Author’s response to reviews

Title: TEX9 and eIF3b Functionally Synergize to Promote the Progression of Esophageal Squamous Cell Carcinoma

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Author’s response to reviews:

Dear respected editors and reviewers,

Thank you very much for your review and suggestions for our article “TEX9 and eIF3b Functionally Synergize to Promote the Progression of Esophageal Squamous Cell Carcinoma”. We have read the comments and suggestions carefully. And we modified our manuscript according to the requirements and suggestions strictly. The questions and advice were answered by us point to point in details. The revision in the manuscript was highlighted with underline. Thank you very much again for your review.
Editor Comments:

Reviewer 1 comments

1. What is the clinical status of the 25 patients in the study? Was the biopsy taken before any treatment? or after neoadjuvant CCRT?

Thank you for your question. We listed the clinicopathological features, including the pTNM stage, of the 25 patients in supplementary table 1 and added the related description in the Material and Methods part “Tissue sample”. The biopsy was all taken from each patient before the treatment and no patient received neoadjuvant therapy.

2. What is the version of TNM staging in the study?

Thank you for your question. We applied the 8th American Joint Committee on Cancer TNM stage in the study. We also revised the description with the precise one in the Material and Methods part “Tissue sample”

3. Would it be possible to know the detailed T and N status of the 25 patients?

Thank you for your suggestion. We added the T and N status information of the 25 patients in supplementary table 1

4. Since TEX9-eIF3b promote the proliferation and migration of cancer cells, is the activity of TEX9-eIF3b correlated with the N stage, or the number of metastatic lymph nodes?

Thank you for your question. We analyzed the correlation between TEX9 protein expression levels with patients’ pN stage and the number of metastatic lymph nodes and found that TEX9 levels were positively correlated with both pN stage (Figure 2C) and the number of metastatic lymph nodes (Supplementary Fig. 2). For the reason that we have proved that eIF3b expression levels was also correlated with TEX9 expression levels (Figure 1C), we speculated that the activity of TEX9-eIF3b correlated with the N stage, or the number of metastatic lymph nodes.

5. Is there any correlation between AKT and TEX9-eIF3b in TCGA data set?

Thank you for your question. We analyzed the correlation between AKT1, AKT2 and AKT3 expression with TEX9/eIF3b according to the TCGA data. The results showed that no significant correlation existed. In our data, we showed knockdown of TEX9 or double knockdown of
TEX9/eIF3b downregulated the pAKT expression, but made no effect on the total AKT expression (Supplementary Fig 3). Therefore, our data was in accordance with TCGA data.

6. In TCGA data set, is a high TEX9-eIF3b predict a worse prognosis?

Thank you for your question. We analyzed the TCGA data and found that no significant prognostic difference when compared with low eIF3b or TEX9 expression subgroup. Also, no significant prognostic difference existed between existed among eIF3b-high/TEX9-high group, eIF3b-low/TEX9-low group and the groups with either high expression of eIF3b or TEX9 (Supplementary Fig 5).

We admit that this study may contain statistical bias due to limited availability of ESCC tissue array and TCGA database. However, future in-depth study will include larger sample sizes to better investigate whether eIF3b-TEX9 can affect the prognosis of ESCC patients. Hoping for your approval. Thank you very much.

With best regards.

Reviewer 2 comments

Manuscript entitled "TEX9 and eIF3b Functionally Synergize to Promote the Progression of Esophageal Squamous Cell Carcinoma". This work contains some interesting biological functional studies, yet the clinical value is not high. The authors are encouraged to performed IHC to see the expression status of TEX9 and eIF3b in a well-characterized cohort to check the correlations of these two proteins and their associations to tumor characters and patient outcome.

