**Author's response to reviews**

**Title:** The role of cytoplasmic p57 in invasion of hepatocellular carcinoma

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**Author's response to reviews:** see over
Author's response to the reviewers

Title: The role of cytoplasmic p57 in invasion of hepatocellular carcinoma

Dear editor,

Thank you for arranging a timely review of our manuscript. Please find enclosed our revised manuscript entitled "The role of cytoplasmic p57 in invasion of hepatocellular carcinoma" by Hui Guo, Yi Li, Tao Tian, Lili Han, Zhiping Ruan, Xuan Liang, Wenjuan Wang, and Kejun Nan. We sincerely appreciate the reviewers’ comments and felt encouraged by their positive feedback. Moreover, we have carefully evaluated the reviewers’ critical comments and thoughtful suggestions, and we have revised the manuscript accordingly. All changes made to the text are in red so that they may be easily identified. Moreover, we have had our manuscript re-edited by American Journal Experts to ensure proper English language usage. Below, we provide a point-by-point response to all comments.

We are looking forward to your response.

Sincerely,

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Editorial request:

1. requesting for copyediting:
We recommend that you copyedit the paper to improve the style of written English. If this is not possible, you may need to use a professional language editing service.
Response
Thank you. We have had our manuscript re-edited by American Journal Experts to ensure proper English language usage, and we have attached the editorial certificate that we received from AJE.

Reviewer 1:

Discretionary revision:
Discretionary additional data could be to report the cytoplasmic p57 alterations in humans hepatocellular carcinoma alcohol associated and in human hepatocellular carcinoma hepatitis B or hepatitis C correlated. (i.e.) divide the 45 patients with hepatocellular carcinoma according to the ethiology (Alcohol related group; B hepatitis related group; C hepatitis related group) and report cytoplasmic p57 Alterations.
Response
Thank you for your suggestion. In our study, we did not enroll HCC patients with hepatitis C because it is low rate of hepatitis C-infection in China, and we lacked information regarding alcohol consumption. Therefore, we did not divide groups according to hepatitis C status or alcohol consumption. Actually, we analyzed the association between cytoplasmic staining of p57 and hepatitis B in HCC patients. The results showed that cytoplasmic staining of p57 was not associated with hepatitis B status in HCC patients (P > 0.05). However, we noted that 42 HCC patients had hepatitis B and only 3 HCC patients did not have hepatitis B. We felt that the distribution of patients was not balanced; thus, we did not include this group in our
Reviewer 2:

Major Compulsory Revisions

1. Nuclear staining of p57 is not evident in Figure 1A. The authors should label nuclei that are considered to be p57-positive with arrows. There seem to be no cofilin-positive cells in Figure 1C. Have the brownish vesicles been considered as cofilin-positive signals? Cofilin IHC-staining of cancer tissues including hepatocellular carcinoma looks different. The reliability of the correlations between nuclear p57 with tumor size, cytoplasmic p57 with metastasis as well as cytoplasmic absence of p57/cofilin with TNM stage and metastasis require improved stainings which should be provided in Figure 1.

Response

We thank the reviewer for raising this critical issue. We have replaced the Figure 1A, C and signed p57-positive cells with arrows. Moreover, the tissue shown in Figure 1C and Figure 1B was from a patient at a later stage (Ⅲ). In our study, we found that p57 was expressed in both the nucleus and cytoplasm of liver cells. In Figure 1, we did not provide an image illustrating the correlations between nuclear p57 and tumor size because p57 is a well-known cell cycle regulator, and we instead focused on the relationship of cytoplasmic p57 with invasion. However, we think that your suggestion is very important. Therefore, we have provided images of nuclear staining of p57 from an HCC patient with a larger tumor (Supplementary Fig S1) and cytopalsmic staining of p57 from an HCC patients with the later stage (Supplementary Fig S3).

2. The authors claim that p57 interacts with LIMK1 and provide co-immunoprecipitation data in Figure 2D. There is, however, no visible effect on “co-immunoprecipitation” of LIMK1 in the hepatoma cell lines after knock-down of p57. The authors should evaluate other mechanisms of p57-mediated regulation of cofilin expression. Realtime PCR analysis for cofilin mRNA should be performed to reveal transcriptional versus post-transcriptional
mechanisms.

Response

We are very sorry for this confusion. The Cip/Kip proteins (p21, p27, p57) have been reported to interact with the RhoA/ROCK1/LIMK1/cofilin pathway. In this pathway, Phospho-cofilin is the unique substrate of LIMK1 and reflects its activity. We want to investigate whether p57 can physically interact with LIMK1 by co-immunoprecipitation. The level of phospho-cofilin was reduced in the cytoplasm, which is the effect of interaction of LIMK1 with p57 in the hepatoma cell lines after p57 knockdown (Figure 3B). In previous study, our results showed that the protein level of total cofilin did not change after knockdown of p57 (Guo et al, 2011). Additionally, we performed cofilin mRNA by realtime PCR analysis. As showed in Fig S2, there is no significant difference in p57 depletion compared to control shRNA.

