Title: Regulation of HSP27 on NF-kappaB pathway activation may be involved in metastatic hepatocellular carcinoma cells apoptosis

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Author's response to reviews: see over
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Dear Editor,

It is our pleasure to send our revised manuscript with revised titled ‘Regulation of HSP27 on NF-κB pathway activation may be involved in metastatic hepatocellular carcinoma cells apoptosis’ to BMC CANCER. All authors have read this revised manuscript, and agree to submit it to your journal.

During the process of metastasis, cells are subjected to various apoptotic stimuli. Aberrant expression of apoptotic regulators often contribute to cell metastasis. Heat shock protein 27(HSP27) is recently confirmed as an apoptosis regulator, but its apoptotic mechanism in metastatic hepatocellular carcinoma (HCC) cells remains unclear. In this report, siRNA-mediated HSP27 knockdown in metastatic HCC cells-MHCC97H resulted in decreased motility and invasion in vitro and increased apoptosis ratio which was detected by Flow Cytometry and TUNEL staining. Through a Human Q Series Signal Transduction in Cancer Gene Array, it indicated the prominently altered signal pathway was NF-κB pathway. Furthermore, inhibition of HSP27 led to significant decreasing of nuclear NF-κB contentration, which was demonstrated by ELISA assay. Co-immunoprecipitation assay showed interaction of IKKα, IKKβ, IκBα with HSP27 and its phosphorylated form in three HCC cells with different metastatic potentials. Immunobloting analysis also showed that inhibition of IKK activity and reducing of the association between IKKβ and IKKα in MHCC97H cells after HSP27 was efficiently depleted. In summary, these findings revealed possible roles of HSP27 in metastatic HCC cells apoptosis, in which regulation of HSP27 molecule on NF-κB pathway activation may be involved. Further studies will
be carried out that may clarify the molecular mechanism of HCC metastasis in detail. It would provide some fundamental information that contributes to manifestation of HCC cells' metastatic phenotype and finding of some potential anti-metastasis therapeutic targets in HCC.

We have revised our manuscript according to the referees’ comments.

We expect your valuable suggestions on the manuscript for the possible acceptance by BMC Cancer. Thank you for your time and consideration.

Sincerely yours,

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The reviewer comments and our response to the reviewer’s critiques are as follows:

**Responses to Reviewer Madhavi Rane 's report:**

**Major Compulsory Revisions**


   **Response:**
   Thank you so much for your valuable suggestions and comments.
   We have revised the associated conclusion according to your comments in the revised sections.

2. Experiments in Figures 1, 2, and 4 were performed how many times? The investigator has to include the statistical analysis of the data represented in Figures 1, 2, 3 and 4.

   **Response:**
   Thanks.
   Experiments in Figures 1, 2, and 4 were performed *triplicately* and corresponding methods of statistical analysis were shown in METHODS SECTION and Figure Legends.

3. Relative ratio of nuclear NF-κB content (fig 3B) in the 3 cell lines tested is identical to relative ratio of IKK activity in the same 3 cell types (figure 4C). Was the relative values of these 2 assays identical or one of the figures is mislabeled?

   **Response:**
   Yes. The increasing tendency of relative ratio of nuclear NF-κB content in the 3 cell lines is coincident with increasing of relative ratio of IKK activity in the same 3 cell types. It indicated there was IKK-dependent NF-κB pathway activation in our
metastatic HCC cell lines.

4. In figure 4 authors show association of Hsp27 with IKKb, IKKa, and IkB. Based on Parcellier’s study, the reviewer is puzzled how IkB is not degraded in the above samples given the fact that activated p65 NF-κB is in the nuclear fraction. Additionally, Figure 4A demonstrates Hsp27-IKK association and Figure 4B demonstrates that this association correlates with IKK activity, which is markedly inhibited upon disrupting Hsp27-IKK interaction by silencing Hsp27 expression. This contradicts the work demonstrated by Park et al JBC (2003) 278:35272-35278) where enhanced Hsp27-IKK association resulted in decreased IKK activity. Have the authors tested the phosphorylation status of Hsp27 in these cells? It would be helpful if the authors could speculate on these differences.