Thank you very much for your suggestion. We performed IHC in the tissue array of ESCC patients. The score of each case was evaluated according to the percentage of immunoreactions and staining intensity. Then the cases were classified into TEX9-low or TEX9-high expression groups (Supplementary Fig 4). We applied the Kaplan-Meier product limit estimator to draw DFS and OS curves and compared the difference between each group with the log-rank test. Unfortunately, no significant prognostic difference between TEX9-low or TEX9-high expression group was found. Furtherly, we took the previous data of eIF3b expression into account and found that no significant prognostic difference existed among eIF3b-high/TEX9-high group, eIF3b-low/TEX9-low group and the groups with either high expression of eIF3b or TEX9. We then check the TCGA database and found the similar results that no significant prognostic difference existed (Supplementary Fig 5).
We admit that this study may contain statistical bias due to limited availability of ESCC tissue array and TCGA database. However, future in-depth study will include larger sample sizes to better investigate whether eIF3b-TEX9 can affect the prognosis of ESCC patients. Hoping for your approval. Thank you very much.

With best regards.

Reviewer 3 comments

1. The upregulated and downregulated proteins were selected by using 1.5-fold change and p<0.05 in overexpressing- and depleted eIF3b ESCC cells. The verified protein list was shown in supplementary Table 1. However, the supplementary table 1 was a antibody list which used in the manuscript. Please show the protein list in the manuscript.

   Thank you for your question. We feel terribly sorry for our fault for listing the wrong table number in the manuscript. We revise the table number and the protein list was shown in the Supplementary Table 3.

2. Please have a rationale that TEX9 was chosen for the study.

   Thank you for your question. Quantitative proteomics was performed in eIF3b-depleted, eIF3b-overexpressed and normal control of EC109 cells. All quantified proteins were summarized in Supplementary Table 2. The results of quantitative proteomics analysis showed that TEX9 was the only one protein, upregulated (ov1:2.29; ov2:2.04) and downregulated (sh1:0.51; sh2:0.49) in two technical replicates with relative quantification 2 fold-changes. In addition, the study focused on TEX9 and esophageal carcinoma is limited up till now. It is very interesting why TEX9 expression is related with eIF3b expression in esophageal squamous cell carcinoma. Thus, TEX9 was chosen based on proteomics analyses in this study.

3. In figure 1C, what is the "normal control"? For overexpressing system, the control should be vehicle (or vector alone). For knockdown system, the control should be siRNA-control or sh-vector. This two system should be use different control.

   Thank you for your advice. The three groups in Figure 1c were the cells that were all transfected with lentiviral vectors. The control group was transfected with the lentiviral vector of irrelative
sequence. It is true that two different controls for two systems were better than only one control for two systems. For the reason that all the three groups received the same transfection procedure and parallel vector, we chose one control group for the two system. In our future study, we would use two controls for two systems. Hoping for your approval. Thank you very much.

4. Please quantitate the protein level in figure 2A.

Thank you for your advice. We measured the protein level in the bottom of Figure 2A. We added the related description “Densitometry analysis was used to quantitate protein expression with Image J (bottom)” in the figure legend of figure 2A.

5. In figure 3B and C, please show the full images.

Thank you for your advice. In Figure 3B, each image is the full image of the 50mm culture plate. In Figure 3D (which was Figure 3C in the previous version), during the experiment, we save the images of 5 random visual field for each group. It is truly a good idea for us to store the full image. We will carry it out in our future experiments. Hoping for your approval. Thank you very much.

6. In figure 3D, the analyzed cell numbers were not the same among these groups. It should be used the same (or similar) cell numbers to analyze the results.

Thank you for your suggestion. We feel sorry for this difference of cell numbers between each group. We re-performed the assay and replaced data in Figure 3E (which was 3D in the previous version). Thank you very much.

7. In figure 4D, the full image should be presented.

Thank you for your advice. In Figure 4D, during the experiment, we save the images of 5 random visual field for each group. It is truly a good idea for us to store the full image. We will carry it out in our future experiments. Hoping for your approval. Thank you very much.

8. Please verify these results in xenograft model.

Thank you for your suggestion. We performed the Tumor xenograft assay. 4-week-old male nude mice were applied for the experiment. Totally, 4×106 cells of si-Control and si-TEX9
groups were injected subcutaneously into either side of mice posterior flank. 3 weeks later, the
mice were executed and the size of tumors were measured by caliper for statistical analysis. The
data showed that si-Control groups could form significantly larger tumors than the TEX9
knockdown groups did, which was consistent to the in vitro results (Fig 3C). And we added the
related description in Results section with underline.

With best regards.