Author's response to reviews

Title: Zinc finger protein ZBTB20 expression is increased in hepatocellular carcinoma and associated with poor prognosis

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

Thank you very much for sending us the comments for the manuscript submitted to your journal. Now we are submitting a revised version, which was carefully revised according to the reviewers’ comments. A point-to-point list of the responses is given at the end of the letter.

All the three reviewers felt this manuscript was interesting and well conducted. The core finding of this manuscript may be that in hepatocellular carcinoma, ZBTB20 was positively associated with poor prognosis in patients with HCC, definitely beyond its role as an AFP suppressor. We are now exploring the related mechanism.

Sincerely Yours

Prof. Hongyang Wang

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Answers to the comment point-to-point

Review 1:

Major points:

1. Although the provided starting results in vitro appear to be consistent, the Western blot images seem like to be manipulated. The ZBTB20 antibody staining has several bands in their gel image. The whole gel images should be displayed plus the positive and negative controls instead of just cut the bands as they wish. The molecular weight should also be marked. This fact precludes the possibility to the specificity of their ZBTB20 analysis in HCC and therefore represents a limitation to the overall significance of authors' findings.

Answer: Thanks. We are sorry for insufficient information on Fig.2. The image was cut from the
whole gel however without any artificial manipulation. Now a whole gel image including three cases of HCC, positive (293T transfected with ZBTB20-expressing plasmid) and negative (naïve 293T) control plus three HCC cell lines was provided as a supporting material, with the main aim to show that the band on gel of Figure 2 is really the signal of ZBTB20.

2. Since the author made the conclusion that “ZBTB20 proved to be a risk factor for tumor recurrence and independent molecular marker of prognosis in HCC and may become a novel molecular target in the strategies for the prediction of tumor recurrence and prognosis or treatment of HCC.” The related mechanistic studies should be performed. The expressions of P450 family members, plus MMP-2 and MMP-9, should be detected in tumor samples.

Answer: In our research, ZBTB20 expression was found to be closely correlated to some parameters indicating recurrence and an independent molecular marker of prognosis in HCC. The mechanism for its action in carcinogenesis is under investigation.

In our previous work (Mol Cell Biol. 2009 May;29(10):2804-15), Zbtb20 KO mice displayed reduced expression of several cytochrome P450 family members. These data indicate that some important liver intrinsic functions are impaired in the Zbtb20 null mice and that complementation via transgenesis significantly increased survival, potentially by restoring the expression of cytochrome P450 family members. MMP-2 and MMP-9 have been shown to be associated with HCC invasiveness. To check whether ZBTB20 play certain roles in HCC partly through regulating cytochrome P450 family members, MMP-2 or MMP-9, the more convictive data is that whether ZBTB20 regulate these molecules directly through in vitro analysis.

3. The ZBTB20 expression between normal and fibrotic/cirrhotic livers should also be analyzed to clarify its dynamic changes during liver injury.

Answer: Thanks. The data concerning the fibrotic/cirrhotic livers have been added in the revised manuscript and the Figure 3 was renewed.

Minor points:

1. Since the information of “Ethics Committee of the Eastern Hepatobiliary Surgery Hospital” could not be obtained on the internet, the approval notice for the current human studies should
be provided as the supplementary information.

Answer: Thanks. The approval notice from our Ethics Committee was attached as a supporting material.

2. All the normal and HCC cells were cultured in DMEM medium, which is not consistent with the recommendations from ATCC for PLC/PRF/5, HepG2, SK-Hep-1 and Hep3B. The explanation should be included.

Response: Thanks for this comment. Yes, EMEM, not DMEM, is the recommendation from ATCC for culture of PLC/PRF/5, HepG2, SK-Hep-1 and Hep3B. In our lab, we once tried changing the basic medium for above cell lines from EMEM to DMEM and checking the possible "phenotype". However, no difference was found among these two groups.

3. The origin of primary anti-ZBTB20 polyclonal antibody should be described in Material and Methods section.

Answer: The anti-ZBTB20 polyclonal antibody was prepared ourselves which has been described in our work published at "Mol Cell Biol. 2009 May;29(10):2804-15", or Ref.39 in our manuscript. We cited this paper in our revised manuscript.

4. AFP expression should be included in Fig. 2.

Answer: Thanks for this comment. After receiving the revising letter, we purchased a new copy of AFP antibody for western blot analysis. Unfortunately, this antibody did not work well after more than four tries. We do believe something was wrong with this antibody. We are now trying an AFP antibody from another supplier.

