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Title: The mechanism of EAF2 and VASH1 mediating STAT3/TGF-β1 crosstalk to regulate invasion, migration and angiogenesis in colorectal cancer

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Dear Editor，

Thank you very much for your attention and the reviewers' comments on our paper *The mechanism of EAF2 and VASH1 mediating STAT3/TGF-β1 crosstalk to regulate invasion, migration and angiogenesis 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 Gastroenterology.* 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 B (Very good)

Language Quality: Grade A (Priority publishing)

Conclusion: Accept (General priority)

Specific Comments to Authors: The authors hypothesised the influence of TGF-β1 on VASH1. The manuscript shows the possible functional role and mechanism of VASH1-mediated TGF-β1 related pathway in CRC The study suggests that EAF2 and VASH1 may serve as new diagnostic and prognostic markers for CRC, and provides a clinical basis for exploring new biomarkers for CRC. This study complements the mechanism of EAF2 in CRC cells, enriches the rol7e and mechanism of CRC cell-derived VASH1, and provides a new possible subtype of CRC as a therapeutic target of STAT3/TGF-β1 pathway

The author's Answer: I am very glad to receive your comment. Thank you very much for your affirmation and encouragement. We will continue to work hard.

Reviewer #2:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors: This study aimed to investigate the expression of Vasohibin 1 (VASH1) in colorectal cancer (CRC) and its correlation with the expression of ELL-associated factor 2 (EAF2). The study found that EAF2 was down-regulated and VASH1 was up-regulated in advanced CRC tissue, and the expression levels of EAF2 and VASH1 were positively correlated. Furthermore, overexpression of EAF2 inhibited the activity of signal transducer and activator of transcription 3 (STAT3)/transforming growth factor beta 1 (TGF-β1) pathway by up-regulating the expression of VASH1, which inhibited cell invasion, migration, and angiogenesis in vitro. These findings suggest that EAF2 and VASH1 may serve as new diagnostic and prognostic markers for CRC, and provide a potential therapeutic target for the STAT3/TGF-β1 pathway in CRC. The study complements the understanding of the mechanism of EAF2 and VASH1 in CRC cells and provides new ideas for the development of targeted therapies for CRC.

Comments: -for all Western blot figures, densitometry readings/intensity ratio of each band should be included; the whole Western blot showing all bands and molecular weight markers should be included in the Supplementary Materials;

The author's Answer: Thanks for your attention and kind suggestion. The Western blot figures shown in this article are unprocessed images including bands and molecular weight markers, such as those presented in the Supplementary material. For each Western blot band, intensity ratios were measured using ImageJ software with GAPDH as the reference and analyzed statistically. We have presented these results and their statistical significance in bar charts in the paper.

gene silencing experiments should use at least two gene-specific siRNAs

The author's Answer: Thanks for your attention and kind suggestion. There are three plasmids targeting for silencing human STAT3 (GenBank no. NM_139276) or silencing human VASH1 (GenBank no. NM_014909) provided by GeneChem (Shanghai, China) in this study. This is explained in the research methods in the paper. In the experiment, we treated the target gene with three gene-specific siRNAs, and analyzed the silencing effect of the three silencing chains on the gene. The siRNA with the most obvious

silencing effect and the highest inhibition rate was selected for subsequent experiments. This has been supplemented with changes in the results section of the text.

And The wound healing scratch assay is a widely used in vitro method to study cell migration and wound closure. However, there are some limitations associated with this assay, including: Variability in wound creation: The scratch wound is created manually, which can lead to variability in the size and shape of the wound. This can affect the reproducibility of the assay and the accuracy of the results. Limited control over cell behavior: The assay does not allow for precise control over cell behavior, such as directionality and migration rate. Cells may also undergo proliferation or apoptosis during the course of the assay, which can affect the interpretation of the results. Insensitivity to subtle changes: The assay may not be sensitive enough to detect subtle changes in cell migration or wound closure, particularly in cases where the cells are slow-moving or the wound is small. Lack of 3D context: The assay is performed in a 2D environment, which does not fully recapitulate the complexity of the in vivo wound healing process, where cells interact with each other and with the extracellular matrix in a 3D context. Difficulty in quantification: Quantifying wound closure can be subjective and time-consuming. There is also a risk of bias or error when manually measuring the width of the wound over time. The limitations of invasion and migration assays include the fact that they do not necessarily represent the true in vivo behavior of cells. In vitro assays cannot fully mimic the complex interactions between cells and the extracellular matrix, and the three-dimensional nature of the in vivo environment. Additionally, the results of these assays can be influenced by factors such as cell seeding density, serum concentration, and experimental conditions. Quantification of cell invasion and migration also has limitations. Manual counting of cells can be time-consuming and subjective, while automated methods may not always accurately distinguish between live and dead cells, or may be affected by artifacts such as debris or scratches. Furthermore, the choice of quantification method can depend on the specific assay being used, and different methods may have varying levels of sensitivity and reproducibility.

