitation of shit study: "There are some limitations in this study. Our team found that ENO1 and PKM2 are directly related, and PKM also appeared in the data of two IP repeats of ENO1 in immunoprecipitation. However, this study has not yet reached a clear conclusion on how ENO1 differentially regulates alternative splicing of PKM2 and the subsequent differences in biological effects." (see Page 11, line 350-354).

Reviewer Comments-Reviewer B

1. In the text, there are totally 23 references cited, but there are 24 references in the references list. Please check and revise.

Reply: We have modified our references as advised: We have revised. There are 24 references in the text and references list.

2. You cited wrong references in below sentences.

338 changes in GC cells (18). In 2007, Hutchinson et al. identified a long-chain non-coding
339 RNA necessary for maintaining the completion of paraspeckle and named it *NEAT1*
340 (19). LncRNA *NEAT1* is overexpressed in GC tissues and cell lines and is related to the

Reply: We have modified our text as advised: We have revised. (see Page 6, line 169),(see Page 10, line 310-312)

3. Please add citation of reference for Gao's study.

97 Zhang et al. (7) and Gao et al. documented the mechanism by which *ENO1* degrades
98 mRNA as an RBP. They found that *ENO1* protein binds to (Ironregulatoryprotein 1,

Reply: We have modified our text as advised: We have revised. (see Page 3, line 86)

4. Please check if the citations of references need to be added in the below 2 sentences since you mentioned the previous "studies".

80 GC cells, promoting apoptosis and increasing their chemosensitivity (4). *In-vivo* studies
81 on the effects of *ENO1* on GC have shown that the deletion of *ENO1* inhibits the
82 tumorigenicity of GC cells in nude mice. Given that *ENO1* is a key enzyme in
83 glycolysis, we used the Seahorse XF96 extracellular flux analyzer to study the effects

301 improved. Previous studies have primarily focused on the occurrence, development,
302 and transformation mechanism of GC. It has been established that changes in energy

Reply: We have modified our text as advised: We have revised. (see Page 3, line 72), the sentences of line 301, I may think it would be better not to add references.

5. If available, please update your reference list by including related literatures published within a year (in 2022). Some of the references are outdated.

Reply: We have modified our text as advised: We have deleted four outdated references and

updated new references. (see Page 14-15, line 460-470), And revised the discussion section. (see Page 9, line 287-294)

Changes in the text: RNA regulates glycolysis and embryonic stem cell differentiation via *ENO1*(14). *ENO1* is a member of the RNA degradosome in prokaryotes, it can bind to LncRNA and participate in the occurrence and progress of tumor(15). Moreover, Huang et al. (16) found that gastric cancer cell line MGC-803 knocked down the expression profile of ENO1 gene, as a result, there were 448 DEG, of which 183 (40.85%) were down-regulated. Deng et al. (17) found that CCDC65 as a new potential tumor suppressor induced by metformin inhibits activation of AKT1 via ubiquitination of ENO1 in gastric cancer.

6. Please cite specific Figure name below. For example, Figure 1A, B…

In addition, Figures should be cited consecutively in the text and numbered in the order in which they are discussed. (For example: Figure 1 contains 4 parts, such as Figure 1A, 1B, 1C, 1D, these parts should also be cited consecutively, unless Figure 1 is already cited before Figure 1A, 1B, 1C, 1D.).

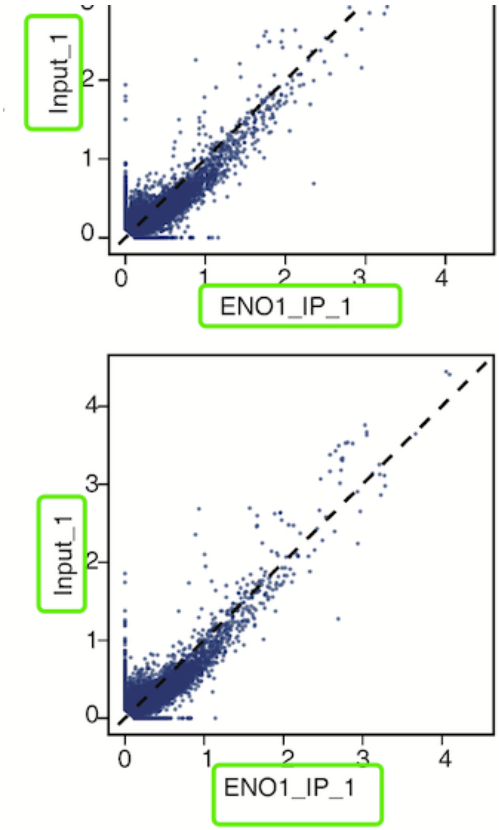
223 the *ENO1* protein in the lysate has not been completely precipitated, and the
224 immunoprecipitation was successful (A,B,D).
225 For the input sample, the IP sample reads were significantly enriched in the 5'UTR,
226 3'UTR, CDS, and intron regions, which showed that *ENO1* has a wide range of RNA-
227 binding activities (E,G). The binding motif of *ENO1* obtained by the 2 experiments was
228 consistent, and *ENO1* was shown to bind to the GACGAGGA motif and CCAAG on
229 the RNA (F). The overlap of the *ENO1* binding peaks obtained by the repeated
230 experiments is shown in the following figure (H). The peak-related genes were repeated
231 in 2 experiments using the Ablife method, clustered (C), and underwent a GO function
232 analysis (I). Interestingly, we found that the consistency of the 2 experiments was very

