Peer Review File

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Reviewer A

This manuscript demonstrated that gene expression of CLEC5A was significantly higher in CRC tissues than in adjacent normal tissues. CLEC5A mRNA expression is higher in HT29, SW620, and SW480 cells compared to colon epithelial cells (NCM460). After constructing the CLEC5A knockdown using HT29 and SW480 cells, cell proliferation capacities, as measured by the CCK-8 assay, colony formation, and EdU tests, as well as cell migration and invasion capacities, were diminished. Reduction in DNA replication and repair assays by PCNA expression and cell cycle process assays by CyclinD1 and CyclinE1 expression in sh-CLEC5A in HT29 and SW480 cells confirmed that CLEC5A facilitated the migration of CRC cells. In addition, EMT and E-cadherin levels were elevated, whereas N-cadherin, MMP2, and MMP9 levels were decreased. In the xenograft model, the efficacy of CLEC5A in regulating tumorigenesis and progression was confirmed. In addition, the function of CLEC5A in tumorigenesis was mediated by phosphorylation of the AKT/mTOR pathway in tumor tissues and CRC cells. CLEC5A may regulate CRC pathogenesis by interacting with target genes of multiple biological processes, such as COL1A1 for ECM formation, via the activation of the PI3K/AKT/mTOR pathway.

The manuscript is well-written. The objectives are clearly stated. This manuscript contains intriguing findings that are supported by substantial evidence. Before the manuscript can be considered for publication, the authors must resolve several important issues, in my opinion. My perspective on these issues is provided in the following commentary:

1. The expression levels of CLEC5A varied among cancer cell types, were higher than in normal cells, and were implicated in tumorigenesis and the progression of cancer cells. Why did HCT116 cells exhibit the same level of CLEC5A expression as NCM460 colon epithelial cells (Figure 1)? Please the authors addressed in the context of the manuscript.

Reply: Based on your valuable feedback, we have rechecked the relevant experimental data and conducted statistical analysis. We have now replaced the modified images, as shown in Figures 1F-revised. The results do indicate that the level of CLEC5A can also be increased in colon cancer cell line HCT116, but its difference is not as significant as HT29 and SW480 cells. Change in the text: Figures 1F.

2. In the Introduction, lines 84-87, the authors must define the information regarding the correlation between the PI3K/AKT pathway and CLEC5A, which promotes tumorigenesis.

Reply: Thank you for your comment. In the introduction section, we cited the studies from Wang Q, Shi M, Sun S, et al (16) and Fan HW, Ni Q, Fan YN, et al (19),confirming that CLEC5A can promote the tumorigenesis and progression of gastric cancer and brain glioblastoma through

the PI3K/ AKT pathway, so we also believed that these two can play a synergistic role in regulating the proliferation and migration of colon cancer.
Change in the text: page 4, lines 19-21.

3. The images in Figure 2A, which depict the expression of the CLEC5A gene, require annotations indicating cell types, as do those in Figures 2D, 2E, 3A, and 3B, which require annotations indicating the control and sh-CLEC5A groups, respectively.

Reply: Thank you for your editing suggestions on image modifications. We have added corresponding cell type annotations in Figure 2A, as well as annotations for the control group and sh-CLEC5A group in Figures 2D, 2E, 3A, and 3B, as shown in Figures 2 and Figures 3.
Change in the text: Figure 2 and Figure 3.

4. Why was there no error in the CLEC5A expression levels of the control group (Figures 2A and B)?

Reply: Thank you for your comment. We apologized for explaining that Figures 2A and B respectively showed the knockdown effici-ency of sh-CLEC5A in HT29 and SW480 cells at the protein level, so the expression levels of CLEC5A in control group were all classified as 1 to calculate its relative expression levels in sh-CLEC5A transfection group. We have replaced corresponding modified images (Figures 2A-revised, and Figures 2B-revised) in the same location.
Change in the text: Figure 2.

5. Figure 4 is titled "CLEC5A knockdown inhibits CRC growth and suppresses the AKT/mTOR pathway phosphorylation in vivo", but sub-figures (D) and (E) contain data from cell lines. Where did the data for sub-figure (G) originate? The in vivo tumor tissues or the cell line? The authors must revise the title of this figure and explicitly indicate in all sub-figures whether the data were obtained from tumor tissues or cell lines.

Reply: Thank you for your comment. We have revised the title of Figure 4 to "CLEC5A knockdown internally inhibits the tumor growth of colon cancer, and suppresses the AKT/mTOR pathway phosphorylation in vitro and in vivo; the interaction between CLEC5A and COL1A1 co-regulates colon cancer progression as well". Among them, sub-figures (D), (F), and (G) contain data from TCGA gene expression dataset, whereas sub-figures (E) contains data from CLEC5A knockdown cells.
Change in the text: Figure 4.

