**Author's response to reviews**

**Title:** The expression level of miR-18b in hepatocellular carcinoma is associated with the grade of malignancy and prognosis

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**Author's response to reviews:** see over
The expression level of miR-18b in hepatocellular carcinoma is associated with the grade of malignancy and prognosis.

Dear editor Miss Nikka Portodo
on behalf of Dr. Xin Chen

Thank you for your prior review of our paper "The expression level of miR-18b in hepatocellular carcinoma is associated with the grade of malignancy and prognosis". We have made substantial changes to the text, tables and figures in accordance with the comments and concerns raised by the reviewers and would like to re-submit our article as an original paper.

Reply for reviewer: Zhen Zhang

-Major Compulsory Revisions

1) There are no asterisks in Figure 1 and 2. If that means no statistically significant difference across the samples, then the conclusions of the paper become very shaky.

> We added the statistical analysis and attached * to the group out of which capable came statistically.

2) Ago-2 Co-IP is the only experiment for testing if TNRC6B is the target of miR-18b. However, the authors didn’t indicate how many independent replicates had been done, and there are no error bars in Figure 1C. The presented data is too weak to support the conclusion that TNRC6B is a target without statistical analysis and at least one alternative method for confirmation.

> This ago2-co-IP experiment was performed three replicate. We added error bars in figure 1C. Alternatively we also added the conventional experiment that co-transfection miR-18b or miR-18b and clone 3’UTR of TNRC6B into luciferase reporter vector. Then we confirmed the TNRC6B was targeted by miR-18b.

3) In both Figure 1B and Figure 2A, Western Blot is better than qRT-PCR to examine the repression of miR-18b on TNRC6B, as miRNA regulation is on both transcript and protein level.

> The expression level of TNRC6B RNA was controlled by miR-18b, however, this
The experimental result of RNA was not able to be reproduced that of TNRC6B protein. The following things can be considered as a cause. (1) The influence by expression of single mRNA where many mRNA(s) have many expression levels of controlling TNRC6B as a target gene and (2) Since there are many expression levels of TNRC6B, the influence on the TNRC6B protein by expression of miR-18b could not be easily observed.

4) The quality of the images in Figure 3 is very poor. They should be replaced by high quality images. In addition, the staining results should be quantified, for example, as the percentage of positively stained cells per slide.

We have replaced Figure 3 with high quality images and we added the information about signal positivity for in situ hybridization of miR-18b and TNRC6B in unstaining in figure 3B.

5) In cell proliferation and adhesion experiments, TNRC6B overexpression and siRNA knockdown share one control. Because the authors used completely different transfection reagents for overexpression and knockdown, each method should have its own negative control (empty plasmid by Fugene 6 for overexpression, and negative control siRNA by RNAiMAX for knockdown). This is critical, especially when the differences between control and treated groups are very small.

We modified the presentation as reviewer suggested. The control was displayed according to the experiment of DNA vector transfection, and the experiment of RNA transfection.

-Minor Essential Revisions

1) The authors referred to Figure 1B for miR-18a, miR-122 and miR-423-5p overexpression and miR-221 suppression. However, only miR-18b data is presented there. The data of miR-22 overexpression and inhibition should also be presented in Figure 1B.

We added miR-22 information in figure 1B.

2) In Figure 1C the y-axis is not the relative expression level of TNRC6B as labeled.

We corrected the label of y-axis in figure 1C. It is now depicted as relative quantity of TNRC6B mRNA in AGO complex.

3) The cell lysis step is missing in the method of Ago2 CoIP.
We added the procedure of Ago2-co-IP in the material section.
1. The authors claimed that TNRC6B is the target of miR-18b. In order to further support this conclusion, the author should clone 3'UTR of TNRC6B into luciferase reporter vector and determine whether miR-18b has effect on luciferase activity after co-transfection of luciferase reporter vector and miR-18b mimic or miR-18b inhibitor.

> We added co-transfection analysis of miR-18b mimic or miR-18b inhibitor and 3'UTR of TNRC6B into luciferase reporter vector and added figure 1C.

2. The authors should mutate the seed region of 3'UTR of TNRC6B and determine whether the mutation impairs the effect of miR-18b mimic or inhibitor on luciferase activity.

> We performed the experiment as reviewer’s recommendation and we added the conventional experiment that co-transfection of miR-18b or miR-18b inhibitor and clone 3'UTR of TNRC6B into luciferase reporter vector. Then we confirmed TNRC6B was targeted by miR-18b.

3. The protein is the real player of cellular processes. How about protein level of TNRC6B? Does overexpression of miR-18b or miR-18 inhibitor have effect on protein level of TNRC6B?

> The expression level of TNRC6B RNA was controlled by miR-18b, however, this experimental result of RNA was not reproducible with the TNRC6B protein. We have outlined the possible reasons for this in our reply to reviewer one but in short the possible causes are: (1) The influence of a single RNA expression may be difficult to observed where there are many mRNA(s) with expression levels controlling TNRC6B as a target gene and (2) Since there are many expression levels of TNRC6B, the influence on the TNRC6B protein by expression of miR-18b could not be identified.