Author's response to reviews

Title: MicroRNA-1246 enhances migration and invasion through CADM1 in hepatocellular carcinoma

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

Thank you for your efforts in our manuscript entitled ‘MicroRNA-1246 enhances migration and invasion through CADM1 in hepatocellular carcinoma’ (Manuscript ID 1779637735119515). We also truly appreciate the constructive comments and helpful suggestions of the reviewers. We have addressed the questions raised by the reviewers and some additional data in our recent experiments are added. The corresponding changes in the manuscript are marked by red-colored text.

Please see the following point-by-point response to reviewers’ comments.

Reviewers' comments:

Reviewer 1:

Major compulsory revisions

Comment: The miR-1246 inhibitors were only tested on cell lines BEL7402 and SMMC7721, but not on Hep12 cells, where miR-1246 expression is highest and which was used for differential expression analysis. Please add data on HEP12 cells treated with the inhibitors or provide an explanation for not including this experiment.

Response: We added data on Hep12 cells treated with the miR-1246 inhibitors. Please see the following figure (which is added in the manuscript as Supple Figure 3). As expected, cell motility in Hep12 was significantly reduced after transfection of the miR-1246 inhibitor compared with inhibitor control.
Inhibition of miR-1246 reduced migration and invasion of Hep12.

Transwell migration (n=4) and invasion (n=4) assays showed that Hep12 cells transfected with the miR-1246 inhibitor (800 nM) had lower invasive and migratory potentials respectively than the control (inhibitor control). (A) is a microscopic image of crystal violet staining; (B) shows the statistical results.

Comment: A Western blot of CADM1 expression in Hep11 versus Hep12 cells as well as in Hep11 cells with and without miR-1246 mimic needs to be included.

Response: We added the western blot results of CADM1 expression as show by the two figures below (which were added as Supple Figure 9 and 6 in the manuscript). The first one shows CADM1 expression in Hep11 versus Hep12 cells...
lanes) and the second one shows CADM1 expression in Hep11 cells with and without miR-1246 mimic.

Minor essential revisions:

**Comment:** The M&M; dual luciferase assay, describes a miR-9 binding site for CADM1 (1st sentence). This probably should be miR-1246.

**Response:** Sorry for the mistake. We have changed it to miR-1246 in the manuscript.

**Comment:** miR-1246 expression in several HCC cell lines is shown in Supple Figure 1. To fully appreciate the relevance the origin of the cell lines HepG2, SMMC-7721, Hep3b and BEL-7402 in relation to tumor stage needs to be described as well. Are they derived from primary or metastatic cancers; how does miR-1246 expression relate to tumor status?

**Response:** miR-1246 expression in several HCC cell lines is now shown in Supple Figure 9 in the revised manuscript. HepG2 cell line was derived from the liver tissue of a 15 year old male with differentiated hepatocellular carcinoma. Hep3b was
derived from an 8 year male. SMMC7721 was derived from a 50 year old Chinese man and BEL7402 from a 53 year old Chinese man. All four cell lines are from primary cancers. However, the tumor stages are not clear.

As to the correlation between miR-1246 expression and tumor status, our results didn’t suggest that miR-1246 expression level in primary hepatocellular carcinoma is correlation with tumor stage. (Please see Supple Table 5 for detailed information). However, due to the limited patient number and the fact that most patients in our study are in stage 1, we couldn’t rule out the possibility that miR-1246 is correlated with tumor status at the current stage. Further studies with more patients are needed.

**Comment:** the alignment of miR-1246 seed sequence in CADM1 shown in Fig 3A is not well displayed.

**Response:** We revised Fig 3A to make it clearer and well displayed.

**Comment:** In M&M reference is made to Table 4 for sequences, this should be Supplementary Table 4.

**Response:** Sorry for the mistake. We have changed it to Supple Table 4 in the manuscript.

**Comment:** In the Discussion reduced CADM1 expression in cervical cancer is mentioned but no reference is included (for example: Overmeer et al. J Pathology, Volume 215, Issue 4, pages 388–397)
**Response:** We added references 21, 22 to the discussion part.

**Comment:** Is any information known on CADM1 methylation in HCC versus miR-1246 up-regulation. Are both involved in CADM1 down-regulation or are they potentially mutually exclusive. Please provide data or elaborate on other mechanisms that may contribute to CADM1 down-regulation in HCC. Similarly, is anything known on a correlation between miR-1246, miR-10b and miR-216, the latter two of which also regulate CADM1.

**Response:** We thank the reviewer for the great comment. Zhang W et al (Aberrant methylation of the CADM1 promoter is associated with poor prognosis in hepatocellular carcinoma treated with liver transplantation. Oncol Rep. 2011 Apr;25(4):1053-62.) reported that aberrant hypermethylation of CADM1 was frequently found in HCC cell lines with decreased CADM1 mRNA. However, we found no information about the roles of methylation and miR-1246 in CADM1 regulation. So whether they are both involved or mutually exclusive remains for further study. Additionally, we didn’t find information about the correlation between miR-1246, miR-10b and miR-216.

**Comment:** On 3rd page of Discussion an invalid reference number is included [22528944,22237452]

**Response:** Sorry for the mistake. We have changed it in the manuscript.
Reviewer 2:

Major revisions:

Comment: The authors showed in the Supple Figure 1 and 2 that miR-1246 level is higher in Hep12, and four other HCC cell lines including HepG2, SMMC-7721, Hep3b and BEL-7402, than Hep11. However, nothing is stated about the metastatic properties of these cell lines. For example, does the miR-1246 endogenous levels in those cell lines correlates with high migratory and invasive behavior of the cells? Please provide the data. Also, what is the effect of miR-1246 knockdown in Hep12 cells on migration and invasion?

