Author's response to reviews

Title: Nrf2 is a potential prognostic marker and promotes proliferation and invasion in human hepatocellular carcinoma

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

We are pleased to answer the questions of the reviewers’ and the manuscript (MS: 5123661121557048) has also been extensively revised according to the comments and the journal style. This paper was proofread by a native English professional with science background. We are appreciating for each editor’s hard work on our manuscript.

Editor's comment:
Thank you for submitting your research article manuscript to BMC Cancer for consideration of publication. Your manuscript has been reviewed by four experts in the field. While most reviewers found this study potentially interesting and publishable, it needs some additional evidence to support your conclusions, and the manuscript needs quite extensive revision. We would like to consider a revised manuscript with the reviewers' comments addressed. When you resubmit the manuscript, please summarize point-by-point responses to the reviewers.

Answer: Thank you for each editor’s hard work on our manuscript and we summarize point-by-point responses to the reviewers as below.

1. Reviewer 1: Qingguang Liu
Reviewer's report:
For this article, the study was designed well and results were convinced. However, the underlying mechanism did not demonstrated clearly. Additionally, it is essential to perform the in vivo experiments to confirm the finding in vitro.

Answer: For the exact mechanism and in vivo experiments, we are doing further research and publish them in next manuscript.

2. Reviewer 2
Reviewer's report
Title: Nrf2 is a potential prognostic marker and promotes proliferation and invasion
The authors have made an interesting observation that the expression Nrf2 is increased in human hepatocellular carcinoma tissues and the high level of Nrf2 expression is significantly correlated with tumor differentiation and lymph metastasis. They further concluded that Nrf2 promoted proliferation by inhibiting apoptosis and enhanced the invasive ability of human hepatocellular carcinoma cells partly through regulating expression of Bcl-xl and MMP-9 depending on in vitro evidences. However, the following concerns need be addressed before a decision on publication can be reached.

1. There are lots of types and grammar errors in the manuscript, for example, Page 10, line 4 “a over-expression” to “an over-expression” Page 11, line 12 “repressed promoted” to “promoted”.
   Answer: Sorry for these types and grammar errors, we have modified them and this paper was proofread by a native English professional with science background.

2. It is clear that the normal liver cell line LO2 also expresses Nrf2 (Figure 1), although slightly lower than malignant cell lines. Is the LO2 metastatic? If the Nrf2 were overexpressed in LO2, what would happen? Given the subcellular location difference between normal (cytosol) and malignant cell lines (mainly in nuclear), the authors should at least discuss the role of subcellular location of Nrf2 in differentiation and metastasis.
Answer: We add two more typical immunohistological features in Fig 1 and discuss the role of subcellular location of Nrf2 in differentiation and metastasis in the result part.

3. The knockdown and overexpression results (Figure 3) are not evident. The authors should conduct extra experiments to have better knockdown or overexpression efficiency.
Answer: Sorry for it, we did extra experiments and change them to new data in figure 3.

4. As shown by the authors that Nrf2 expression correlates HCC cell lines differentiation and metastasis in vitro and could be an independent diagnostic biomarker for survival. However, the authors did not provide in vivo data to confirm the important roles of Nrf2 in HCC cells differentiation and metastasis.
Answer: For the exact mechanism and in vivo experiments, we are doing further research and publish them in next manuscript.

3. Reviewer 3: Moo Jun Baek
Discretionary Revisions
Nuclear factor (erythroid-derived 2)-like 2 is more commonly known as NFE2L2 rather than Nrf2. I recommend to replace the gene name Nrf2 to commonly known NFE2L2 in the manuscript.
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests.
Answer: Nrf2 or NFE2L2 is both ok, but we had published some articles used
“Nrf2” before, so we choose Nrf2 rather than NEF2L2.

Nrf2 is a potential therapeutic target in radioresistance in human cancer.

The noncytotoxic dose of sorafenib sensitizes Bel-7402/5-FU cells to 5-FU by down-regulating 5-FU-induced Nrf2 expression.

Propofol induces proliferation and invasion of gallbladder cancer cells through activation of Nrf2.

The effects of nrf2 on tumor angiogenesis: a review of the possible mechanisms of action.

Correlation of Nrf2, HO-1, and MRP3 in gallbladder cancer and their relationships to clinicopathologic features and survival.