Reply: We have modified our text as advised: (see Page 7, line 207-215)

Changes in the text: immunoprecipitation was successful (Figure 1A, Figure 1B, Figure 1D). For the input sample, the IP sample reads were significantly enriched in the 5'UTR, 3'UTR, CDS, and intron regions, which showed that *ENO1* has a wide range of RNA-binding activities (Figure 1E, Figure 1G). The binding motif of *ENO1* obtained by the 2 experiments was consistent, and *ENO1* was shown to bind to the GACGAGGA motif and CCAAG on the RNA (Figure 1F). The overlap of the *ENO1* binding peaks obtained by the repeated experiments is shown in the following figure (Figure 1H). The peak-related genes were repeated in 2 experiments using the Ablife method, clustered (Figure 1C), and underwent a GO function analysis (Figure 1I).

7. Figure 1:

The x-axis and y-axis in below two images are the same one. Please check.



Reply: We have modified our figure as advised: (see Page 16)

9. Figure 2-4:

Please indicate the meaning of **, *** in Figure 2 legend, ** in Figure 3 legend and *** in Figure 4 legend.

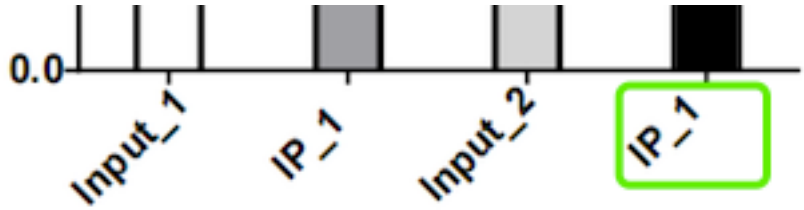
Reply: We have modified our text as advised: (see Page 19, line 542), (see Page 20, line 551), (see Page 22, line 572)

Changes in the text: **, p<0.05; ***, p<0.001; in Figure 2 legend;
**, p<0.05; in Figure 3 legend;
***, p<0.001; in Figure 4 legend;

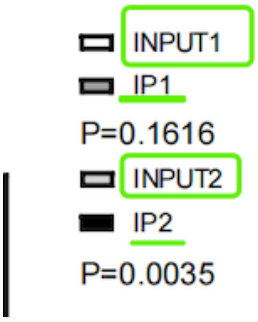
10. Figure 6:

1) Please indicate the meaning of *, **, ***, ****, ns in Figure 6 legend.

2) Please revise all "IP_1" like below to "IP_2".



3) Please revise all "INPUT1" to "Input_1", "INPUT2" to "Input_2" and "IP1" to "IP_1", "IP2" to "IP_2".



Reply:

1) We have modified our text as advised: (see Page 24, line 592-593)
2) We have revised all “IP_1” like below to “IP_2”. (see Page 24)
3) We have revised all “INPUT1” to “Input_1”, “INPUT2” to “Input_2” and “IP1” to “IP_1”, “IP2” to “IP_2”.2) (see Page 24)

Changes in the text:

1): *, P<0.01; **, p<0.05; ***, p<0.001; ****, p<0.0001; ns, not significant; in Figure 6 legend.

2): We have revised all “IP_1” like below to “IP_2”.

3): We have revised all “INPUT1” to “Input_1”, “INPUT2” to “Input_2” and “IP1” to “IP_1”, “IP2” to “IP_2”.2)