

**Dear Editors:**

Thank you for giving us the opportunity to revise our manuscript. We appreciate the comments and suggestions from the reviewers. These comments are all valuable and very helpful for revising and improving our paper. We have studied the reviewers' comments very carefully and tried our best to improve the manuscript. Our point-by-point responses to the original reviewers' remarks are provided underneath each comment.

**Dear reviewer 1 (03491790):**

Thank you for giving us the valuable opportunity to revise our manuscript. We sincerely appreciate the valuable comments and suggestions, which are very helpful for revising and improving our paper. We have studied your comments very carefully and tried our best to improve the manuscript. Our point-by-point responses to the original remarks are provided underneath each comment.

**Comments to the Author:**

1. Fig. 1, the authors should indicate both circ\_0000375 and circ\_0011536 in Fig. 1A. What rationale of choosing these two circRNAs for further study? What about other circRNAs with high fold change and confidence?

**Response:**

This is a very valuable suggestion. We completely agree with you and note circ\_0000375 and circ\_0011536 in Figure 1A. As we mentioned in our manuscript, we identified a circRNA-miRNA-mRNA network that might be involved in the diagnosis and prognosis of colorectal cancer in our previous study <sup>[1]</sup>. Among the five circRNAs in that comprehensive network, circ\_0000375 and circ\_0011536 exhibited the top 2 highest fold changes, indicating promising high values as colorectal cancer biomarkers. Under this circumstance, we selected these two circRNAs for further examination in this study. For other circRNAs with high fold change and confidence, it is necessary to explore the diagnostic and prognostic value for colorectal cancer in the future.

**Reference**

[1] Yin TF, Zhao DY, Zhou YC et al. Identification of the circRNA-miRNA-mRNA regulatory network and its prognostic effect in colorectal cancer. *World J Clin Cases*. 2021 Jun 26;9(18):4520-4541.

2. For wound healing assay, did the authors use TScratch to quantify the migrating areas?

**Response:**

We sincerely thank you for this kind question. However, TScratch is not available on the Internet. In this study, the wound area was quantified using ImageJ software, as we mentioned in the Materials and Methods section. The main purpose of ImageJ is to calculate the area and pixel value. As a simple and powerful tool to quantify and process images, ImageJ is widely used for quantifying migrating areas in wound healing assays in some studies <sup>[1-4]</sup>. In summary, it should be scientific to quantify the migrating areas using ImageJ.

**References**

- [1] Peng L, Sang H, Wei S, Li Y, et al. circCUL2 regulates gastric cancer malignant transformation and cisplatin resistance by modulating autophagy activation via miR-142-3p/ROCK2. *Mol Cancer*. 2020 Nov 5;19(1):156.
- [2] Sherman AB, Gilger BC, Berglund AK, et al. Effect of bone marrow-derived mesenchymal stem cells and stem cell supernatant on equine corneal wound healing in vitro. *Stem Cell Res Ther*. 2017 May 25;8(1):120.
- [3] Pitzurra L, Jansen IDC, de Vries TJ, et al. Effects of L-PRF and A-PRF+ on periodontal fibroblasts in in vitro wound healing experiments. *J Periodontal Res*. 2020 Apr;55(2):287-295. doi: 10.1111/jre.12714. Epub 2019 Nov 28.
- [4] Mendes C, Dos Santos Haupenthal DP, Zaccaron RP, et al. Effects of the Association between Photobiomodulation and Hyaluronic Acid Linked Gold Nanoparticles in Wound Healing. *ACS Biomater Sci Eng*. 2020 Sep 14;6(9):5132-5144.

3. Fig 4 and 5 can be combined as they represent the same results but in different cell lines.

**Response:**

We appreciate your instructive comments and admire your profound research experience. The results of Figure 4 and Figure 5 have been combined into a new Figure 4 in the revised manuscript to reveal the function of overexpression of circ\_0000375 and circ\_0011536.

4. Have the authors performed the analysis based on both circRNAs? ie. will both yield better ROC or diagnosis power compared to each single one?

**Response:**

Thank you very much for your valuable question. We further examined the diagnostic efficiency based on both circRNAs. According to the ROC curve analyses, the AUCs of the combined circRNAs were 0.928 in tissues and 0.997 in serum samples of CRC, indicating that better diagnostic powers were obtained based on both circRNAs. These results are shown in Figure 3, and more details about this issue have been added to the revised manuscript.

5. Table 2, are the survival data available? If so, the authors should perform survival plots based on these two circRNAs.

**Response:**

This is a good question which should be addressed. We entirely agree with you that prognostic value should be explored for these two circRNAs. However, we did not collect the survival data of these patients with colorectal cancer in this study. The effect of these two circRNAs as prognostic biomarkers should be examined with large-scale cohorts in the future, which is also mentioned in the Discussion of our manuscript.

**Dear reviewer 2 (05776245):**

Thank you for giving us the valuable opportunity to revise our manuscript. We sincerely appreciate the valuable comments and suggestions, which were very helpful for revising and improving our paper. We have studied your comments very carefully and tried our best to improve the manuscript. Our point-by-point responses to the original remarks are provided underneath each comment.

**Comments to the Author:**

1. Add hyphen between “miRNA” and “binding” in second paragraph of Introduction.

**Response:**

We sincerely thank you for your careful review. We apologize for the ambiguous statement and truly appreciate the opportunity to explain it.

2. Consider changing the last sentence of Introduction: delete “that” and change “CRC and provided” to “CRC, providing”.

**Response:**

We entirely agree with you, and the corresponding statement has been modified in the revised manuscript as you suggested.