**Response:**

Thank you so much for your valuable suggestions.

It is well-known that HSP27 needs to be dissociated from large oligomers into small oligomers and these dimers are thought to bind the unfolded proteins. In cells, the oligomeric size of HSP27 seems to be regulated by stress-dependent post-translational modifications (Bryantsev et al., 2007). The most prominent modification is stress-induced phosphorylation of HSP27. Phosphorylation promotes dissociation of the large HSP27 oligomers to the smaller ones and eventually to tetramers and dimmers (Kato et al., 1994, Lambert et al., 1999). Moreover, the chaperone activity is not restricted to the cytoplasm, but is also seen in the nuclear compartment. This activity and nuclear translocation are influenced by phosphorylation, but HSP27 phosphorylation status is insufficient for both events (Bryantsev et al., 2007). According to the reviewer’s comments and suggestion, we have tested basal phosphorylation level of HSP27 in Hep3B, MHCC97L, MHCC97H cells and its phosphorylation in co-immunoprecipitant with IKKa antibody from these three cells by western blotting, the result showed although basal level of phosphorylated HSP27 and IKK activity increased consecutively from Hep3B to MHCC97L, MHCC97H cells, phosphorylated HSP27 which associated with IKK complex reduced from
Hep3B, MHCC97L to MHCC97H cells. In addition, it was also found in our study that IKK complex in HCC cells was not only associated with phosphorylated HSP27, but also with HSP27 molecule. These results indicate that phosphorylation of HSP27 may have a negative effect on activation of IKK complex through interfering IKK complex activation by some intercellular kinases, consistent with some literatures (Bhattacharyya et al., 2008). While non-phosphorylated HSP27 may maintain IKK signalosome specific conformation to be activated by upstream stimuli, which can be implied through decreasing activity of IKK in HSP27 knockdown metastatic HCC cells. The phenomenon contradicts some researchers’ work which be mentioned by reviewer. It could be due to specific cell line and logical experimental conditions. We hypothesized there is a feedback loop which includes the switch of regulated role of non-phosphorylated HSP27 and phosphorylated HSP27 in IKK complex activity. However, the mechanism needs to be elucidated in further study.

Secondly, it is also recognized that there is a complex network of various signal transduction pathways activation in HCC cells. The association of NF-κB activation with HCC promotion, progression, and metastasis is well documented. In higher metastatic MHCC97H cells, in which activity of NF-κB pathway is higher than MHCC97L and Hep3B cells, we found HSP27 was associated with IKKα, IKKβ and IκBα, not with activated NF-κB p65. That IκBα was detected in co-immunoprecipitates with HSP27 monoclonal antibody could be due to these whole cell extracts from three HCC cells were used in our study. In other words, intricate signal transduction regulation mechanism may result in NF-κB pathway partly activation in our metastatic HCC cells, not all of IκBα in the cytoplasm had been degraded. Moreover, we assumed IκBα protein was not immediately degraded when associating with HSP27 might result from phosphorylation regulation of HSP27 oligomers. This provides a study target in future research.

References
Bhattacharyya, S., Dudeja, P. K. and Tobacman, J. K., 2008. Carrageenan-induced NFkappaB activation depends on distinct pathways mediated by reactive oxygen...
species and Hsp27 or by Bcl10. *Biochim Biophys Acta.* 1780, 973-982.


**Minor Essential Revisions**

1. Experiment performed in Figure 1 is not described in detail. How long were the cells cultured in the presence of control or Hsp27 siRNA? Were the cells cultured with siRNA overnight or over a longer period?

**Response:**

Thanks. In our study, HCC cells were cultured for 24hr to detect mRNA level and for 48hr to detect protein level in the presence of control or HSP27 siRNA, because siRNA duplex are generally depleted in less than three days at when the interference effect of siRNA will be weak.

