**Ms. Ref. No.:** 77761

**Title:** Overexpression of EAF2 suppresses invasion, migration and angiogenesis via STAT3/TGF-β1 crosstalk in colorectal cancer

**World Journal of Gastrointestinal Oncology**

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**Dear Editor，**

Thank you very much for your attention and the reviewers' comments on our paper *Overexpression of EAF2 suppresses invasion, migration and angiogenesis via STAT3/TGF-β1 crosstalk in colorectal cancer*.

We have revised the manuscript according to your kind advices and the reviewers' detailed suggestions. Enclosed please find the responses to the reviewers. We sincerely hope this manuscript will be finally acceptable to be published on *World Journal of Gastrointestinal Oncology*. Thank you very much for all your help and looking forward to hearing from you soon.

Here blow is our description on revision according to the reviewers' comments.

**Response to Reviewer:**

Reviewer #1:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors: Manuscript ID 77761 describes the role of EAF2 in several biological functions of CRC cells including their angiogenesis, proliferation, and invasion.

Although very interesting the following points should be clarified:

* In the introduction section, the authors used future tense for their aims. Please change all of them to past tense.

**The author's Answer**: Thanks for your kind advice and we revised it according to the comment.

* The authors have mentioned that they used DMEM for culturing HUVECs and other endothelial cells. This medium is the correct choice for endothelial cells since it does not contain all the necessary growth factors including VEGF, IGF, EGF, FGF and etc. The classic medium is EGM2. This can significantly impact the results. (Please refer to PMID: 33344453 and https://doi.org/10.1016/j.jocit.2018.09.004)

**The author's Answer**: Thanks for your attention and kind suggestion. We read this article carefully. We cited the article in the Results section (Result 6.) in our paper.

* Concerning the wound healing test with RKO cells, it is not clear why the authors did not continue the experiment in order to have a closed wounded area?

**The author's Answer**: Thanks for your attention and kind suggestion. For wound healing test with RKO cells, we plated 2 × 105 RKO cells/well into 6-well plates. Scratch wounds were generated using 200 μL pipette tip when the cells reached 90% confluence. And then incubated in serum-2% medium. In the experiment we photoed the scratch wounds at 0, 6h, 12h, 24h, 48h, 72h, 96h in five selected regions. The results showed that there were significant differences in wound healing area of RKO cells at 72h, but there was no closure phenomenon. However, some cells could float when cultured to 96h. There was also no obvious closure phenomenon. Therefore, we selected culture to 72h to calculate the healing rate.

* Please mention how many times each experiment was performed.

**The author's Answer**: Thanks for your attention and kind suggestion. The experiments were repeated at least three times under the same experimental conditions. It is also mentioned in Materials and methods section.

* In lines 322 and 323 are the authors sure about the figure citation?

**The author's Answer:** Thanks for your attention and kind suggestion. The figure quoted in this section is correct.

* The representative images in figure 7 are not acceptable. Please use some better-quality photos. Moreover, it is not clear what have the authors measured

in the tube formation test? Please refer to PMID: 33344453 for a better analysis of networks.

**The author's Answer**: Thanks for your attention and kind suggestion. The images in Figure 7 have been replaced with clearer typical pictures and re-analyzed. We analyzed the images for evaluating the branch points in the tube formation test. This is supplemented in the Materials and methods section.

* I am not sure if the HUVECs are the best endothelial model in this experiment knowing that endothelial progenitor cells (EPCs) are involved in cancer angiogenesis. I suggest the authors comment on this. PMID: 33627177

**The author's Answer**: Thanks for your attention and kind suggestion. We read this article carefully. We cite the article in the Discussion section in our paper.

* It has been shown that TGF-β1 treatment can induce long non-coding RNA expression mostly through regulation of FOXP1-IT1 and RAD21. It would be interesting to discuss the role of lncRNA in CRC as it is a hot topic. PMID: 35194111

**The author's Answer**: Thanks for your attention and kind suggestion. This is indeed a current research hotspot, and it will be of great significance to study and discuss relevant issues. In the following experimental research work, we will further explore the relevant regulatory effects of lncRNA on EAF2 and downstream pathways.

Reviewer #2:

Scientific Quality: Grade C (Good)

Language Quality: Grade C (A great deal of language polishing)

Conclusion: Major revision

Specific Comments to Authors: This study investigated the relationship between EAF2 and colorectal cancer progression. All results indicate an increased risk of colorectal cancer due to decreased EAF2 expression, and it is also well verified in the analysis of clinical samples.

However, there are major and minor issues to address.

1. In table 1, the expression of EAF2 did not show any correlation with clinicopathologic characteristics. It should be focused more on the association with tumorigenesis, but did not.

