Peer Review File

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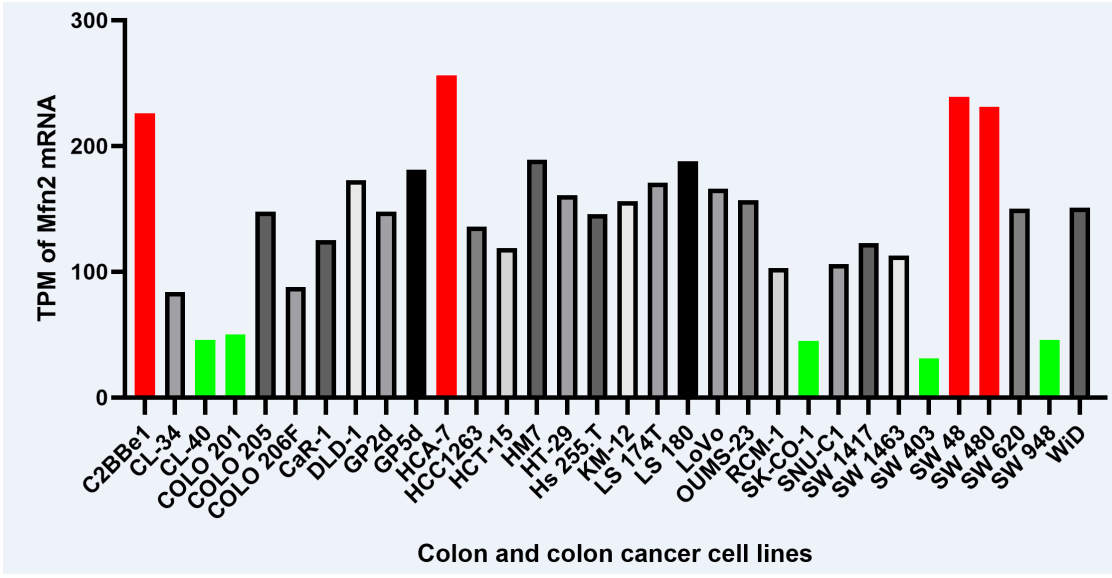
Reviewer comments:

1. Response to comment: "*1. Although the authors examined the MFN2 expression in colon cancer tissues, it is still unclear which types of cells express MFN2 in normal and cancer tissues. The authors need to provide the data that show which types of cells express MFN2 in the normal tissues and which cells show a decreased expression of MFN2 in the tumor tissues. It is important to analyze the difference between normal epithelial cells and colon cancer cells in vivo. The reviewer thinks this is one of the limitations of this study.*"

Response:

Thank you for pointing out this very important issue. We revised it as follow.

We explored the Expression Atlas database (https://www.ebi.ac.uk/gxa) for more information on MFN2 expression in normal colon and colon cancer cell lines. MFN2 expression was shown to be high in cell lines C2BBe, HCA-7, SW48, and SW480, but low in CL-40, COLO201, SK-CO-1, SW403, and SW948. These results indicated that MFN2 expression was also heterogeneous in different colorectal cancer cell lines (fig 2a).



2. Response to comment: "*2. Although the authors analyzed the pathways associated with MFN2 expression levels using GSEA, it is still unclear the MFN2 function in these pathways. The authors need to show the role of MFN2 in these pathways in Results and Discussion.*"

"*Although it is supposed that MFN2 regulates mitochondrial function, the authors did not show the function was decreased in cancer tissues. The authors need to show the correlation between MFN2 and mitochondrial function using GSEA.*"

Response:

Thank you for pointing out this very important issue. MFN2 is an important protein involved in mitochondrial fusion, and MFN2 performs many of its functions through mitochondria. There are some overlaps between the two parts, so we reply them together. We revised it as follow.

Results:

Using GSEA, we found that the high expression of MFN2 in colon cancer may be related to three metabolic signaling pathways, respectively KEGG GALACTOSE METABOLISM and KEGG GLYCOLYSIS GLUCONEOGENESIS. In addition, the high expression of MFN2 in colon cancer may be related to apoptosis and VEGF

signaling pathways. However, GESA revealed only one signaling pathway KEGG_RIBOSOME, which may be related to the low expression of MFN2 in colon cancer.