The author’ s Answer: Thanks for your attention and kind suggestion. In the experiment, the influence of cell growth on scratch experiment can be reduced to a certain extent by means of isodense simultaneous culture of the same batch of cells. Measurement of its width using ImageJ software reduced the quantization error to some

extent. *In vitro* experiments do have some limitations. More objective and scientific experimental methods should be combined in vivo and in vitro to further comprehensively discuss and evaluate the functional effects of EAF2 and VASH1 on CRC, so as to make up for the limitations and one-sidedness of this study which only conducted in vitro invasion, migration and angiogenesis studies. Additional changes have been made in the discussion section of the paper. Thank you for your advice and help. We will make further improvements in the future research work.

Overall, the authors aimed to investigate the correlation between the expression of Vasohibin 1 (VASH1) and ELL-associated factor 2 (EAF2) in colorectal cancer (CRC) and their potential as diagnostic and prognostic markers and therapeutic targets. The study found that VASH1 was upregulated and EAF2 was downregulated in advanced CRC tissue, and their expression levels were positively correlated. The overexpression of EAF2 inhibited the activity of the STAT3/TGF-β1 pathway, which inhibited cell invasion, migration, and angiogenesis in vitro. These findings suggest that EAF2 and VASH1 may serve as new diagnostic and prognostic markers for CRC, and provide a potential therapeutic target for the STAT3/TGF-β1 pathway in CRC. The study suggests that EAF2 and VASH1 may serve as potential therapeutic targets for the STAT3/TGF-β1 pathway in CRC. It is important to note that targeting immunomicroenvironment (i.e. CTLA-4) also aim at novel approaches related to the authors findings: is a type of immunotherapy that aims to enhance the immune system's ability to fight cancer by blocking an inhibitory checkpoint. Therefore, targeting CTLA-4 may have a complementary effect on the therapeutic targets identified in the first study, as it could enhance the immune response against CRC cells and potentially increase the efficacy of therapies targeting EAF2 and VASH1. However, further research is needed to investigate the potential synergistic effects of these different therapeutic approaches in the context of CRC (please refer to PMID: 34067631 and expand accordingly).

The author’s Answer: Thanks for your attention and kind suggestion. After fully reading the paper (PMID: 34067631), we have a certain understanding of immunotherapy in CRC. Inhibition of cytotoxic T lymphocyte protein 4 (CTLA-4) can reactivate immune cells, especially T cells, in patients with CRC, thereby enhancing the ability of immune cells to fight tumors. In addition, Regulatory T cells (Tregs) in tumor microenvironment can mediate mechanisms such as immune escape, and may also participate in the regulation of ovarian cancer angiogenesis through VEGFA and

VASH1. The synergistic effect of VASH1-related pathway anti-angiogenesis therapy and immunotherapy may become a new idea for CRC treatment. Additional changes have been made in the discussion section of the paper.

Reviewer #3:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: The article is within the scope of the journal. It deals with an interesting topic. It is well written. The reading is fluent. Presents an experiment describing the materials, methods, results as well as a discussion.

The article should be improved in several aspects: a) Extend the state of the art. b) Establish some conclusions and lines of future work. c) Improve the discussion to compare the work presented with other similar works, establishing the advances and limitations of the work presented.

The author’s Answer: Thanks for your attention and kind suggestion. We have carefully revised the relevant sentences and sentence patterns in artical. The discussion compare this study and other related studies are supplemented in the discussion section. With the help of your valuable comments, we have revised and supplemented the relevant discussion part, more comprehensively expounded the significance and innovation of this research work in related fields, and analyzed the shortcomings of the research once again, and made constructive arrangement for the future research work combined with the latest research progress. Additional changes have been made in the discussion section of the paper.