Author's response to reviews

Title: Grp78 promotes the invasion of hepatocellular carcinoma

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Author's response to reviews: see over
Cover letter:

Dear editor:

We have revised our manuscript as your request and re-submitted to BMC cancer for your consideration. All the reviewers’ comments have been thoroughly considered in revision process. In the revised manuscript, we explored the mechanism of overexpression of Grp78 negatively regulated Rock kinase activity. This problem has been concerned by all the reviewers. We have modified our manuscript and corrected the spelling and grammar mistakes. This revised manuscript has been reviewed by a foreign expert from USA. The Ethics Committee of Liaoning Medical College approved and supervised the specimen collection procedures (EC: 2007011). We have got the permissions of all the patients. This data have been introduced in the materials and methods.

Dr Rongjian Su designed the experiments, analyzed the data and wrote the manuscript. Zhen Li, Hongdan Li, Huijuan Song, Cuifen Bao and Jia Wei performed the experiments. Professor Liufang Cheng found the patients with HCC, collected the clinicopathological data and analyzed these data.

Sincerely
Rongjian Su

E-mail: rongjiansu@hotmail.com
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Dr Jin-zhang Zeng’s comments

Dear Dr Zeng, thank you for your attention.

1. The expression of Grp78 in HCC. It is not clear that the immunostaining of Grp7 protein is associated tumor grades as indicated in Fig. 1. HE staining for indication of tumor grades should be provided. In addition, it is better to include staining of both tumor and the corresponding nontumor tissues in one picture.

Western blotting analysis should be also performed.

Response: According to Dr Zeng’s advice, we have supplemented the images of HE staining to reveal the differentiation extents of HCC tissue samples. But we cannot examined Grp78 and FAK levels in tissue samples by western blot because all the tissue samples we get are formalin-fixed paraffin-embedded.

2. The SMMC7721 was the only cell line for mechanistic study. However, this cell line endogenously expresses high level of Grp78 (Fig. 2A). They should conduct experiments in various HCC cell lines to see whether Grp78 expression levels are associated with tumor invasive phenotypes. Using those cell lines with low or undetectable of Grp78 in their transfections will help to demonstrate the oncogenic activity of Grp78, while knockingdown Grp78 in those cell lines with high level of Grp78 will contribute to the conclusion.

Response: Thank you for your advice. Actually, we have examined Grp78 levels in Chang liver cells, QGY-7703 cells and SMMC7721 cells before we initiated our research. The results revealed that Grp78 levels were up-regulated in the three cell lines and
SMMC7721 showed a relatively lower Grp78 level than Chang liver cells and QGY-7703 cells. We also reviewed the references which indicated that SMMC7721 is widely used in the research of hepatocellular carcinoma cells invasion.

3. **Transwell assay in Fig. 2C. They should also provide the staining results.**

Response: As you have seen, this article contained too many pictures. For the consideration of length, we did not provide the images of Hocheast 33258 staining.

4. **The Grp78-transfected cells did not show a significant increased spreading Feature (Fig. 3).**

Response: Fig 3 evaluated the status of cell spreading and cell polarity; the data we collected have been statistically analyzed by one way ANOVA. The statistical analysis revealed that the difference is significant.

5. **The phospho-FAK staining should be costained with anti-Grp78 to compare their association (Fig. 4A). Grp78 transfection did not obviously increase the levels of phosphor-FAK (Fig. 4B). These experiments need to be repeated.**

Response: (1): Costaining of Grp78 and phospho-FAK is very difficult, even impossible technically in western blot analysis for the restriction of the quality of antibodies. Even antibodies from Chemicon and abcam have cross interactions with other closely related proteins. Sometimes without the guidance of protein marker, it is very difficult for the researchers to identify the results of western blot stained with one antibody. (2): As Dr Zeng’s request, we have repeated these experiments and revised our pictures.

Sincerely

Rongjian Su
Dr Weizhong Wu’s comments

Dear Dr Wu, thank you for your attention.

1. **The diagnostic criteria for differentiation of HCC should be described in detail.**

   Response: Thank you for your comments about the diagnostic criteria for differentiation of HCC. We classified the differentiation extents of HCC tissue samples according to Edmondson-Steiner grading system. We have mentioned this criteria in revised manuscript but did not describe it in details for the restriction of length. Pardon me please!

2. **List all clinicopathologic features of 44 HCC patients enrolled in this study and analyze the correlation of Grp78 levels and tumor venous invasion, intrahepatic metastasis and microvascular invasion if possible.**

   Response: As you can see, we have revised table 1 in our revised manuscript, portal verin invasion and intrahepatic metastasis have been supplemented. The correlations of Grp78 levels and tumor venous invasion, intrahepatic metastasis have been analyzed. But we cannot find the descriptions of microvascular invasion status in the medical charts of the patients. So we did not analyze whether Grp78 levels are correlated with microvascular invasion.

3. **List the sources of important materials such as antibodies.**

   Response: As your quest, we have listed the important materials in the section of method under the subtitle reagents and antibodies.

4. **Improve English language and correct many solecisms in the manuscript.**

   Response: As you can see, we have re-write this article. The revised manuscript has been reviewed by a foreign teacher from USA.
5. Re-plot the figures, change the titles of X or/and Y axis and add p value when it is significant.

Response: As your request, we have e-plotted the figures and replaced X/Y as the ratio of long axis/short axis. The symbols of * or # have been added in the pictures when the difference is significant.

6. describe the role of dominant negative Rock pasmid KDIA in HCC invasion and metastasis, and explain possible reasons that knockdown of Grp78 expression activates endogenous Rock activity in the present posture of dominant negative Rock pasmid.