4. **Reviewer 4: Yunpeng Hua**

Nrf2, or nuclear factor E2-related factor 2, is a master transcriptional activator of genes encoding enzymes that protects cells from oxidative stress and xenobiotics by induction of a transcriptional regulation of several antioxidant enzymatic pathways and also various drug efflux pumps-members of multidrug resistance protein. However, some reports have shown that drugs that activate Nrf2 can promote cell growth and an increasing number of works point to a potential role for Nrf2 and its transcriptional target genes in tumorigenesis. Recently, a new paper, published in the magazine of Hepatology, indicated the onset of Nrf2 mutations is a very early event, likely essential for the clonal expansion of preneoplastic hepatocytes to HCC, and functional experiments demonstrate that
Nrf2 is an oncogene, critical for HCC progression and development. But now there is no any report for the role of Nrf2 in the prognosis of HCC patients. I believe that the conclusion of this article, which Nrf2 is a potential prognostic marker and promotes proliferation and invasion in human hepatocellular carcinoma partly through regulating expression of Bcl-xL and MMP-9, is useful to our clinic and basic scientists.

Major Compulsory Revisions:

1. In this study, only 80 cases were involved in the study from January 2005 to December 2009. Why are there so few cases recruited during 5 years?
   Answer: There are all course more cases in the two hospitals during 5 years, these 80 cases are only part of them and these patients are well follow up. Sorry for our terrible description and we modified it.

2. Today is April 4, 2015. Why were Patients only followed up closely until December 2011? I believe it is better if you follow up patients until now.
   Answer: Sorry for it, but most of follow up data are 5 years survival data. We think it is enough for the research of prognostic value of Nrf2 in HCC.

3. Can you tell me if the resection for HCC is curable in this study? It is well-known that tumor size, tumor number, tumor differentiation, vascular invasion, resection margin status, tumor recurrence, etc. are predictive for tumor recurrence after curative resection. Why didn’t the authors analyze the relationship between Nrf2 and the above factors?
   Answer: Yes, it is curative resection. And we add tumor size and tumor number into our paper, while tumor differentiation and vascular invasion (included in metastasis) was already in the manuscript.

4. In my clinical experience, the HCC cases with Lymph metastasis are few. Why is the proportion of Lymph metastasis close to half in the clinical study?
   Answer: Sorry for the error, it should be “metastasis” rather than “lymph
metastasis”, including lymph, vein, distance metastasis.

5. Is the survival disease free survival (DFS), or overall survival (OS)? I feel it is better to analyze respectively the correlation of DFS and OS with Nrf2 in the HCC patients.
Answer: We added the DFS data in the manuscript.

6. In page 6, raw 10-13, Nrf2 immunoreactivity was predominant in the nucleus. The number of positive-staining cells showing immunoreactivity on the membrane for L1CAM in ten representative microscopic fields was counted and the percentage of positive cells was calculated. I don’t understand what does mean. Is Nrf2 located in the nucleus or on the membrane? What is L1CAM?
Answer: Sorry, it was a type error, we delete the “The number of positive-staining cells showing immunoreactivity on the membrane for L1CAM in ten representative microscopic fields was counted and the percentage of positive cells was calculated.”

7. How about the level of Nrf2 expression in the normal liver and liver cirrhosis? Can you show the data of the Nrf2 expression in Lymphatic metastatic tumor?
Answer: We do not know the level of Nrf2 expression in the normal liver and liver cirrhosis, since we have no specimens of normal liver and liver cirrhosis. But in our result, normal liver cells LO2 also has Nrf2 expression but mainly in cytoplasm. So we can guess that normal liver and liver cirrhosis also have Nrf2 expression, but the levels and location needs more research. In our research and opinion, Lymphatic metastatic tumor had high expression Nrf2 and nuclear location as shown in Fig 1 B.

8. Why did you choose these three HCC cell lines, Hep3B, Bel-7402, and HepG2? You found that the level of Nrf2 are different in these cell lines. Can
you tell me how about their abilities of proliferation and invasion, whether their abilities are associated with Nrf2 level?

Answer: The reason for us to choose these three HCC cell lines in to find HCC cells with lowest/highest expression Nrf2, not to clarify the relationship between Nrf2 and malignant behavior. But in one of our previous article, the Bel-7402 with highest expression Nrf2 and nuclear location proliferates faster than other two cells, even under DDP pretreatment.

[Expression and distribution of Nrf2 in several hepatocellular carcinoma cell lines].  
Ma RQ, Zhang MX, Wang JS, Cai H, Yeer MK, Duan XY.  
PMD: 21651857

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests: I declare that I have no competing interests

Thank you very much for consideration!