Author's response to reviews

Title: Functional microRNA high throughput screening reveals miR-9 as a central regulator of liver oncogenesis by affecting the PPARA-CDH1 pathway

Authors:

Alexandra Drakaki (adrkaki@mednet.ucla.edu)
Maria Hatziapostolou (MHatziapostolou@mednet.ucla.edu)
Christos Polytarchou (CPolytarchou@mednet.ucla.edu)
Christina Vorvis (cvorvis@ucla.edu)
George A Poultsides (gpoultsides@stanford.edu)
John Souglakos (johnsougl@gmail.com)
Vassilis Georgoulias (georgouliasv@gmail.com)
Dimitrios Iliopoulos (diliopoulos@mednet.ucla.edu)

Version:3 Date:20 May 2015

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

We are re-submitting our manuscript entitled “Functional microRNA high throughput screening reveals miR-9 as a central regulator of liver oncogenesis by affecting the PPARA-CDH1 pathway” by Alexandra Drakaki et al. for consideration for publication in BMC Cancer. The revised version of our manuscript has addressed all the comments suggested by the reviewers.

Specifically, in the revised version of the manuscript, we have included pictures from both the invasion and colony formation assays that we performed in miR-9 overexpressing liver cancer cells. Furthermore, we have included in the methods section additional information requested by the reviewers related to microRNA and gene expression data normalization. In addition, we found out that miR-9 expression levels correlate with HCC tumor stage, suggesting the involvement of miR-9 in HCC tumor progression. Importantly, we have constructed mutant (area of miR-9 binding site) vectors harboring the 3’UTRs of PPARA and CDH1 genes and showed that miR-9 overexpression does not affect their luciferase activity, suggesting the direct interaction between miR-9 and PPARA and CDH1 3’UTRs. Finally, we found that miR-9 inhibition results in up-regulation of PPARA and CDH1 mRNA levels.

For all these reasons, we strongly believe that our manuscript will be found to be novel, of interest to the broad readership of BMC Cancer and that you will consider it for publication.

Sincerely,

Dimitrios Iliopoulos, Ph.D
Associate Professor of Medicine
Director, Center for Systems Biomedicine
David Geffen School of Medicine at UCLA
Response to Reviewer’s #1 Comments

Comment 1: Background: In general this section is poorly descriptive. The authors should highlight the most important studies on the field of microRNAs and HCC, instead of a brief description as stated in sentence 70-71 in page 3.
Response: We would like to thank the reviewer for this comment. In the revised version of our manuscript, we have included in the background section (2nd paragraph) several key studies related to the role of microRNAs in hepatocellular oncogenesis and included all these studies in the references. Specifically, we are discussing the role of microRNAs in HCC properties and mechanisms, such as cell cycle, cell proliferation and metastasis and also their role as blood biomarkers in HCC.

Comment 2: Methods: The authors should mention the method used to analyze real-time PCR expression data.
Response: We would like to thank the reviewer for this comment; this is important information that was missing from our initial manuscript. In the revised version of our manuscript, we have included this information in the methods section (real-time PCR analysis) and in also in the related figure legends. Specifically, microRNA expression levels were normalized to the levels of U6 small nuclear snRNA. The normalized microRNA levels were quantified relative to the levels of a given control tissue. Gene expression levels were normalized to the levels of Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. Normalized gene expression levels were quantified to the respective control.

Comment 3: Results: In the introductory paragraph of this section, the authors have mentioned that the microRNA screening was performed in human hepatocytes. The authors should mention the origin of these human hepatocytes.
Response: We apologize that we have not mentioned the origin of the hepatocytes that we performed the microRNA screening. In the revised version of our manuscript, we have included this information and mentioned that we performed the screen in SNU-449 liver cancer cells. This is also shown in Figure 1B.

Comment 4: Results: The authors should show pictures in Fig. 1 (cells stained with crystal violet) showing high invasion levels when cells were transfected with miR-9 in comparison with negative controls.
Response: As suggested by the reviewer, in the revised version of our manuscript we have included pictures from the invasion assays. Specifically in Figure 1D, we are showing pictures of cells stained with crystal violet. These images show that cells transfected with miR-9 have higher invasiveness relative to cells transfected with microRNA negative control, verifying our hypothesis that miR-9 overexpression increases the invasive phenotype of liver cancer cells.

Comment 5: Results: The authors quantified the expression levels of miR-9, miR-224 and miR-21 in HCC tumors and control tissues. As abovementioned, the authors should mention what was the method used for these analyses and refer in the y-axis of graphs (figure 2) if miR-9 expression were simply normalized to the housekeeping or were quantified relative to the expression levels of control samples.
Response: As suggested by the reviewer, we have included in the methods and figure legend, information regarding the normalization of microRNA expression levels and altered the y-axis of graphs in Fig. 2 accordingly. Specifically, microRNA expression levels were normalized to the levels of U6 small nuclear snRNA. The normalized microRNA levels were quantified relative to the levels of a given control tissue.
Comment 6: Results: It would be nice if the authors correlate the expression of these microRNAs with some clinicopathological characteristics of HCC patients and tumors in order to better understand the clinical importance of miR-9, miR-224 and miR-21 in HCC.

