Author's response to reviews

Title: 14-3-3sigma Induces Heat Shock Protein 70 Expression in Hepatocellular Carcinoma

Authors:

Chia-Chia Liu (car01711213liu@nhri.org.tw)
Yee-Jee Jan (yejan@vghtc.gov.tw)
Bor-Sheng Ko (kevinkomd@gmail.com)
Yao-Ming Wu (wyaoming@gmail.com)
Shu-Man Liang (shu-man@nhri.org.tw)
Shyh-Chang Chen (jesica2700@yahoo.com.tw)
Yen-Ming Lee (velociraptorpixy@yahoo.com.tw)
Tzu-An Liu (ann.liu@nhri.org.tw)
Tzu-Ching Chang (ctching9@mail.cmu.edu.tw)
John Wang (shengw@seed.net.tw)
Song-Kun Shyue (skshyue@ibms.sinica.edu.tw)
Li-Ying Sung (liyingsung@ntu.edu.tw)
Jun-Yang Liou (jliou@nhri.org.tw)

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Enclosed please find a revised manuscript entitled “14-3-3# Induces Heat Shock Protein 70 Expression in Hepatocellular Carcinoma” which we wish to be considered for publication of the BMC Cancer. We appreciate the constructive comments made by the reviewers. In response to the critiques of reviewers, we have performed additional experiments and revised the manuscript extensively. Changes in the revised manuscript are marked by “Red” color in the text and the English is extensively polished. Point-by-point responses to each reviewer’s comments are attached.

Thank you for your consideration.

Sincerely Yours,

Jun-Yang Liou Ph.D.
Institute of Cellular and System Medicine,
National Health Research Institutes,
Response to the reviewers' comments:

Reviewer 1:

We appreciate the constructive comments and have performed additional experiments as suggested. We have performed the Western blotting to show the expression of 14-3-3# in non-HCC cells. We have added the information of microarray in the supplementary results. We have corrected the mistakes and extensive revised the manuscript. Following is point by point response.

1. Fig 1A to demonstrate that the expression is increased in tumors a region of non-tumor should be stained and compared as a control. Fig. 1B, there is no control sample, a non HCC cell line.

Response: We thank for the reviewer’s suggestion. We have added the labeling of the non-cancerous part as “N” and tumor part as “T” of HCC tissue (Figure 1A, right panel) and described in the Figure legends (Page 22, line 4). For non HCC cell lines, we have added the Western blotting of 14-3-3# expression in HUVECs and 293 cells. We found that the 14-3-3# expression level is undetectable in these cells (Supplementary Figure S1) and description is added in the Results section (Page 10, lines 4-6).

2. Fig. 1C. MTT assay is not described or referenced in Materials and Methods.

Response: We thank reviewer’s thoughtful comment. We have added the description of MTT assay (Cell proliferation assay) in the Materials and methods (Page 7, paragraph 3) of this revised version.

3. Page 9, State that the expression level in HCC cell lines is strong (except Huh-7) but there is no control cell line or tissue for comparison. Possible that the other cell lines have normal or control expression and Huh-7 is lower than normal or control.

Response: The same as the point 1, we added the results of other non-HCC cells in Supplementary Figure S1. The expression of 14-3-3# in Huh-7 is low but is undetectable in HUVECs and 293 cells (described in Page 10, lines 4-6).

4. Page 10, please explain how the gene expression profile analysis was done. Was this done on the Huh-7 cells that over expressed 14-3-3#? This information should at the least be put into the Materials and Methods or in Supplementary information.

Response: We thank for reviewer’s suggestion. The gene expression profile analysis was done on the Huh-7 stables cells. The information has been added in the Supplementary Table S3 (increased genes) and Table S4 (decreased genes).

5. Fig, 2B, 2C. It should be made clear what cells were used.

Response: We thank for reviewer’s comment. To make it consistent, we have described that “clone 2 of 14-3-3# and clone 1 of the control were used for the following experiments” in Page 10, line 11 of the revised manuscript.
6. Fig. 4B. This figure is confusing. The authors must provide more information about this figure in the legend. Knockdown is stated to have reduced migration, yet the figure shows in one case stimulation and the other appears to be no effect. Could the labels on the graph be incorrect?

