Response to Reviewers

Dear Editors,

We are submitting the revised manuscript "***KIFC3 promotes proliferation, migration, and invasion in esophageal squamous cell carcinoma cells by activating EMT and β-catenin signaling***" (**Manuscript Number: 73309**) according to your suggestions.

We would like to thank the editors and reviewers for their hard work and insightful comments on our manuscript. Overall, we appreciate the constructive comments made by the editors and reviewers, which have helped us to improve our manuscript. We have addressed all the concerns raised by the reviewers. The point-by-point responses, as well as changes to the manuscript, are as follows:

Reviewer #1: I am grateful for the opportunity to review this interesting manuscript entitled: "KIFC3 promotes proliferation, migration and invasion in esophageal squamous cell carcinoma by activating EMT and β-catenin signaling". This report is very interesting because the significance of KIFC3 for ESCC was evaluated multidirectionally using IHC, lentiviruses transfection, western blotting and in vivo experiment. However, there are several problems which should be revised or reconsidered in your manuscript. Problem list was summarized as below.

Minor comments:

1. Page 7, Line 28 Please show the method of positive cell counting.

   Response: Thank you for your suggestion. The proportion of positive tumor cells was scored as follows: 1 (<10% positive tumor cells), 2 (10–50% positive tumor cells), 3 (50–75% positive tumor cells), and 4 (>75% positive tumor cells). The intensity of staining was graded according to the following criteria: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). The staining index was calculated as the product of the proportion of positive cells times the staining intensity score (range from 0 to 12). The median of staining index was used as the cut-off value; staining index higher than the cut-off value was identified as high

expression, while that less than the cut-off value was identified as low expression. We have added the method of positive cell counting in the revised manuscript. Thank you again for your advice.

2. Page 8, Line 8 You should show the background of 34 patients with ESCC and compare clinicopathological factors between KIFC3 positive group and negative group.

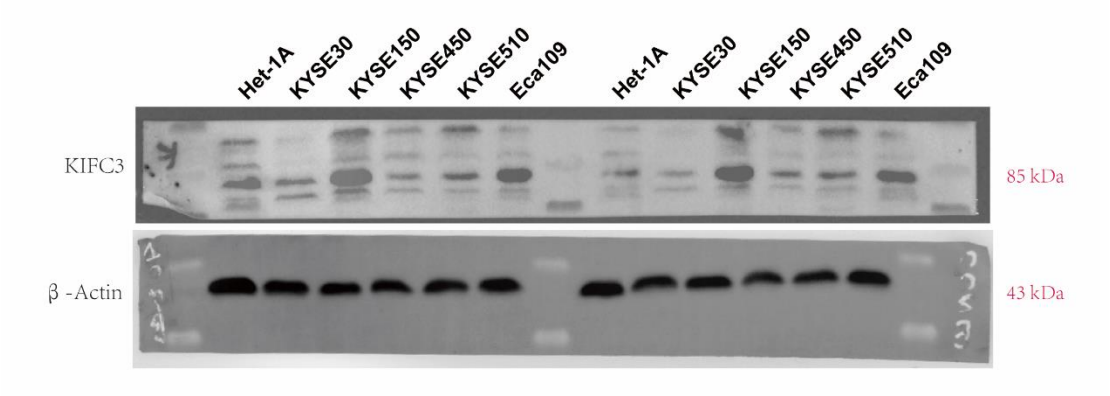
Response: This is a very accurate and helpful suggestion. We have adopted your advice, and the results are as follows. Due to the small sample size, only N phase shows significant difference between KIFC3 positive group and negative group. M phase also shows a difference, but not significant. Luckily, data from Kaplan-Meier Plotter (https://kmplot.com/analysis) revealed that a lower expression of KIFC3 was associated with better overall survival in patients with ESCC (Figure 1B), which help us confirm the role of KIFC3 in ESCC. We will try to collect more samples in our hospital and aim to get more reliable results using our data in the next study. Thank you again for your useful suggestion.

Characteristics		Total (n=34)	KIFC3		Chi-square test
			Low expression (n=17, 50.0%)	High expression (n=17, 50.0%)	
Gender	male	26	13(50.0%)	13(50.0%)	0.666
	female	8	4(50.0%)	4(50.0%)	
Age (years)	＜60	17	7(41.2%)	10(58.8%)	0.084
	≥60	17	10(58.8%)	7(41.2%)	
T	2	18	9(50.0%%)	9(50.0%%)	0.319
	3	14	8(57.2%)	6(42.8%)	
	4	2	0(0%)	2(100%)	
N	0	17	13(76.5%)	4(23.5%)	0.02
	1	13	3(25.0%)	10(75.0%)	
	2	3	1(33.3%)	2(66.7%)	
	3	1	0(0%)	1(100%)	
M	0	31	17(54.8%)	14(45.2%)	0.07
	1	3	0(0%)	3(100%)	

3. Figure 1 C and D Please show negative control (normal cell?) in western blotting.

Response: This is a very accurate and helpful suggestion. We have adopted your advice and reported the results.