2. How long were the cells cultured in the siRNA before apoptosis assays were performed? Were cells treated with Hsp27 siRNA for the same time as in Figure 1? A modest increase in apoptosis (10%) is seen in Hsp27 siRNA transfected cells compared with control and mock transfected cells. Does a longer timeline of Hsp27 siRNA transfection increase cellular apoptosis?

**Response:**

Thanks. The cells were cultured in the siRNA for 24hr before apoptosis assays, when mRNA level of HSP27 was only 18% of that in control group (Figure 1B). In
addition to TUNEL-staining, we attempted to confirm apoptotic rates by measuring the mitochondrial membrane potential changes using JC-1-staining, because the loss of mitochondrial membrane potential is an early indicator for apoptosis (Smiley et al., 1991, Moudy et al., 1995). Both results showed apoptosis increased in HSP27 siRNA-treated MHCC97H cells. Due to viability of HSP27 siRNA-treated HCC cells reduced in 36hr, shown in our previous study (Guo et al., 2008), we detected apoptosis in 24hr. Of course, the suggestion is our target in future research. Thanks again.

References


3. After discussing data represented in Figure 3 authors state “These results suggested that Hsp27 has an effect on NF-κB activation and there is a relationship between nuclear NF-κB activation and metastatic potentials of HCC cells”. While this may be the case in MHCC97H cells (Figure 3A), increased NF-κB activation from Hep3B>MHCC97L>MHCC97H is not correlated with increased Hsp27 expression. Also was this increase in p65 nuclear localization normalized to total protein load in
each lane? Hsp27 immunoblot from these 3 cell lysates will address this question. Equal loading will also need to be demonstrated by anti-actin or anti-GAPDH immunoblotting.

Response:
Thank you so much for your valuable suggestions and comments. In the study, same amount of nuclear proteins from three HCC cell lines were subjected to measurement of nuclear NF-κB by ELISA, it indicated increased NF-κB activation from Hep3B>MHCC97L>MHCC97H. In addition, according to your comments, we detected HSP27 protein level in three cell lysates, which was normalized to GAPDH level in cells. The detail is shown in revised manuscript. Thanks again.

4. Authors need to indicate source of all antibodies used in the study with catalogue numbers.

Response:
According to your comments, the source of all antibodies used in the study with catalogue numbers has been added in the METHODS SECTION.

5. Measurement of Phospho-IκB by ELISA: did the authors normalize sample values with total cell protein? This needs to be included in the methods section.

Response:
Yes, total cell protein from each sample was normalized and was same amount which had been subjected to ELISA analysis. Phospho-IκBα (Ser32) Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of Phospho-IκBα (Ser32) protein. An IκBα Mouse mAb has been coated onto the microwells. After incubation with cell lysates, both non-phospho- and phospho-IκBα proteins are captured by the coated antibody. Following extensive washing, Phospho-IκBα (Ser32) Antibody is added to detect the captured phospho-IκBα (Ser32) protein. HRP-linked anti-rabbit antibody is then used to recognize the bound detection antibody. HRP substrate, TMB, is added to develop
color. The magnitude of optical density for this developed color is proportional to the quantity of phospho-IκBα (Ser32) protein.

The revision has been added in the METHODS SECTION.

6. In Immunoprecipitation protocol please indicate how many mg of appropriate antibodies were utilized for the assay? Where were these antibodies purchased from? Were isotype control IP’s performed? Data represented in Figure 4 suggests that IgG control immunoprecipitations were performed but this is not included in the methods section.

Response:
Thanks. According to your comments, we have clarified the description of immunoprecipitation protocol in this revised manuscript. These details can be found in METHODS SECTION.

Responses to Reviewer L Catley's report:
The paper makes the hypothesis that HSP27 contributes to metastatic potential in hepatocellular carcinoma cells. The data presented uses solid methods to demonstrate some important events, namely:
- SiRNA knockdown
- HSP27 RNA and protein down-regulated
- Nuclear NFKB activation decreased
- Apoptosis demonstrated by flow cytometric cell cycle analysis

However, to address the hypothesis that HSP27 is involved with migration, the methods describe the migration assay using transwell migration assay, but this data is not presented. In addition, if this assay is used to measure migration, the authors would need to demonstrate that the cells are viable i.e. reduced migration is not due to apoptosis, which has already been demonstrated in the first part of the paper. I suggest the authors address these issues or modify the conclusions and hypotheses.