Discussion:

Mitochondria have long been regarded as the cell's "powerhouse," as they produce more than 90% of ATP in aerobic settings via oxidative phosphorylation. To maintain optimal mitochondrial activities, each cell maintains a precise balance between fusion and fission. Mitofusin-1 (MFN1), mitofusin-2 (MFN2), and optic atrophy 1 (OPA1) are the three primary dynamin-related proteins that mediate mitochondrial fusion in mammals(1). Abnormal MFN2 function can cause mitochondrial fusion dysfunction, leading to a variety of diseases, such as diabetes, obesity, cardiovascular and cerebrovascular diseases, neurodegenerative diseases, malignant tumors and so on(2).The increased glucose intake and increased glycolytic rates suggest that metabolic changes help tumor cells grow faster(3). Mfn2 reduces cancer cell growth by interacting with pyruvate kinase 2 (PKM2) via the N-terminus and restricting metabolic flow to aerobic glycolysis; phosphorylation of Mfn-2 strengthens this connection(4). The loss of Mfn2 activity induces metabolic changes in mitochondria, including decreased mitochondrial membrane potential, cellular oxygen consumption, and substrate oxidation. Our study also found that high expression of MFN2 in colon cancer may be involved in KEGG GALACTOSE METABOLISM and KEGG GLYCOLYSIS GLUCONEOGENESIS pathway, suggesting that the development of colon cancer may be related to the abnormal mitochondrial energy metabolism caused by MFN2.

In the early stages of intrinsic apoptosis, mitochondria are the principal target(5). Dynamic changes in the mitochondrial location of numerous proteins, including MFN2 and Drp1, as well as the apoptosis regulators Bcl-2 associated X (BAX) and Bcl-2 antagonist/killer (BAK), cause mitochondrial fragmentation during apoptosis(6). Overexpression of either MFN1 or MFN2 can also delay apoptosis by delaying the activation of downstream caspases and apoptosis(7). MFN2 location on the outer mitochondrial membrane is known to be mediated by BAX, while BAK binds to both MFN1 and MFN2(8). Several investigations have shown that MFN2-induced apoptosis

is most likely mediated through the PI3K-Akt pathway(9,10). Our GSEA showed that high expression of MFN2 may promote apoptosis in colon cancer cells, but the specific inhibition needs to be further elucidated. Vascular endothelial growth factor (VEGF) upregulates MFN2 expression in human umbilical vein endothelial cells, and knocking down MFN2 reduces VEGF-induced human umbilical vein endothelial cell migration and differentiation, indicating that MFN2/VEGF connection in endothelial cells plays a role in angiogenesis(11). Another study showed that overexpressed MFN2 has an inhibitory effect on pancreatic cancer, which may be related to the VEGF signaling pathway(12). Our analysis also found that high expression of MFN2 may be involved in the VEGF signaling pathway in colon cancer. VEGF plays a key regulatory role in the growth and invasion of colorectal cancer. The VEGF inhibitor bevacizumab provides a significant survival benefit for advanced colorectal cancer treatment. Clarification of the relationship between MFN2 and VEGF in colon cancer cells is likely to bring new ideas for targeted therapy of colorectal cancer.

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6. van der Bliek AM, Shen Q, Kawajiri S. Mechanisms of mitochondrial fission and fusion. Cold Spring Harb Perspect Biol 2013;5.
7. Srinivasan S, Guha M, Kashina A, et al. Mitochondrial dysfunction and mitochondrial dynamics-The cancer connection. Biochim Biophys Acta Bioenerg 2017;1858:602-14.
8. Brooks C, Wei Q, Feng L, et al. Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins. Proc Natl Acad Sci U S A 2007;104:11649-54.
9. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91:231-41.
10. Kim NH, Kim K, Park WS, et al. PKB/Akt inhibits ceramide-induced apoptosis in neuroblastoma cells by blocking apoptosis-inducing factor (AIF) translocation. J Cell Biochem 2007;102:1160-70.
11. Lugus JJ, Ngoh GA, Bachschmid MM, et al. Mitofusins are required for angiogenic function and modulate different signaling pathways in cultured endothelial cells. J Mol Cell Cardiol 2011;51:885-

93.
12. Lin Z, Lin X, Chen J, et al. Mitofusin-2 is a novel anti-angiogenic factor in pancreatic cancer. J Gastrointest Oncol 2021;12:484-95.

3. Response to comment: "*The authors examined the effect of MFN2 expression on overall survival in colon cancer using TCGA and GEPIA. However, the reviewer thinks GEPIA is one of the applications for analyzing TCGA. In other words, the authors may be analyzing data from the same patients. Did they use different patient data?*"