Response: We appreciate the reviewer to point out the labeled mistakes. We apology for the mistakes and have corrected the labeling in the revised version.

7. Fig. 4D. Not clear what this figure is showing. This experiment must be clarified. Again, are the bar graphs labeled correctly?

Response: Same as Point 6, we apology for the mistake and have corrected the labeling in the revised manuscript.

8. The conclusion that the findings indicate 14-3-3# participates in promoting HCC cell migration and tumor development via regulating #-catenin/HSF-1#/HSP70 pathway is mostly based on the experiments done with cell lines. To make such a conclusion experiments would have to be done on tumor growth in animals.

Response: We thank reviewer’s thoughtful suggestion. To perform the animal experiments definitely will provide strong support for this study. In this study we demonstrated the data of molecular level and clinical relevance. The in vivo part to establish the animal model will be investigated in the continued study.

9. Figure S1. This blot was probed with anti- Flag antibody but probing with anti-14-3-3# antibody would give the reader an idea of the endogenous levels and how much higher the levels are with transfection. The question is “how much more 14-3-3# protein is needed to get the effect”. Are these levels produced by transfection physiological or pharmacological?

Response: This point is very well taken. We have determined the expression of 14-3-3# using anti-14-3-3# antibody according to the reviewer’s suggestion. We found that either the moderate (clone 1 and 2) or extremely higher (clone 3 and 4) expression of 14-3-3# (Supplementary Figure 2B) upregulate the expression of HSF-1# and HSP70 (Figure 2A and 2B). These results suggest that the appropriately increased 14-3-3# expression may be sufficient to regulate HCC tumor progression and an excess of 14-3-3# have no further effect on HSF-1# and HSP70 expression (Figure 2A and 2B). The description is added in the Discussion section (Page 14, Paragraph 2).

Minor Essential Revisions:

1. Page 2, first line, “Garduate” should be Graduate.
Response: It is corrected in the revised manuscript.

2. Page 9, “selected with G418 for 4 weeks”
Response: It is corrected in the revised manuscript.

Reviewer 2:
We thank for the very positive comments. Following is point by point response.
1. If the final goal of the authors is to suggest the use of 14-3-3# as a diagnostic marker, which is not supported by their data, they need to compare its expression in a (large) cohort of pre-malignant hepatocellular nodules (mainly high grade dysplastic nodules).

Response: We thank for reviewer’s thoughtful comments. The current results of this study only support that 14-3-3# serves as a potential prognostic marker, but not diagnostic marker. Therefore, we have deleted the description of diagnostic marker in this revised manuscript.

2. If the authors are suggesting to use this marker as a prognostic one, which indeed seems strongly supported by the study, they need, in keeping with international guidelines, to validate its expression in a completely different series of patients.

Response: We thank for reviewer’s suggestion. Although we have provided evidence that 14-3-3# potentially serve as one of the prognostic marker of HCC, however, it may be needed to combine with other factors. Thus, further investigation is needed before we made the conclusion. Further work includes different series of patients may be investigated in the future study.

3. If the authors are simply reporting a novel (and interesting) molecular link in HCC, they can avoid to suggest any clinical conclusion.

Response: This point is very well taken. We agree with the reviewer that claim the clinical conclusion is beyond the scope of this study. We have eliminated the conclusion that 14-3-3# is a diagnostic marker and therapeutic target of HCC. We have only described that 14-3-3# is a potential prognostic marker of HCC in the revised manuscript.

Reviewer 3:

We appreciate the constructive comments and have performed additional experiments according to the reviewer’s suggestions. Following is point by point response.

1. Results, p9: “… increased 14-3-3# expression was significantly associated with surgical margin (P=0.008), capsular formation (P=0.028) and …” The implications of these findings must be explained.

Response: We thank for the reviewer’s suggestion. We have added the description “These results indicate that expression of 14-3-3# is associated with a more aggressive tumor behavior and a poor prognosis” in the Result section (Page 9, lines 19-20).

2. Results, p9 and p11: the term “marginal significance” is very misleading and must be deleted as it is a range without statistical significance

Response: The term “marginal significance” is deleted in the revised manuscript.