**The author's Answer**: Thanks for your attention and kind suggestion. In our study, statistical results showed that the expression of EAF2 protein in CRC tissue was negatively correlated with distant metastasis ($r = -0.268$, $p = 0.025$) and CEA ($r = -0.249$, $p = 0.038$), but not with other clinical characteristics, such as age, sex, primary tumor site, tumor size, tumor histological differentiation, degree of differentiation, angiolymphatic and/or perineural invasion, tumor stage, tumor invasion depth, lymph node status, and CA19-9, P53 and CDX2 (Table 2). This may have something to do with not having enough cases in the study. More cases and more comprehensive clinical studies are needed to further explore this question. This is also discussed in the paper.

2. In figures 3 and 5, a heterogeneous expression pattern of GAPDH was shown between the groups. The bar graphs for the RKO and HT29 groups in Figure 3 and the EAF2-OA-T group in Figure 5 are unreliable.

**The author's Answer**: Thanks for your attention and kind suggestion. The relevant images in Figure 3 and 5 have been have been replaced with clearer typical pictures and re-analyzed.

3. Information on the conditioned media utilized in the study in figures 7 and 8 is missing. Producing method and the purpose of its use should be described in detail.

**The author's Answer**: Thanks for your attention and kind suggestion. We have corrected and added information on the conditioned media and the purpose of its use in the article.

4. It should be clarified whether the increase in the risk of CRC is due to a decrease in EAF2 expression or a decrease in detection due to an EAF2 mutation when using an ordinary antibody. The progression of CRC induced by EAF2 mutation is well established, and the authors' point of view is the same as that according to the described background.

**The author's Answer**: Thanks for your attention and kind suggestion. In this

study, our findings suggest that EAF2 protein is underexpressed in cancer tissue of patients with advanced CRC. However, further studies are needed to determine the mechanism of decreased EAF2 protein expression in CRC. In the following research experiments, we will further explore the mechanism of reduced EAF2 expression in CRC. This discussion has been supplemented in the paper.

5. Depending on the results, decreased expression of EAF2 increases the risk of CRC. If so, it is wondering how to increase the EAF2 in patients with CRC? It must be discussed because the authors argued that EAF2 is possible to consider as a therapeutic target.

**The author's Answer**: Thanks for your attention and kind suggestion. This discussion has been supplemented in the paper.

6. It needs a great deal of English correction.

**The author's Answer**: Thanks for your attention and kind suggestion. And we improved the English grammar with the help of an English speaker.

Reviewer #3:

Scientific Quality: Grade E (Do not publish)

Language Quality: Grade D (Rejection)

Conclusion: Rejection

Specific Comments to Authors: In its current form the manuscript cannot be properly reviewed since the quality of the English language is in parts not good enough to prevent misunderstandings and wrong judgement. However, the research itself might be of interest. This is why I will suggest to reject but recommend resubmission after lagnuage check.

The author's Answer: Thanks for your attention and kind suggestion. And we improved the English grammar with the help of an English speaker.

Reviewer #4:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: Feng et al. investigated the role of EAF2 in colorectal cancer. They revealed that EAF2 suppresses cell motility and angiogenesis via the stat3/TGF-b1 pathway. This is a well planned and performed study. Also, the paper is relatively well written.

I have some minor comments on it.

1. Statistical analysis in materials and methods is not described sufficiently. Applications of Wilcoxon signed-rank test and ROC analysis are not explained. What is the outcome (dependent variable) for determining the cut-off of EAF2 as an independent variable in the ROC analysis?

**The author's Answer**: Thanks for your attention and kind suggestion. This is supplemented in the Materials and methods section and Results section.

2. Method of multivariate analysis is not explained. How did you select variables subject to multivariate analysis from univariate analysis? P values of histologic type and CA19-9 are both 0.378, but the former was entered into multivariate analysis but the latter was not. Please explain the reason.

**The author's Answer**: Thanks for your attention and kind suggestion. The clinicopathological characteristics with $p < 0.3$ in univariate analysis were included in multivariate analysis to assess the independent prognostic effects of EAF2 protein on OS by adjusting for confounding factors. We have corrected Table 3.

3. (ll.259-260 & Table 2) According to my calculation, p value of distant metastasis is not 0.025 but 0.466 by chi-square test or 0.709 by chi-square test with Yates' correction. P value of CEA is not 0.038 but 0.728 by chi-square test or 0.943 by chi-square test with Yates' correction. Please review the Table 2.

**The author's Answer**: Thanks for your attention and kind suggestion. The data in Table 2 have been corrected after checking.

4. ITH in l.364 should be spelled out.

**The author's Answer**: Thanks for your attention and kind suggestion. ITH has been spelled out in Introduction section.

5. There are some grammatical errors. Please revise English.

**The author's Answer**: Thanks for your attention and kind suggestion. And we improved the English grammar with the help of an English speaker.