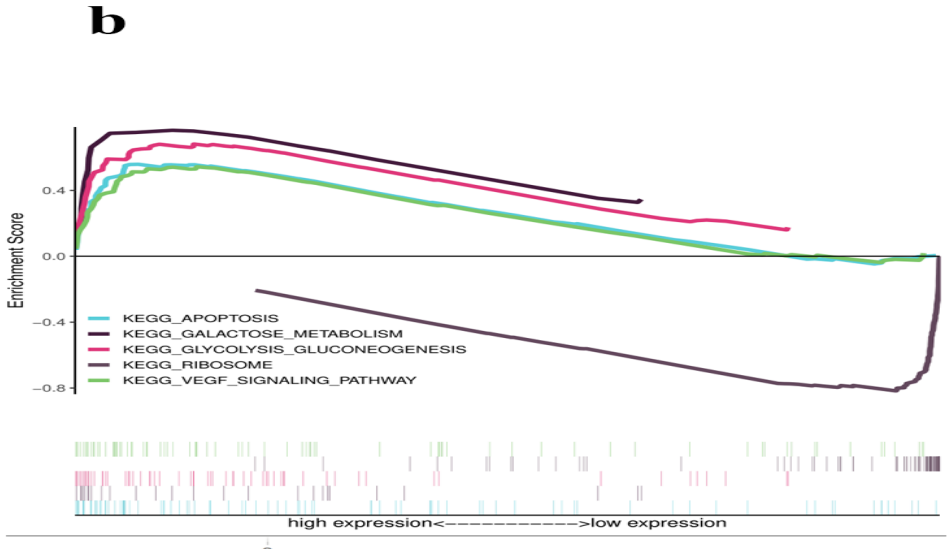
Response:

Thank you for pointing out the mistakes. The patients in GEPIA database and TCGA database are the same, so we have deleted the corresponding pictures.

4. Response to comment: "*In figure 3b, the curve charts and explanations are complicated to understand. It is better to show only more focused pathways directly related to the function of MFN2.*"

Response:

Based on your valuable comments, we have streamlined the signaling pathways that may be involved in MFN2.



5. The authors should examine the MFN2 expression in tumor tissue and normal tissues by immunohistochemistry using specific anti-human MFN2 antibodies.

Response:

Method:

Immunohistochemistry (IHC) Staining

Immunohistochemical pictures of MFN2 protein expression studies were done in normal and CRC tissues from the HPA (http://www.proteinatlas.org/) to determine differences in MFN2 expression at the protein level. For IHC, the anti-body HPA030554 was employed.

Result:

IHC findings were acquired and processed from the HPA to measure MFN2 expression at the protein level. MFN2 IHC staining was medium to high in colon normal tissues (Figure 2A), but MFN2 IHC staining was faint or not identified in CRC tumor tissues (Figures 2B).

Figure legend

Figure 2. The MFN2 expression detected by immunohistochemistry and single cell

RNA-seq analysis of the colon cancer. Representative immumohistochemical stainings in colon normal tissues (A) and colon cancer tissues (B); (C) From dataset GSE139555, an t-SNE plot showing 11 cell clusters (up) and MFN2 expression levels in various clusters (down); (D) The t-SNE plot showing 9 cell clusters (up) and expression levels of MFN2 in different clusters from dataset GSE146771 (down).

6. The authors should examine the MFN2 expression using single RNA-seq data sets of colon tumor tissues and normal colon tissues available from public data (GEO or something).

Response:

Method:

Single cell RNA-seq data processing

GEO datasets (https://www.ncbi.nlm.nih.gov/gds) were used to download single cell RNA-seq data associated with colon cancer (GSE139555 and GSE146771). To identify key cell types, researchers used the Seurat tool in R software. PCA was performed using highly variable genes, and primary components were computed using t-SNE analysis.

Result:

We employed single cell RNA seq data from the GSE139555(1) and GSE146771(2) datasets to explore MFN2 expression in a variety of cells, including colon cancer cells, immunological cells, stromal cells, and vascular endothelial cells. MFN2 expression was either low or non-existent in distinct clusters, according to differential gene analysis (Figure 2C-D).

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Figure legend

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of MFN2 in different clusters from dataset GSE146771 (down).

1. Wu TD, Madireddi S, de Almeida PE, et al. Peripheral T cell expansion predicts tumour infiltration and clinical response. Nature 2020;579:274-8.
2. Zhang L, Li Z, Skrzypczynska KM, et al. Single-Cell Analyses Inform Mechanisms of Myeloid-Targeted Therapies in Colon Cancer. Cell 2020;181:442-59 e29.

7. In Figure 2, the figure legend is poor. Which are normal cells and cancer cells?

Response:

Thank you for your valuable advice. We have redrawn figure 2 and described it.

8. Material and Methods, it is still unclear how the authors performed. The address is missing. Which dataset did the authors use??

Response:

Thank you for your valuable advice.

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Immunohistochemical pictures of MFN2 protein expression studies were done in normal and CRC tissues from the HPA (http://www.proteinatlas.org/) to determine differences in MFN2 expression at the protein level. For IHC, the anti-body HPA030554 was employed.

Single cell RNA-seq data processing

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