In Figure 1C, we evaluated the expression of KIFC3 in ESCC cells and normal esophageal epithelial cell line Het-1A. We can see that the expression of KIFC3 in Het-1A is lower than that in ESCC cells but not the lowest, and the expression of KIFC3 shows obvious difference even in ESCC cell lines. This result may be because every cell line has its unique epigenetic characteristics, and not all genes show the same effect in different ESCC cell lines. We can still make the conclusion that KIFC3 is overexpressed in most ESCC cell lines.



In Figure 1D, we evaluated the expression of KIFC3 in normal ESCC cell lines and the cell lines transfected with empty vectors or target gene vectors. The results are shown in the revised Figure 1D.

We appreciate you greatly for your advice that has made our results more reliable.

Reviewer#2

Little is known about the mechanism of squamous cell carcinoma of the esophagus. The kinesin superfamily (KIF) proteins that play a crucial role during mitosis and meiosis present various functions in tumor pathology. More particularly, KIFC3 plays a role in the positioning and integration of Golgi and apical transport in epithelial cells. This work concerns the study of the expression and the role of KIFC3 in the toral

progression of squamous cell carcinoma of the esophagus in order to provide elements of understanding of the mechanisms involved in this pathology. For this, the authors examined tumors from patients, cell lines from esophageal squamous cell carcinoma, tumor xenografts in mice. This article brings useful elements to the understanding of tumor progression in the case of esophageal squamous cell carcinoma but before considering its publication, I would make a few remarks concerning the manuscript.

Minor comments:

1. Page 6, Immunofluorescence: are there negative controls? How were the negative controls prepared (by omitting the first antibody or others)?

   Response: Thank you for your question. Normal goat serum was used as a negative control, and we have added the details in the revised methods and materials.

2. Page 7, Immunohistochemistry: same remarks: are there negative controls? How were the negative controls prepared (by omitting the first antibody or others)? Who are the suppliers for the biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin.

   Response: Thank you for your question. Normal goat serum was used as a negative control. Biotinylated secondary antibody was supplied by Aspen, and horseradish peroxidase-conjugated streptavidin was supplied by Beyotime. We have added these details in the revised methods and materials section.

3. Page 8, Results. “ESCC tissues and adjacent non-tumor tissues were collected from 34 patients with ESCC”: how many males and females? what age ranges are the patients in?

   Response: Thank you for your question. We have checked our sample, and there are 34 patients (26 males and 8 females, aged 47–72 years) with ESCC. We have added these details in the revised manuscript.

4. Page 20, figure 1 A: specify the color obtained for the tissues concerned; specify the value of the scale bar

   Response: Thank you for your suggestion. In IHC, the tissues with high expression of KIFC3 were considered positive, and the positive cells were yellow, yellow-brown, or brown. We also illustrated the color in the Methods and Materials section. In the ESCC tissue, the proportion of KIFC3 positive cells (which were yellow, yellow-brown or brown) was significantly higher than

that in the normal esophageal tissue, which means that KIFC3 expression is higher in the ESCC tissue. The scale bar in Figure 1A means 500 μm, and we have added the explanation in the figure legend.

5. Page 23, figure 4 D: explain the picture; specify the color obtained for the cells detected by the Ki67 (or add these details in the materials and methods); add a scale bar to micrographs

   Response: Thank you for your advice. Ki67 is a marker reflecting cell proliferation, and high expression of Ki67 usually means active tumor progression. In IHC, the tissues with high expression of Ki67 are considered positive, and the positive cells were yellow-brown or brown. After KIFC3 knockdown, the proportion of KIFC3 positive cells decreased significantly, which means KIFC3 knockdown inhibits tumor proliferation. We have added scale bars on the images of Figure 4D.

6. Page 24. Figure 5: add a scale bar on the images of lines A and B.

   Response: Thank you for your advice. We have added scale bars on the images of Figure 5A and B.

7. Page 25, figure 6 C: add a scale bar

   Response: Thank you for your advice. We have added scale bars on the images of Figure 6C.

Finally, we appreciate you for your precious time in editing our manuscript, and the reviewers for their valuable suggestions and comments. We did our best to improve the manuscript and made some changes in the manuscript. We would be glad to respond to any further questions and comments that you may have.

Sincerely,

Feng Xu