6. CLEC5A promotes tumorigenesis and the pathogenesis of cancer by activating the PI3K/AKT/mTOR pathway. This study demonstrates the AKT/mTOR pathway and its downstream phosphorylation, including the expression of p-mTOR, p-P70S6K, and p-S6, but not PI3K levels. Without PI3K expression data, the Y-axes of sub-Figures 4C and 4E were also labeled with PI3K/AKT/mTOR. Please clarify this unclear data.

Reply: Thank you for your comment. We were sorry about mistakenly equating the AKT/mTOR pathway with the PI3K/AKT/mTOR pathway, thereby leading to the incorrect Y-axes labeling in sub-Figures 4C and 4E. We have now revised it to "Phosphorylated AKT/mTOR pathway expression", as shown in Figures 4C-revised, and Figures 4E-revised.
Change in the text: Figure 4.

7. The effect of CLEC5A knockdown on cancer cell proliferation, colony formation, and EdU assays in cell lines appears to be less significant than the effect on tumor size and volume of xenograft tumor tissues. Please elucidate this discrepancy, authors.

Reply: Thank you very much for your valuable question. In previous experiments, we purchased a total of ten nude mice to construct tumor xenograft mice models for colon cancer. However, due to the fact that we artificially selected four pairs of xenograft tissues with strong differences for statistical analysis, there were discrepancies between the differences and cell experiments. We deeply apologize for this, and randomly select five pairs of xenograft tissues for statistical analysis again. The corresponding modified images have been replaced, as shown in Figures 4A-revised, and Figures 4B-revised.
Change in the text: Figure 4.

8. The effect of CLEC5A knockdown on cancer cell proliferation, colony formation, and EdU assays in cell lines appears to be less significant than the effect on tumor size and volume of xenograft tumor tissues. Please clarify this discrepancy.

Reply: Thank you very much for your valuable question. Same as the answer to question 7 above.

Reviewer B

The paper titled "CLEC5A regulates the proliferation and migration of colon cancer via the AKT/mTOR signaling pathway" is interesting. CLEC5A may promote the development and migration of CRC by triggering the AKT/mTOR signaling pathway both in vivo and in vitro. Moreover, COL1A1 could serve as the target gene of CLEC5A. 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: Thank you for your comment. We have supplemented the methods (see Page 2, line 17-21), results (see Page 3, line 3-5), and background (see Page 2, line 12-15) section of abstract in this article, in particular illuminated the clinical needs of research focus in the background section.
Change in the text: Abstract.

2) The title used in this study is colon cancer, but the manuscripts use colorectal cancer (CRC). Suggest a unified name, which may be more rigorous.

Reply: Thank you for your sincere suggestion. We have replaced all the "colorectal cancer (CRC)" with "colon cancer" in this article to avoid unnecessary confusion.

3) What is the correlation between the expression of CLEC5A and the prognosis of CRC patients? How to gain in-depth understanding through bioinformatics? It is recommended to add relevant content.

Reply: Following your valuable suggestion, we have added a survival analysis based on the clinical follow-up data of colon cancer patients downloaded from TCGA database (see Figure 1E), suggesting that no significant correlation exists between the expression levels of CLEC5A and prognosis of colon cancer patients.
Change in the text: Page 10, line 15-19.

4) There are still some weak points in this paper. It is suggested that the author increase the inhibitor or agonist of signaling pathway. This is more conducive to support the conclusions of this study.

Reply: We agree that more studies would be useful to understand the details of interaction between AKT/mTOR signaling pathway and CLEC5A in colon cancer pathological process. However, we do not have the necessary tool-set to study the effect of inhibitor or agonist in AKT/mTOR signaling pathway on colon cancer. We hope to employ the related experiments in the future to further determine the detailed mechanism between CLEC5A and this signaling pathway.
Change in the text: None.

5) Figure 2D,3 and 4 are not clear enough. It is recommended to provide clearer figures again.

Reply: Thank you for your comment. We have revised the clarity of corresponding images in Figure 2D,3 and 4.

6) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "TRPV3 inhibits colorectal cancer cell proliferation and migration by regulating the MAPK signaling pathway, J Gastrointest Oncol, PMID: 36388700". It is recommended to quote the articles.