5. The legends of Fig. 1, Fig. 3 and Fig. 5 should be enriched.

Answer: The legends of Fig. 1, Fig. 3 and Fig. 5 have been enriched in the revised manuscript.

6. The manuscript is too ordinary to have three 1st authors and 2 correspondence authors. The rational for the unusual strategy should be explained in Authors’ contributions section.

Answer: In authors’ contribution section, what the authors did for this work was presented and all of the listed authors reached the common sense that the first three authors contributed equally and
the last two authors are equally responsible for the overall work.

Review 2:

1. Are the data sound? Yes, with the exception of figure 4. The correlation of AFP staining to ZBTB20 staining in the inverse is not well demonstrated with the figure presented. Figure 4 c/d does not appear to be different in overall intensity compared to Figure 4 a/b, where less ZBTB20 appears to correlate with more AFP staining. If this is the best example, it calls into question the methodology of staining quantification.

Answer: Thanks for this comment. We have renewed with another case as example in Figure 4 c/d.

2. Are the discussion and conclusions well balanced and adequately supported by the data? Yes, but limited based on the fact that this is a completely descriptive study. Although a clear correlation between ZBTB20 expression and clinical parameters appears to be established, there is a lack of any related mechanism, in terms of role of ZBTB20 in disease progression or why it results in more rapid death of HCC patients. The authors have at their disposal the ZBTB20 knock-out mouse line. This would beg the question, for example, of the response of this ZBTB20 KO to DEN induced HCC. Do these mice have more tumors, more rapidly progressing, and larger, with more metastatic disease?

What then might be the relationship between a potential AFP repressor and tumor progression? Without further investigation, this manuscript is very limited in relevance.

Answer: nAs shown in previous papers, besides AFP, ZBTB20 regulated various target genes. The overall role of this molecule in stepwise carcinogenesis of HCC is believed to be complicated, which needs in-detail research. Our initial data on ZBTB20 KO to DEN induced HCC showed that hepatocyte-specific knockout of ZBTB20 alleviated the HCC genesis. Identify the direct target and related pathway(s) possibly responsible for tumor-promoting role of ZBTB20 is underway.

In our opinion, the fact that ZBTB20 is an AFP repressor did not preclude the possibility of its role as a tumor promoter. What is more, in liver, AFP level is believed to be regulated by some factors including but more than ZBTB20.
3. Can the authors explain the lack of correlation between serum AFP level and ZBTB20 level? This seems to go against their prior publication of ZBTB20 regulating AFP, and should be addressed more clearly.

Answer: Yes, our results did not show any correlation between serum AFP level and tissue ZBTB20 level. We suggested that the serum AFP, secreted / leaked from the liver hepatocytes, is controlled or influenced by some other factors, which may result in inconsistency of its level between the serum and in situ tissue. Which have been demonstrated in the result section, ie, “while the serum AFP level had no significant correlation with the tissue AFP IRS (P= 0.538)”.

4. Is the writing acceptable? The writing needs attention.

Answer: We have double checked our writing.

Minor essential revision:

1. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Yes, but this could be highlighted better. Xie et al. PNAS. 105(31) 10859-10864, described a ZBTB20 knock-out mouse which demonstrated increased AFP expression

Answer: Thanks. This paper has been covered in our reference as No.38 in the list. Moreover, we mentioned the earlier discovery that ZBTB20 negatively regulates AFP expression with this paper as a citation reference.

Review 3:

I have read carefully the paper. It is clearly written and the statistical analysis seems sound throughout. I have also checked a few p-values from chi-square test and they are correct.

One statement of the paper seems in error. In the Section "Correlation between ZBTB20 Expression and Prognosis", the paper stated "The 1-, 3-, and 5-year OS rates were 77.5%, 53.5%, and 45.9%, respectively; and the 1-, 3-, and 5-year DFS rates were 94.1%, 69.2%, and 44.4%, respectively".

The authors need to double check on this since DFS (the interval between the dates of surgery and recurrence) is generally shorter than OS (the interval between the dates of surgery and death). The one year OS rate is supposedly Pr(OS>1) and the one year DFS rate is Pr(DFS>1). It follows that Pr(OS>1) > Pr(DFS>1).
The same is true for year 3 and 5. This is clear from the survival curves in Figure 5.

Answer: We are very sorry for mistakes made during calculating the value of DFS. After second check, the result should be as follows:” 1-, 3-, and 5-year DFS rates were 52.8%, 33.4%, and 26.5%, respectively”, which has been renewed in our revised manuscript.