Response: We found that there was a statistically significant correlation between miR-9 levels and HCC tumor stage (data shown in Figure 2D), suggesting that miR-9 expression correlates with HCC progression. Regarding miR-21, we found that it was expressed higher in late stages (II, III, IV) relative to stage I tumors. Also, we did not found any statistical significant between miR-224 expression levels and HCC tumor stage. The data related to miR-21 and miR-224 are shown in Suppl. Figure 1. Taken together, these data suggest that miR-9 is the microRNA more relevant to human disease.

Comment 7: Results: The authors should quantify the expression levels of miR-9 before and after transfection. It would be nice if the authors show some pictures to complement the graphs.

Response: As suggested by the reviewer, we have evaluated miR-9 expression levels before and after transfection in both SNU-449 and HepG2 cells. MiR-9 was found to be 6-fold up-regulated in SNU-449 after its transfection (Figure 3A) and was found to be 4.3-fold up-regulated in HepG2 cells (Suppl. Figure 2A).

In addition to the pictures that we included in Figure 1D, we have added pictures with stained cells from the invasion assay in Figure 3C. Furthermore, in Figure 3D, we have included pictures from the soft agar colony assay in SNU-449 cells transfected with microRNA control (miR-Ctrl) and miR-9 (Figure 3D). Also, we have included pictures from the invasion assay in SNU-449 cells treated with siRNA negative control or siRNA against PPARA (Figure 5E) and also treated with antisense-microRNA negative control or antisense-miR-9 (Figure 5B).

Comment 8: Results: To demonstrate that PARA and CDH1 are direct downstream targets of miR-9, the authors should mutate the binding sites in the 3'UTR of PPARA and CDH1.

Response: This is an important point raised by the reviewer and in the revised version of our manuscript we have included data related to these luciferase assays. Specifically, we have mutated the binding sites for miR-9 in the 3'UTRs of PPARA and CDH1 and performed luciferase assays in the presence of microRNA-9. Our data revealed that the mutant constructs have resulted in loss of the suppression of the luciferase activity in both cases (Figure 4B), suggesting the direct interactions between miR-9 and the 3'UTRs of PPARA and CDH1.

Comment 9: Results: Figures 4C,D,E,F,G – the authors should mention if these values were relative or normalized expression levels.

Response: As suggested from the reviewer, in the revised version of our manuscript, we have included the information that gene expression levels were relative to controls.

Comment 10: Results: To demonstrate that inhibition of PPARA and consequent reduction of CDH1 expression is not cell line dependent, the authors should perform these assays in another cell line (e.g HepG2).

Response: As suggested by the reviewer, we have evaluated CDH1 mRNA levels in HepG2 cells after inhibition of PPARA by using an siRNA. This experiment revealed that PPARA inhibition results in reduction of CDH1 mRNA expression (Figure 4G), proposing that these findings are not cell line dependent.
Minor comment 1: To conclude that PPARA and CDH1 are or not direct targets of miR-9 luciferase assays with mutant binding sites must be performed.
Response: We have performed this experiment (described in comment 8), as suggested by the reviewer (Figure 4B).

Minor comment 2: To conclude that miR-9 controls CDH1 expression directly through binding of its 3’UTR and indirectly by controlling PPARA expression, the authors should inhibit CDH1 and check if PPARA expression levels were not affected.
Response: We have performed the experiment suggested by the reviewer and specifically, we inhibited CDH1 expression by an siRNA against CDH1 and evaluated PPARA mRNA expression levels 48h post transfection. Our data showed that inhibition of CDH1 does not affect PPARA mRNA expression levels (Suppl. Figure 3). Thus, there is not a bi-directional interaction between PPARA and CDH1, but only PPARA inhibition is able to affect CDH1 levels.

Minor comment 3: It would be nice if the authors demonstrated that upon inhibition of miR-9, the expression levels of CDH1 and PPARA were recovered.
Response: As suggested by the reviewer, we evaluated CDH1 and PPARA mRNA expression levels in liver cancer cells transfected with anti-miR-9. We found that miR-9 inhibition resulted in up-regulated of CDH1 and PPARA mRNA levels (Figure 4C), suggesting that miR-9 regulates their expression.

Minor comment 4: Discussion: In this section, the authors mentioned that high miR-9 expression levels were previously correlated with poor prognosis of HCC patients (reference 28). Concerning the current study did the expression levels of miR-9 associate with clinicopathological variables?
Response: As we have discussed above (comment 6), we have found that miR-9 expression levels positively correlate with HCC tumor stage (Figure 2D). These findings were consistent with the included reference.

Minor comment 5: It would be nice if the authors refer and comment their results in relation to a previous publication by Hao-Xiang et al. “MicroRNA-9 reduce cell invasion and E-cadherin secretion in SK-Hep-1 cell”, Med Oncol. 2010 Sep;27(3):654-60.
Response: We have included (reference 37) and discussed this reference that was suggested by the reviewer (discussion section).

Response to Reviewer’s #2 Comments

Reviewer #2 did not have any comment and did not request further revisions.