Reviewer 3:

This manuscript by Guo et al investigates the role of cytoplasmic p57 in hepatocellular carcinoma (HCC). The paper contains some interesting observations but it is rather descriptive. The authors tried to demonstrate that cytoplasmic p57 is involved in HCC invasion however, no invasion assay are reported in the manuscript to demonstrate the role of p57 in this mechanism.

Response

We are appreciated your suggestions. In previous study, our results showed that p57 downregulation accelerated the invasion of HCC cells in vitro and in vivo by controlling the activity of LIMK1. (Guo et al, 2011). Notably, we found p57 was localized to both the nucleus and the cytoplasm of HCC cells, which is different from the function of a cell cycle regulator. In this study, we further investigated the role of cytoplasmic p57 in HCC.

Several points need to be addressed:

a) Figure 1A has to be replaced, cytoplasmic staining is moderate and look like Background.
Response

We thank the reviewer’s suggestions, and Figure 1A has been replaced. In our study, we found that p57 was expressed in both the nucleus and cytoplasm of liver cells. In Figure 1, we provided an image in which cytoplasmic p57 was more apparent because we were focusing on the relationship of cytoplasmic p57 with invasion.

b) Figure 1: magnification can’t be 400x as described in the methods.
Response

We are very sorry for this mistake. At first, we used 400x, but my mentor felt that this magnification level was too low. Therefore, we further magnified the images. I have recovered 400x in the revised manuscript.

c) Authors need to show p57 and p-cofilin staining in the same area at least in three HCC cases and surrounding non tumour tissues.
Response

Thank you for your suggestions. The tissue samples shown in Figure 1B (p57 staining) and Figure 1C (cofilin staining) are from a patient at a later stage (III). Moreover, we presented tissue samples from three HCC cases with surrounding non-tumor tissues (Supplementary Fig S3)

d) Figure 2A: p57 expression in BEL7402 and SMMC7721 cells is not moderate but high.
Response

We are very sorry that our description was not clear. p57 expression in BEL7402 and SMMC7721 cells is moderate in contrast with that in the normal liver cell line LO2. In HCC cell lines, p57 expression is high in BEL7402 and SMMC7721 cells. We corrected this description on Page 5 (line 107) in the Results. The sentence now reads, “We selected the BEL7402 and SMMC7721 cell lines for the follow-up experiments because they exhibit moderate p57 expression, in contrast with the normal liver cell line LO2 (Fig 2A).”

e) In methods authors report two shRNA sequence for p57 silencing however, throughout the manuscript only one shRNA is reported. Both shRNA-p57 have to be used in at least some experiments to role out off-target effects.
Response

We are very sorry that our description was not clear. Actually, we designed four p57 shRNA sequences. We selected the most effective shRNAs based on p57 downregulation as measured by RT-PCR and western blot, and we added these data to the manuscript (Figure 2B,C). In our manuscript, one of the sequences is sense, and the other is anti-sense. The target is as follows:

CACCGCTTTAAGAGTCATTATATTCAGAGATATATAATGACTTTAAAGCTTTTTTG

We have revised the sentence on Page 10 (line 220-224) in the Methods.

f) Figure 2D does not show difference in LIMK1 expression in p57 silenced cells compared to NC cells.

Response

We thank the reviewer for raising this critical issue. Actually, the previous study showed that p57 did not alter the protein level of LIMK1 but rather regulated the activity of LIMK1 (Guo et al, 2011). Therefore, we did not provide results regarding LIMK1 expression, and we used the level of p-cofilin to reflect the activity of LIMK1.

g) Figure 3A: BEL7402-shp57 are not silenced since no difference in p57 expression is evident between sh-p57 and shNC cells.

Response

We are very sorry that our description was not clear. Green fluorescence represents the transfected plasmid (GFP-shRNA), red fluorescence shows target protein (p57 and p-cofilin) expression, and blue fluorescence (DAPI) shows the nucleus. In the BEL7402-shNC cells, we observed that the red fluorescence was strong. However, the red fluorescence was very weak in the BEL7402-shp57 cells. We are appreciated your question and we will further improve the quality of fluorescence in the future.

h) Figure 3B: an internal housekeeping control is necessary also for nuclear proteins (for example Histones).

Response

Thank you for your suggestion. Lamin A was used as an internal control for nuclear proteins (Figure 3B).