Response: Supple Figure 2 showed miR-1246 level in five HCC cell lines including HepG2, SMMC-7721, Hep3b and BEL-7402 and Hep11. Hep11 has the lowest expression level of miR-1246 and SMMC-7721 has the highest. We compared the metastatic properties of these cell lines (Please see the following figure). The endogenous miR-1246 expression level is almost consistent with the migratory and invasive behavior of the six cell lines. We think the metastatic property of the cells is a combinatorial effect of various factors and miR-1246 is one of them. So to examine the roles of miR-1246 in HCC migration and invasion, we need to analyze in one cell line.

Migration and invasion of Hep12 were significantly reduced after transfection of the
miR-1246 inhibitor compared with inhibitor control. Please see the following figure (which is added in the manuscript as Supple Figure3).

Inhibition of miR-1246 reduced migration and invasion of Hep12.

Transwell migration (n=4) and invasion (n=4) assays showed that Hep12 cells transfected with the miR-1246 inhibitor (800 nM) had lower invasive and migratory potentials than the control (inhibitor control). (A) is a microscopic image of crystal violet staining; (B) shows the statistical results.

Comment: For figure 3, please compare CADM1 protein levels between different HCC lines. This information would substantiate the finding that miR-1246 suppresses CADM1 in HCC. Also, did authors observe suppression of CADM1 expression with
miR-1246 overexpression in Hep11 cells? Please provide the data. It was mentioned in the discussion that CADM1 gets methylated or lost its expression by LOH, so it would be interesting to know whether miR-1246 targets CADM1 in these HCC cell lines directly. Please perform Luciferase assay.

**Response:** We added western blot result showing CADM1 protein levels between different HCC lines. Please see the following figure (which is added as Supple Figure 9 in the manuscript). We observed suppression of CADM1 expression with miR-1246 overexpression in Hep11 cells. Please see the following figure (which is added as Supple Figure 6 in the manuscript).

We didn’t perform Luciferase assay in the six HCC cell lines because these cells have relatively high levels of endogenous miR-1246 which would interfere with the results. So we conducted a standard luciferase reporter assay in 293T cells which have very low levels of endogenous miR-1246 and found that CADM1 is a direct target of miR-1246. Please see figure 3 in the manuscript. As any cellular process is controlled by complex signaling pathways and multiple regulatory factors, it is possible that the regulation of CADM1 expression is a multi-factorial process too. Besides the
posttranscriptional regulation of miR-1246, there may be other mechanisms contributing to the regulation of HDAC6 expression at mRNA/transcription level directly or indirectly. We searched the literature and found that CADM1 could get methylated or lost its expression by LOH. So we mentioned it in the discussion part.

Comment: In Figure 4, the authors perform knockdown of CADM1 only in one cell line i.e. SMMC, yet concluded in the discussion that CADM1 knockdown results in increase in migration and invasion of HCC cell lines. This conclusion is incorrect. To strengthen this statement, please perform CADM1 knockdown in Hep11 and HepG2 cells and perform migration and invasion assays.

Response: We thank the reviewer for the suggestion. In Figure 4, we performed knockdown of CADM1 in SMMC7721 and BEL7402 cell lines. CADM1 knockdown in both cell lines results in increase in migration and invasion. We forgot to mention BEL7402 cell line in the result and we added it in the revised manuscript. Additionally, we perform CADM1 knockdown in Hep11 and perform migration and invasion assays. Similar results were obtained. Please see the following figure (which is added as Supple Figure 10). However, we didn’t perform these experiments in HepG2 cell lines as they express very low levels of CADM1.
CADM1 knockdown in Hep11 promote migration and invasion.

(A) Western blot assay showed that CADM1 were downregulated in Hep11 cells transfected with the CADM1 siRNA. (B, C) Transwell migration (n=4) and invasion (n=4) assays showed that Hep11 cells transfected with the CADM1 siRNA had greater invasive and migratory potentials than the control (siRNA control). (B) is a
microscopic image of crystal violet staining; (C) shows the statistical results.

**Comment:** It is shown in figure 6C and 6D that miR1246 and CADM1 levels can stratify HCC stage 1 patients for disease free survival. However it is unclear whether these two parameters are exclusive from each other or can be used together on those samples. Please clarify.

**Response:** Our results showed that miR-1246 expression is negatively correlated to CADM1 expression (P=0.003). To examine whether these two parameters are exclusive from each other or can be used together to stratify HCC stage for disease free survival, we need to classify tumor samples into four groups: low miR-1246 high CADM1, low miR-1246 low CADM1, high miR-1246 low CADM1, high miR-1246 high CADM1 and analyze the correlation. However, only 38 patients were recruited in our study and 25 were in stage 1. If we divided these patients into the abovementioned four groups, the patient number is too small for us to get a statistically meaningful conclusion. So at the current stage, we don’t know whether miR-1246 and CADM1 are exclusive from each other or can be used together to stratify HCC stage for disease free survival.

**Minor revisions:**

**Comment:** In Dual Luciferase assay method section, it was stated that “an 66 bp fragment of CADM1 3’UTR containing the predicted site for miR-9 was synthesized”-please clarify.
Response: We are sorry for the mistake. We changed miR-9 to miR-1246.