Response:
In our previous study, it showed the important effect of HSP27 on metastatic hepatocellular carcinoma (HCC) cells motility in vitro. So this same phenomenon and associated data has not presented in this original manuscript. Considering your suggestions and according to your comments, we added these data and more clarified the conclusions and hypotheses in the revised manuscript. Thanks again.

**Responses to Reviewer Laura Vargas-Roig's report:**

This manuscript by Guo et al., describes the regulation of Hsp27 on NF-κB pathway activation and its participation in the metastatic potential of hepatocarcinoma cells.

**Major Compulsory Revision**

The authors have omitted to present the results of In vitro Invasion and Migration Assays. These results are very important because in the title the authors mention the metastatic potential of the studied cells.

**Response:**

In our previous study, it showed the important effect of HSP27 on metastatic hepatocellular carcinoma (HCC) cells motility in vitro. So this same phenomenon and associated data has not presented in this manuscript. Of course, according to your kind suggestions, we added the data in revised manuscript.

**Minor Essential Revisions**

**ABSTRACT**

Background: Omit the word recently because the antiapoptotic effect of Hsp27 has been established since several years ago.

**Response:**

Yes, we have revised it according to your suggestion.

Conclusion: Omit the word intercellular.

**Response:**
Yes, we have revised it according to your suggestion.

INTRODUCTION

I suggest to change reference 2 because there are more appropriate publications related to metastatic cells and the decreased sensitivity to apoptosis.

**Response:**

Yes, we have revised it according to your suggestion.

It is necessary to include some paragraph about the signalling pathway of NF-kB to introduce the molecules studied by the authors.

**Response:**

Yes, we have added it according to your advices. Thanks.

METHODS

The methods are appropriate but not all of them are well described. I recommend to describe RT-PCR of Hsp27 and not only mention a reference of some of the authors. In the description of Western blot it is also important to mention the source of the antibodies used.

**Response:**

Yes, we have added the description of HSP27 RT-PCR and the source of the antibodies used according to your suggestion. The associated revision can be found in METHODS SECTION.

RESULTS

Page 8, line 26: “... the result showed NF-kB activation increased from Hep3B to MHCC97H cells.” I suggest to mention that there was a tendency to an increase because the differences obtained were not statistically significant (Figure 3B).
**Response:**
Yes, we have revised it according to your advices. Thanks.

**DISCUSSION**

Page 9, line 24: Omit the word Recent because the reference #14 was published in 2001.

**Response:**
Yes, we have omitted it according to your advices. Thanks.

Page 9, line 26: The reference #15 is not appropriate for the sentence about Hsp27 and apoptosis.

**Response:**
Yes, we have revised it according to your advices. Thanks.

Page 10, line 10: Omit the word recently because the reference #17 was published in 1999.

**Response:**
Yes, we have added appropriate reference according to your advices. Thanks.

Page 11, lines 4 and 5: Omit the word intercellular.

**Response:**
Yes, we have omitted it according to your suggestion. Thanks.

**FIGURE LEGENDS:**

Fig.1: The description of B corresponds to C. The authors must add the description of B and D.

**Response:**
Yes, we have added the description. Thanks.
Fig. 2: C does not correspond to the description mentioned in the figure legend.

**Response:**

Yes, we have revised it according to your suggestion. Thanks.

FIGURES:

Figure 1C: Is the molecular weight of GAPDH correct? Why does it appear below Hsp27?

**Response:**

Yes, the molecular weight of GAPDH is right. We have revised the position of picture. Thanks.

Figure 4: Change D by C and vice-versa.

**Response:**

Yes, we have revised it according to your suggestion. Thanks.