Revision-reviewer #1:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Accept (General priority)

Specific Comments to Authors: All concerns have been well addressed. There is no issue to raise.

**The author's Answer**: I am very glad to receive your comment. Thank you very much.

Revision-reviewer #2:

Scientific Quality: Grade D (Fair)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors: The revised version is now in an English language quality which allows reviewing it. However, in order to gain overall quality, several points have to be adressed to justify publication: 1. The authors refer to MSI-H in the background/introduction, but do not give these data for the 70 cases included.

**The author's Answer**: Thanks for your attention and kind suggestion. There are scholars have found the inactivation of EAF2 in MSI-H CRC, suggesting that EAF2 maybe correlated with the occurrence and development of CRC. However, there are few studies on the expression and role of EAF2 protein in CRC. In this study, we investigated the expression and clinical value of EAF2 protein in CRC. And our findings suggest that EAF2 protein is underexpressed in cancer tissue of patients with advanced CRC. Besides, the survival rate of the group with high EAF2 levels was higher than that of the group with low EAF2 levels. Next, we will further explore the relationship between microsatellite instability and EAF2 activity in CRC, as well as study at the gene level.

2. A Table of all data available for the cases included should be given as Supplementary information. It should explicitely also include the expression levels obtained by immunohistochemistry and - where applicable - W-Blot analysis. Also highlight these cases in order to allow the potential reader to easily follow your case selection.

**The author's Answer**: Thanks for your attention and kind suggestion. Analysis of the data is presented in the Supplementary materials. In this study, 70 pairs of histological sections of colorectal adenocarcinomas and corresponding paracancerous tissue were selected for immunohistochemical study. In addition, we selected another 8 pairs of fresh cancer and adjacent tissue for Western blot assay. None of patients received radiotherapy or chemotherapy prior to surgical resection. It has been revised and supplemented in the article.

3. Speaking of - all original W-Blots must be provided for review.

**The author's Answer**: Thanks for your attention and kind suggestion. The W-blots shown in this article are unprocessed images, such as those presented in the Supplementary material.

4. All cell line experiments must be performed with at least two cell lines - this is now a general rule and not a cruel argument. At best with cell lines in known, low passage numbers.

**The author's Answer**: Thanks for your attention and kind suggestion. In this study, we first preliminarily explored the expression of EAF2 and downstream proteins in a variety of cell lines (human CRC cell lines (SW480, RKO, HCT116, HT29 and HIEC) and normal colorectal epithelial cells (NCM460)), and selected RKO as the cell line for the study. Thank you very much for your suggestion. In the future research work, we will choose at least two cell lines to discuss.

5. In the conclusion: "recombination of proteins, transfection of overespressed genes," - it is not clear what exactly this should mean. Either re-phrase or delete.

**The author's Answer**: Thanks for your attention and kind suggestion. We have corrected in the article.

6. In the Table 2, please modify by dividing the colon cancer cases into right-sided and left-sided cases. Also: please define what is "normal" for those cases where this term is used (CEA, CA19-9, P53 and CDX2).

**The author's Answer**: Thanks for your attention and kind suggestion. In this study, we divided the tumor location into colon and rectum, and only 34.29% of the cases were colon. Therefore, we did not continue to group the cases with colon location. In future studies, we will pay more attention to cases with colonic localization, and further group analysis of colonic localization to explore its clinical significance. We defined low expression as normal. Low expression of them (CEA, CA19-9, P53 and CDX2) were defined as a score of < 3.

Minor points: 1. All sentences used in the abstract are re-used somewhere in the manuscript body - this must be changed!

**The author's Answer**: Thanks for your attention and kind suggestion. We have corrected in the article.

2. Style for mentioning the suppliers should be Company name, City, Country - at first mentioning and Company name subsequently. Please do everywhere.

**The author's Answer**: Thanks for your attention and kind suggestion. We have corrected in the article.

3. There is sometimes a problem with "°C" - I see it sometimes as "a" please check. **The author's Answer**: Thanks for your attention and kind suggestion. We have corrected in the article.

4. Introduction of abbreviations is only recommended when used subsequently at least two more times.

**The author's Answer:** Thanks for your attention and kind suggestion. We have corrected in the article. 5. Legend of Figure 9: GAPDH mentioned - but cannot be found in the scheme. The author's Answer: Thanks for your attention and kind suggestion. We have corrected in Figure 9.

Revision-reviewer #3:

Scientific Quality: Grade B (Very good)

Language Quality: Grade A (Priority publishing)

Conclusion: Accept (General priority)

Specific Comments to Authors: The authors have addressed my concerns and questions. I have no further comment.

**The author's Answer**: Very glad to receive your comment. Thank you very much.

Thank you and all the reviewers for the kind advice.

Sincerely yours,

Mingjun Sun