Reply: Thank you for your comment. We have fully supplemented the introduction section of this article in accordance to suggestions (see Page 3, line 20-33; Page 4, line 2-18), and the similar papers have been cited, such as "CLEC5A promotes the proliferation of gastric cancer cells by activating the PI3K/AKT/mTOR pathway, Biochem Biophys Res Commun, PMID: 32033754", "C-type lectin domain family 5, member A (CLEC5A, MDL-1) promotes brain

glioblastoma tumorigenesis by regulating PI3K/ Akt signalling, Cell Prolif, PMID: 30834619" and so on.

7) There are many genes that regulate the CRC. Why did the author choose CLEC5A for research? Please describe the reason.

Reply: Thank you for your comment. According to the TCGA gene expression data, we screened up-regulated genes by the criteria of "P<0.05 and log2 (FC)>2", in which the P-value of CLEC5A was 4.18×10^{-42}, and log2 (FC) was 3.65, indicating a significant up-regulated gene of colon cancer samples. Additionally, the similar papers have demonstrated the oncogenic role of CLEC5A in gastric cancer and brain glioblastoma (16,19), so we choose the CLEC5A gene for research.

8) What are the relevant characteristics of the tumor microenvironment of CRC? What is the correlation between CLEC5A and the tumor microenvironment? What are the possible goals of future drug development? It is recommended to add relevant content to the discussion.

Reply: Thank you for your comment. In the discussion section of this article, we have elaborated the relevant characteristics of the tumor microenvironment (TME) in colon cancer (see Page 13, line 21-31), and illustrated the mechanism of CLEC5A in immune-inflammatory reactions (see Page 13, line 32-34). Moreover, the possible goals of future drug development in colon cancer was further complemented in the conclusions section (see Page 16, line 4-6).

Reviewer C

1. Animal source

Please provide the source of animal in the method section.

Reply: Thank you for your comment. The source of animal has been provided in the methods section.

2. Figure 1

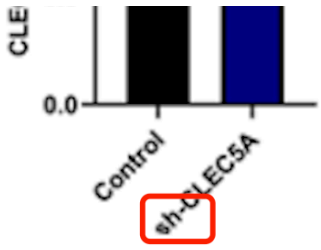
Please provide the scale bar in the figure or magnification in the legend for 1D.

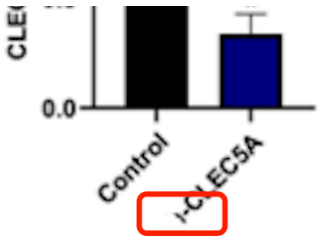
Reply: Thank you for your comment. The scale bar has been provided in the figure 1D.

3. Figure 2

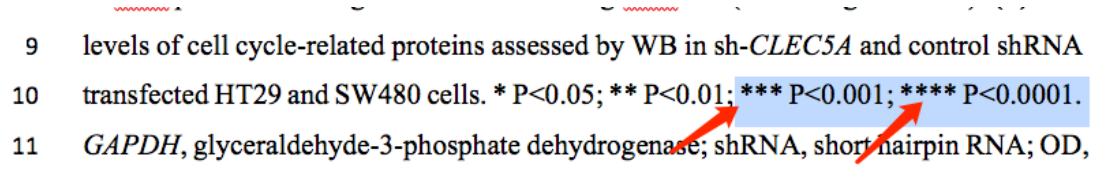
a) Please provide a clearer version of figure 2.

b) The figure is not complete, please revise.





c) NO symbols “***” and “****” in figure 2, but they are indicated in the legend. Please check and revise.


levels of cell cycle-related proteins assessed by WB in sh-*CLEC5A* and control shRNA transfected HT29 and SW480 cells. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; shRNA, short hairpin RNA; OD,

Reply: Thank you for your comment.
a) A clearer version of figure 2 has been provided.
b) Figure 2 has been revised.
c) We have deleted symbols “***” and “****” in figure 2 legend.

4. Figure 3

a) Please provide a clearer version of figure 3.
b) As there are no symbols “***, ****” in the figure, please delete the explanations in the legend.
Reply: Thank you for your comment.
a) A clearer version of figure 3 has been provided.
b) The symbols “***, ****” in legend have been deleted.

5. Figure 4

a) Please provide a clearer version of figure 4.
b) Please explain WB in the legend.
c) NO symbol “****” in figure 4, but it is indicated in the legend. Please check and revise.
Reply: Thank you for your comment.
a) A clearer version of figure 4 has been provided.
b) WB has been defined in legend.
c) We have deleted the symbol “****” in figure 4 legend.

6. Table 1

As there are no symbols “**, ***, ****” in the table, please delete the explanations in the table footnote.
Reply: Thank you for your comment. “**, ***, ****” have been deleted.