Dear Editors,

We are submitting the revised manuscript "***STAT3-mediated activation of mitochondrial pathway contributes to antitumor effect of dihydrotanshinone Ⅰ in esophageal squamous carcinoma cells***" (**Manuscript Number: 68218**) according to your suggestions.

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

Reviewer #1: This manuscript by Qi et al is quite interesting, well written, the findings are original and of relevance. The methods to achieve the objectives are numerous and well described.

Minor comments:

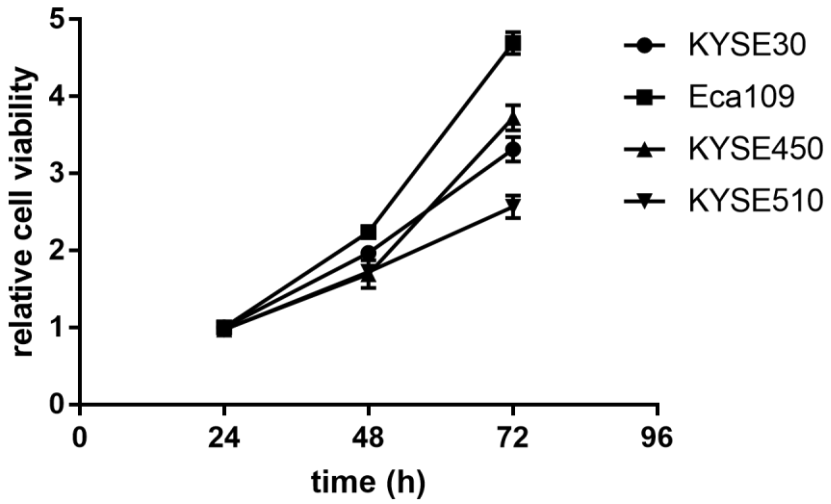
1. Final concentration of DMSO employed must be appointed.

   Reply: Thanks a lot for your suggestion. In consideration of the toxicity of DMSO, DHTS was dissolved in DMSO at a stock concentration of 10 mM and was diluted with RPMI-1640 medium or DMEM medium to perform assays. As a result, final concentration of DMSO in the medium was no more than 10 μL/mL in all assays (including assays performed in Het-1A cells which used a high concentration of DHTS), which would not show obvious toxicity to cells. In control group, DMSO without DHTS was used as negative control, and the final concentration of DMSO in the medium was same as that in the group treated with high concentration of DHTS. We have stressed the final concentration of DMSO in our manuscript. Thanks again for your rigorous academic advice.

2. It is recommended the introduction of a control without treatment in the proliferation assay.

   Reply: This is a very accurate and helpful suggestion. We have adopted your

advice and performed the proliferation assay without treatment in KYSE30, Eca109, KYSE450 and KYSE510 cells. CCK-8 was used to detect cell viability and the results are as follows.



3. KYSE30 cells line needs lower concentration of DHTS to achieve the same inhibition of proliferation. Please, discuss this point.

   Reply: The question is very interesting and deserves discussion. In our research, different concentrations of DHTS is needed to achieve the same inhibition of proliferation and the IC_{50} is different in different cell lines. There are samples that the same drug shows different IC_{50} in different cell lines of the same cancer in other studies [1,2], which indicates that different cell lines show different sensitivity to the same drug. In our research, the IC_{50} of DHTS showed more significant difference among different cell lines. We think it is because that gene expression profiles are not totally consistent in different cell lines of the same cancer, and genes determine the absorption, transportation, and metabolism of drugs. The key molecules including p-glycoprotein, multidrug resistance-associated protein 1, topoisomerase II, glutathione S-transferase-π and matrix metalloproteinase may have different expression among different cancer cells, which may result in different sensitivity to drugs. We speculate that the IC_{50} of DHTS are different because of different gene

expression profiles. Similar to our hypothesis, there is a research demonstrating that higher expression of EGFR, HER2, EGF, HRG1-α and HRG1-β is significantly associated with resistance to 5FU, carboplatin and oxaliplatin resistance by Pearson's correlation [2]. For the mechanism is complicated, maybe a new research is needed to clarify the exact mechanism of IC_{50} difference of DHTS. We appreciate you greatly for your interesting question and great inspiration.