Response: As we mentioned in the manuscript, KDIA transfection decreased the invasion of HCC. Based on your comments, we have supplemented the following experiments:

(1) We have examined the levels phospho-p190RhoGAP in Grp78 overexpressing cells. The result revealed that overexpression of Grp78 in SMMC7721 cells enhanced the phosphorylation of p190RhoGAP.

(2) FAK siRNA knockdown in Grp78 overexpressing cells decreased the level of phospho-p190RhoGAP. The result showed that Grp78 negatively regulated Rock activity in a FAK dependent manner which promoted the phosphorylation of p190RhoGAP when activated.

7. All data come from one cell line, it is suggested to conduct the functional studies in a greater number of HCC cell lines#

Response: we also knockdown Grp78 levels in BEL7402 cells and observed that Grp78 knockdown by siRNA inhibited the invasion of hepatocellular carcinoma. These articles have been published or accepted in the Chinese of cell biology (2 pieces have been
published and 1 piece have been accepted) and Acta Anatomica Sinica (1 piece will be published in No 6, 2009).

Sincerely

Rongjian Su
Dr Fuguo’s comments

Dear Dr Fu, thank you your attention.

1. In methods section, in Cell Culture subheading, the authors should list the researcher’s name who provided the SMMC7721 cell line and put the affiliated institutes in brackets. The issue in Transfection subheading, please name who provided the pCAG-KDIA construct.

Response: we have revised our manuscript as your request. The name and institution have been added.

2. In Fig 2A, 4B, 4C, and 5B, if doable, the authors should normalize the expression level of targeted protein (e.g Grp78) to control protein (e.g Actin) and show a bar graph underneath. This can be easily done by scanning the film, calculating the bands density, and normalizing with control protein.

Response: we have normalized our data as your request. But the diagrams were not showed in this paper for the restriction of length. Alternatively, we showed these data by adding the increased or decreased fold in brackets in the results section.

3. In Fig 2C, 2D, 3B, 3C, 4D, and 5D, wherever the authors claimed a significant difference, they should show the relative P value in both text and figures.

Response: we have added the symbols of * and # in the figures you mentioned when the difference is significant.

4. The rationale and interconnections were not explained clearly in Results
section subheading Grp78 negatively regulates Rock activity. The author should make it clearer.

Response: Based on your comments, we have supplemented the following experiments:

(1) We have examined the levels phospho-p190RhoGAP in Grp78 overexpressing cells. The result revealed that overexpression of Grp78 in SMMC7721 cells enhanced the phosphorylation of p190RhoGAP.

(2) FAK siRNA knockdown in Grp78 overexpressing cells decreased the level of phospho-p190RhoGAP. The result showed that Grp78 negatively regulated Rock activity in a FAK dependent manner which promoted the phosphorylation of p190RhoGAP when activated.

5. In the figure legends, when applicable, the authors should state how many experiments or repeats were done

Response: All the experiments have been repeated for three times in triplicate. We have noted these facts in figure legends.

Sincerely

Rongjian Su
Dr Weidong Zhao’s comments

Dear Dr Zhao, thank you for your attention.

Major point:

In Fig. 4, the relationship between Grp78 and FAK was obscure based on the presented data. Maybe the authors should pay more attention to the negative regulation of RhoA by Grp78, which is demonstrated by Fig. 5.

Response: Based on your comments, we have supplemented the following experiments:

(1) We have examined the levels phospho-p190RhoGAP in Grp78 overexpressing cells. The result revealed that overexpression of Grp78 in SMMC7721 cells enhanced the phosphorylation of p190RhoGAP.

(2) FAK siRNA knockdown in Grp78 overexpressing cells decreased the level of phospho-p190RhoGAP. The result showed that Grp78 negatively regulated Rock activity in a FAK dependent manner which promoted the phosphorylation of p190RhoGAP when activated.

Minor point:

1. Scale bar should be marked on all the figures.

Response: we have revised our manuscript as your request and scale bar has been added in appropriate position.

2. In Fig. 3B, the unit for “area” should be marked and the x-axis labels in Fig. 3B and Fig. 3C is not suitable.

Response: As your request, the unit for areas has been marked in the Y axis in Fig3B, and the X or Y in Fig3C has been replaced by the ratio of long axis/Y-axis. The symbols
of * and # have been added in the pictures when the difference is significant.

3. **For Fig. 5C, the labeled text seems confusing and the figure legend is not Clear.**

   Response: Thank you for your advice. As your guidance, we have checked Fig.5C and the mistake have been corrected. The confusing labels of A, B, C, D have been replaced by a, b, c, d.

4. **The expression level of Grp78 after the knock-down of Grp78 by siRNA in SMMC7721/Grp78 cells should be examined.**

   Response: Based on your comment, we have added this result in our revised manuscript as Fig 2D.

5. **The negative regulation of RhoA by Grp78 should be mentioned in the discussion part.**

   Response: we have discussed the negative regulation of RhoA by Grp78 in the section of discussion part. Based on our supplementary experiments, we proposed that overexpression of Grp78 in hepatocellular carcinoma cells enhanced the activation and activity of FAK which negatively regulated Rock kinase activity via promoted p190RhoGAP level.

6. **The possible role of Grp78 during the development of hepatocellular carcinoma should be discussed in the presented manuscript.**

   Response: Based on your advice, we have revised table 1 in our revised manuscript, portal verin invasion and intrahepatic metastasis have been supplemented. The correlations of Grp78 levels and tumor venous invasion, intrahepatic metastasis have been analyzed.
7. Grammar and typos should be corrected and it is suggested that the manuscript should be modified by an English-speaking researcher for resubmitting.

Response: the revised manuscript has been reviewed by the foreign teacher from USA. The spelling and grammar mistakes have been corrected before submitting.

Sincerely

Rongjian Su