Reviewer's report

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

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Reviewer: Joana Carvalho

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This manuscript by Drakaki et al., pinpointed a novel microRNA signalling pathway, miR-9/PPARA/CDH1 in Hepatocellular carcinoma (HCC). By integrating highthroughput microRNA screening analysis and functional assays, the authors found that miR-9 was upregulated in HCC in comparison with control liver tissues and induces cancer cell growth and invasion by regulating CDH1 and PPARA target genes.

Although several studies have been reported the involvement of microRNAs in HCC, including miR-9, its role and target genes have been poorly described. From that point of view, alone, this manuscript is interesting. However, after having carefully reviewed the manuscript and figures, I feel that several points should be clarified.

The authors should considering the following:

Major compulsory Revisions

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 page 3.

Methods

The authors should mention the method used to analyse real-time PCR expression data.

Results

In the introductory paragraph of this section, the authors have mentioned that a microRNA screening was performed in human hepatocytes. The authors should mention the origin of these human hepatocytes.

Identification of microRNAs regulating HCC invasiveness by performing a human microRNAome library screen in liver cancer cells

To complement Figure 1B and 1C, it would be nice to see some pictures (cells stained with crystal violet) showing high invasion levels when cells were transfected with miR-9 in comparison with negative controls.

Expression levels of microRNA, acting as invasion inducers, in HCC patient
tissues
The authors quantified the expression levels of miR-9, miR-224 and miR-21 in HCC tumours 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 levels were simply normalized to the housekeeping or were quantified relative to the expression levels of control samples.

Besides these analyses, it would be nice if the authors correlate the expression levels of those microRNAs with some clinicopathological characteristics of HCC patients and tumours in order to better understand the clinical importance of miR-9, miR-224 and miR-21 in HCC.

MiR-9 is an inducer of HCC cancer cell properties
It would be nice if the authors show some pictures (invasion, colonies in soft agar and spheres) to further complement the graphs. In addition, the authors should quantify the expression levels of miR-9 before and after transfection.

PPARA and E-cadherin (CDH1) as direct downstream targets of miR-9 in HCC
To demonstrate that, in fact, PPARA and CDH1 are direct targets of miR-9, the authors should mutate the binding sites in the 3’UTR of PPARA and CDH1 and see that, after overexpression of miR-9, the luciferase activity was recovered. Otherwise, miR-9 can lead to downregulation of CDH1 and PPARA but it does not mean that those genes are direct targets of miR-9.

Figure 4C, D, E, F and G – the authors should mention if these values were relative or normalized expression levels.

To demonstrate that inhibition of PPARA and consequent reduction of CDH1 expression is not cell line dependent, the authors should perform these assays in other cell line e.g. HepG2.

The authors should consider the following:
1- To conclude that PPARA and CDH1 are or not direct targets of miR-9, luciferase assays with mutant binding sites must be performed.
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 (in both cell lines).

Suppression of the miR-9 signalling on HCC cell properties
It would be nice if the authors demonstrated that upon inhibition of miR-9, the expression levels of CDH1 and PPARA were recovered.

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
some of the clinicopathological variables and prognosis of HCC patients?
Did the authors know which mechanism or mechanisms are underlying miR-9 overexpression in HCC?


Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

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