4. Figure 3: A and B footnote needs improve to better understand each image.

   Reply: We are so sorry that the footnotes of DHTS concentration were missing. We have added the footnotes in Figure 3A and B in the revised manuscript.

5. Description of primers employed in RT‐qPCR is missing.

   Reply: Thank you very much for your kind reminder. Sequences of primers employed in RT‐qPCR are as follows: forward sequence 5'-CAGCAGCTTGACACACGGTA-3' and reverse sequence 5'-AAACACCAAAGTGGCATGTGA-3' for STAT3; forward sequence 5'-AAATCCCATCACCATCTTCC-3' and reverse sequence 5'-TCACACCCATGACGAACA-3' for GAPDH. We have added the information of primers to "MATERIALS AND METHODS" in the revised manuscript.

6. As the convenience of GADPH as endogenous control in RT-PCR has been recently questioned (doi: 10.1002/pmic.201400577), inclusion of a second loading control gene would be desirable.

   Reply: Thanks a lot for your interesting question. As for the endogenous control in RT-PCR, we have read the article that you mentioned (doi: 10.1002/pmic.201400577) carefully. The article found that expression of GAPDH was significantly up-regulated in cancer tissue comparing with the adjacent normal tissue. And since the article published in 2015, there are still articles about esophageal squamous cell carcinoma that used GAPDH as

endogenous control [3, 4]. Maybe more time is needed for a traditional housekeeping gene such as GAPDH to withdrawal from the role of endogenous control and more reliable evidence is needed. In our research, we used RT-qPCR to verify the knockdown and overexpression efficiency in esophageal squamous cell carcinoma cell lines, only the cancer cell lines were used and we think that the expression of GAPDH should be stable in the same cancer cell line. We still thank you for your advice and we will take more concentration about the housekeeping genes in esophageal squamous cell carcinoma cells and use a loading control gene without dispute in the future.

Major comments:

1.Overall, to improve the relevance of the results obtained, it is highly recommended the inclusion of a normal epithelial cell line (for example: Het-1A) as control in the proliferation, cell cycle and apoptosis assays...

Reply: Thank you very much for your question. As your concern about a novel therapeutic agent, not only anti-tumor effect is needed, but also the safety to normal cells. Following your constructive suggestion, we used normal esophageal epithelial cell line Het-1A as a control. In CCK8 assay, DHTS did not show obvious proliferation inhibition in Het-1A even when the concentration of DHTS was 64 μM after DHTS treatment for 36 h (Figure 1E in the revised manuscript). In annexin V-PE/7-AAD double staining assay for apoptosis, the apoptosis rate of DHTS-treated Het-1A cells showed no significant difference with control group ($P > 0.05$)(Figure 4 in the revised manuscript). These results indicate that DHTS did not inhibit proliferation or induce apoptosis in normal esophageal epithelial cells, DHTS shows no obvious toxicity to normal cells. Thanks again for your objective comment.

Finally, we appreciate very much for your precious time in editing our manuscript and the reviewer for his/her 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.

References

1. Jin F., R. Wang, Y. Zhu, J. Chen, W. Cao, Y. Wang, Y. Wu, X. Song, Y. Huang, J. Dong,Z. Ren, *A novel quinolinylmethyl substituted ethylenediamine compound exerts anti-cancer effects via stimulating the accumulation of reactive oxygen species and NO in hepatocellular carcinoma cells.* Eur J Pharmacol, 2020. **885**: p. 173497.
2. Hamzehlou S., M. Momeny, Z. Zandi, B. Kashani, H. Yousefi, A.R. Dehpour, J. Tavakkoly-Bazzaz,S.H. Ghaffari, *Anti-tumor activity of neratinib, a pan-HER inhibitor, in gastric adenocarcinoma cells.* Eur J Pharmacol, 2019. **863**: p. 172705.
3. Liu J.H., Q.F. Wu, J.K. Fu, X.M. Che,H.J. Li, *Obesity Potentiates Esophageal Squamous Cell Carcinoma Growth and Invasion by AMPK-YAP Pathway.* J Immunol Res, 2020. **2020**: p. 6765474.
4. Zhang H., Y. Wang, W. Zhang, Q. Wu, J. Fan,Q. Zhan, *BAALC-AS1/G3BP2/c-Myc feedback loop promotes cell proliferation in esophageal squamous cell carcinoma.* Cancer Commun (Lond), 2021. **41**(3): p. 240-257.