3. Could you please clarify why RKO cells were not subjected to transfection and not investigated throughout the study the same as HCT116 and SW480?

**Response:**

Thanks for the comment. Many researchers choose to confirm the results in two cell lines to elevate the validity of studies in vitro <sup>[1-6]</sup>. SW480 <sup>[1-3]</sup> and HCT116 <sup>[1, 4-6]</sup> are two cell lines commonly used in many studies for colorectal cancer. Therefore, we chose these two cell lines to explore the function of the two circRNAs.

**References**

[1] Ma Z, Han C, Xia W, Wang S, Li X, Fang P, Yin R, Xu L, Yang L. circ5615 functions as a ceRNA to promote colorectal cancer progression by upregulating TNKS. Cell Death Dis. 2020 May 11;11(5):356.

- [2] Zeng W, Liu Y, Li WT, Li Y, Zhu JF. CircFNDC3B sequesters miR-937-5p to derepress TIMP3 and inhibit colorectal cancer progression. *Mol Oncol*. 2020 Nov;14(11):2960-2984.
- [3] Li XN, Wang ZJ, Ye CX, Zhao BC, Li ZL, Yang Y. RNA sequencing reveals the expression profiles of circRNA and indicates that circDDX17 acts as a tumor suppressor in colorectal cancer. *J Exp Clin Cancer Res*. 2018 Dec 27;37(1):325.
- [4] Peng W, Li J, Chen R, Gu Q, Yang P, Qian W, Ji D, Wang Q, Zhang Z, Tang J, Sun Y. Upregulated METTL3 promotes metastasis of colorectal Cancer via miR-1246/SPRED2/MAPK signaling pathway. *J Exp Clin Cancer Res*. 2019 Sep 6;38(1):393.
- [5] Li Z, Yao H, Wang S, Li G, Gu X. CircTADA2A suppresses the progression of colorectal cancer via miR-374a-3p/KLF14 axis. *J Exp Clin Cancer Res*. 2020 Aug 15;39(1):160.
- [6] Weng W, Wei Q, Toden S, Yoshida K, Nagasaka T, Fujiwara T, Cai S, Qin H, Ma Y, Goel A. Circular RNA ciRS-7-A Promising Prognostic Biomarker and a Potential Therapeutic Target in Colorectal Cancer. *Clin Cancer Res*. 2017 Jul 15;23(14):3918-3928.

4. What threshold was used for differential expression analysis visible in Figure 1A? Could this be enclosed? I am interested in fold-change and p-value. This should be included in Materials and Methods.

**Response:**

We apologize for not providing the fold change and *P* value thresholds in the differential expression analysis of Figure 1A. The cutoff criteria of differentially expressed circRNAs in Figure 1A were set at a *P* value < 0.05 and  $|\log_2(\text{fold change})| > 1$ , which have been added to the Materials and Methods of the revised paper.

5. Consider standardizing the visualization of statistical significance – “a/b/c” symbols (representing  $P < 0.001$ ;  $P < 0.01$ ;  $P < 0.05$ , respectively) are either above the specific

bar (Fig1E or F) or between bars (above the line; Figure 1B or C). I would suggest to use the latter method in all figures.

**Response:**

We sincerely appreciate your kind comment and used the latter method to visualize the statistical significance in all figures of the revised manuscript according to your suggestion. Thank you again for careful review.

6. Could you please explain what the inward and outward arrows mean in Figure 2A and B?

**Response:**

Thank you for this good question. The outward arrows indicate the sequence of the exon on the precursor linear mRNA. After back-splicing to form a circRNA, the inward arrows present the sequences of exons in precursor gene meet together to form a splice junction of the circRNA. This indication refers to some studies to explain the biogenesis of circRNAs <sup>[1-3]</sup>.

**References**

- [1] Zeng W, Liu Y, Li WT, Li Y, Zhu JF. CircFNDC3B sequesters miR-937-5p to derepress TIMP3 and inhibit colorectal cancer progression. *Mol Oncol*. 2020 Nov;14(11):2960-2984.
- [2] Peng L, Sang H, Wei S, Li Y, Jin D, Zhu X, Li X, Dang Y, Zhang G. circCUL2 regulates gastric cancer malignant transformation and cisplatin resistance by modulating autophagy activation via miR-142-3p/ROCK2. *Mol Cancer*. 2020 Nov 5;19(1):156.
- [3] Luo Z, Rong Z, Zhang J, Zhu Z, Yu Z, Li T, Fu Z, Qiu Z, Huang C. Circular RNA circCCDC9 acts as a miR-6792-3p sponge to suppress the progression of gastric cancer through regulating CAV1 expression. *Mol Cancer*. 2020 May 9;19(1):86.

7. In supplementary table 3, is number “1” means “true”, confirming that the mRNA from specific gene was identified in the database as target for each miRNA?

**Response:**

Yes, it is. We apologize for the unclear presentation and have corrected “1” to “Yes” in the revised Supplementary Table 3.

8. Please double-check whether all gene symbols are italicized. I spotted “TP53” symbol not italicized in Discussion.

**Response:**

Thank you very much for your kind suggestion. We have italicized the “P53” symbol in the Discussion and checked other gene symbols carefully in our revised paper.

Thank you again for the careful review and kind comments.

We would like to express our great appreciation to you again for valuable comments on our paper.

Thank you and best regards!

Yours sincerely,

*Shukun Yao*