3. Results, p10: the expression profiling must be made available in supplementary data.
Response: We thank for reviewer’s suggestion. The gene expression profile analysis has been added in the Supplementary Table S3 (increased genes) and Table S4 (decreased genes).

4. Fig 2A: a Western blot is shown with numbered lanes from 1 to 4. I assume these numbers account for different clones of Huh7 cells overexpressing either 14-3-3# or a control construct. However, this is nowhere described properly.

Response: We thank for reviewer’s suggestion and have made a clear description in the revised manuscript. We have added “Lanes 1-4 indicate as 4 different stable clones selected from the single colonies” in the Figure legends (Page 22, lines 10-11).

5. Fig 2B: show HSF-1 and HSP70 expression by qPCR for all 4 controls and 4 cell lines overexpressing 14-3-3#.

Response: The expression of HSF-1 and HSP70 analyzed by q-PCR for all clones of control and 14-3-3 cells has shown in revised Figure 2B.

6. Fig 2: it is nowhere explained which clones were used for the further analysis (Fig 2B/C/D/E).

Response: We thank for reviewer’s comment. To make it consistent, we have described that “clone 2 of 14-3-3# and clone 1 of the control were used for the following experiments” in Page 10, line 11 of the revised manuscript.

7. Fig 4: it can only be assumed that overexpression or knockdown cells are Huh7, as this is nowhere stated. Fig 4B must be wrong as it shows an induction of migration due to 14-3-3# knockdown, which is the opposite of what the text says. Interestingly it contains 2 variants of knockdown and control that are supposedly significantly different from one another, however, again it is nowhere stated what these might be.

Response: We have added the Huh-7 cells in the Figure legends of Figure 4. In addition, we appreciate the reviewer to point out the labeled mistakes. We apology for the mistakes and have corrected the labeling in the revised manuscript.

8. Same is true for Fig 4D.

Response: Same as Point 6, we apology for the mistake and have corrected the labeling in the revised manuscript.

9. Fig 5 tries to explain the dependency of HSF-1 expression on the presence of #-catenin. Now interestingly in the case of 14-3-3# overexpression and strongly elevated HSF-1 protein levels, the knockdown of #-catenin causes complete loss of HSF-1. However, knockdown of #-catenin does not significantly change HSF-1 expression in control cells. This is a striking discrepancy and questions the role of #-catenin in the expression of HSF-1 and therefore clearly needs to be addressed by the authors.

Response: We appreciate the reviewer to point out this point. We have repeated this experiment and have replaced a more representative result in the revised
10. The effect of intervention with β-catenin signaling (β-catenin siRNA) should be shown on the migration of cells used in Fig 5 (Huh7 cells overexpressing 14-3-3# and SK-Hep1).

Response: The reviewer raised a very important issue. We have performed the migration assay of β-catenin knockdown in 14-3-3# stable and SK-Hep1 cells according to the reviewer’s suggestion. Knockdown of β-catenin significantly reduced the cell migration induced by 14-3-3# (Figure 5D and 5E). We have added the description “We next examined whether β-catenin is involved in regulating cell migration of HCC. The 14-3-3#/control cells and SK-Hep1 cells were transfected with siRNA of β-catenin and the migration ability was determined by a trans-well assay. Knockdown of β-catenin significantly reduced cell migration of 14-3-3#/control cells (Figure 5D) and SK-Hep1 cells (Figure 5E)” (Page 12 last 3 lines and Page first 2 lines) in the revised manuscript.

11. Discussion: The discrepancy between effects on cell migration and invasion must be discussed. The activation of the HSF-1 promoter by β-catenin should be considered (putative TCF/Lef binding sites?).

Response: This point is very well taken. We discuss this issue with potential reasons such as multiple factors or the tumor microenvironments involves in the regulation of migration/invasion (Discussion, Page 13, last 4 lines and Page 14 first 6 lines). Moreover, although the posttranslational regulation of HSF-1 is well elucidated, the regulation of HSF-1 promoter remains unclear. Thus, whether β-catenin combines with the co-factors to directly work on the HSF-1 promoter needs further investigation. We have added “Whether the potential TCF/LEF binding motifs found on the promoters of HSF-1 and GS are regulated by β-catenin needs further investigation” in the Discussion (Page 14, lines 22-23).