Author's response to reviews

Title: Upregulation of MicroRNA-19b Predicts Good Prognosis in Patients with Hepatocellular Carcinoma Presenting with Vascular Invasion or Multifocal Diseases

Authors:

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Author's response to reviews: see over
Dear Dr. Cherry Battad, academic editor of BMC Cancer and reviewers:

This is a revision and resubmission of our manuscript. We have made some major modifications in response to the comments and suggestions of the reviewers. Changes in the revised manuscript are highlighted with yellow color and the response to reviewers file will be uploaded with revised manuscript files.

We are grateful for the opportunity to revise our work MS: 5180553861641647 entitled "Upregulation of MicroRNA-19b Predicts Good Prognosis in Patients with Hepatocellular Carcinoma Presenting with Vascular Invasion or Multifocal Diseases" and thank the academic editor and reviewers for the time and effort they took to critique our work and offer suggestions for improving it. We hope that you now find our manuscript suitable for publication in the BMC Cancer.

We look forward to your reply.

Sincerely yours,

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Corresponding Author

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Editor comments:

1. The study would gain considerably if the levels of the miR-19b target genes are also determined in the patient material. Please supplement, if possible.

Reply: Thanks for your comments. We chose 20 HCC samples from our cohort and determined the expression levels of miR-19b target genes as well as analyzed the correlation between these target genes with miR-19b. The results were supplement in supplementary figure 1 and supplementary table 5. We found a trend toward negative correlation between miR-19b with MAPK14 and HIF1A, 2 of the 5 putative target genes in our cell line microarray. Small sample number may result in the statistical insignificance. We had revised the manuscript based on your advice.

Through direct or indirect regulation, miR-19b may suppress the function of EPCAM, MAPK14, HIF1A, as well as HMGB2, and promote the effect of NDRG1, thus suppressing HCC recurrence after curative surgery. In human tumor samples, we also revealed that there was a trend toward negative correlation between the expression of miR-19b with MAPK14 and HIF1A, as shown in supplementary table 5 and supplementary figure 1 and 2. Such findings also supported that miR-19b targeted MAPK14 and HIF1A in vivo.

**Supplementary Table 5** Correlation between the expressions of putative target genes and miR-19b in 20 tumor samples.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Pearson's correlation analysis</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDRG1</td>
<td>-0.253</td>
<td>0.282</td>
</tr>
<tr>
<td>EPCAM</td>
<td>0.232</td>
<td>0.325</td>
</tr>
<tr>
<td>HIF1A</td>
<td>-0.219</td>
<td>0.352</td>
</tr>
<tr>
<td>HMGB2</td>
<td>0.064</td>
<td>0.788</td>
</tr>
<tr>
<td>MAPK14</td>
<td>-0.229</td>
<td>0.332</td>
</tr>
</tbody>
</table>
Additional Editorial Request:  □ □

Requesting deposition of data.

Reply: Thank you for comments. We had deposited our microarray data in Gene Expression Omnibus. The miRNA microarray was accessible through GEO Series accession number GSE69580; the cell line mRNA microarray was accessible through GEO Series accession number GSE69519.

Reviewer: Albrecht Piiper

Essential:

1. Does miR-19b add additional prognostic information to the established prognostic scores?

Reply: Thanks for your comments. Currently, there are several staging systems that provide power in predicting the prognosis of hepatocellular carcinoma. Among them, BCLC staging system is one of the most wildly used and validated systems. In our study, we included patients in either BCLC B (patients with no vascular invasion) or C (tumor with vascular invasion). Patients in early stage, such as BCLC stage 0 or A, were not included. In multivariate analysis, we revealed that higher expression of miR-19b significantly correlated with better disease-free survival and overall survival after curative surgery. In other words, after adjusting the effect of BCLC staging, miR-19b expression can still offer prognostic effect in patients receiving curative surgery. We also separately analyzed survival in BCLC stage B and C. Nineteen patients belonged to BCLC stage B, and 62 patients belonged to BCLC stage C. In BCLC stage C cohort, higher miR-19b showed a trend in correlation with better DFS and OS (Log-rank test, P = 0.075 and 0.052, respectively). Small patient number may affect the results. Therefore, we proposed that miR-19b expression level may add additional prognostic information to
BCLC stage and thus help in deciding whether to receive surgery or not in patients with BCLC stage B or C. We had revised the manuscript based on your suggestion. The revised contents was highlighted in yellow color.

(Original manuscript, Page 7, Line 4)

We retrospectively investigated 81 patients diagnosed with HCC and HBV who were either Barcelona Clinic Liver Cancer (BCLC) stage B or stage C without extrahepatic metastases and received curative surgery between June 2007 and October 2013 at National Cheng Kung University Hospital. For each case, the diagnosis, histologic grade, and presence of liver cirrhosis were confirmed by pathologists.

(Original manuscript, Page 16 Line 10)

In further analysis, we showed that in patients with HBV-associated HCC presenting with multiple tumors or vascular invasion, a high expression level of miR-19b predicted better disease-free survival after curative surgery when compared with those with a low expression level. High expression of miR-19b also predicted better disease-free survival and overall survival in the Cox multivariate analysis. Interestingly, one of the variables in multivariate analysis is the vascular invasion, as shown in table 2. Since we included only patients with HCC of BCLC stage B or C without exhepatic metastases, presence of vascular invasion represented stage C disease, whereas absence of vascular invasion was equivalent to stage B. The results of multivariate analysis indicated that miR-19b expression level correlated with both DFS and OS independent of patients’ BCLC stage. Based on these results, miR-19b may be a useful marker for clinical decision-making, in terms of whether or not to perform surgery, or the frequency of follow-up after surgery.

2. Are there correlations between the levels of miR-19b and the putative target mRNAs in tumor material?

Reply: Thanks for your comments. We chose 20 patients from our cohort and analyzed the expression of the putative target genes listed in table 3. The results were added in the supplement data as supplementary table 5 and supplementary figure 1. We found that there were a trend toward negative correlation between the expression of HIF1A with miR-19b (Pearson's correlation, \( r = -0.219, P = 0.352 \)) and MAPK14 (Pearson's correlation, \( r = -0.229, P = 0.332 \)). It supported the finding in our cell line microarray, that HIF1A and MAPK14 were putative targets of miR-19b. The statistical insignificance may result from smaller patient number. We had revised the manuscript based on your advice.

(Original manuscript, Page 18, Line 2)

Through direct or indirect regulation, miR-19b may suppress the function of EPCAM, MAPK14, HIF1A, as well as HMGB2, and promote the effect of NDRG1, thus suppressing HCC recurrence after curative surgery. In human tumor samples, we also revealed that there was a trend toward negative correlation between the expression of miR-19b with MAPK14 and HIF1A, as shown in supplementary table 5 and
supplementary figure 1 and 2. Such findings also supported that miR-19b targeted MAPK14 and HIF1A in vivo.

**Supplementary Table 5** Correlation between the expressions of putative target genes and miR-19b in 20 tumor samples.

<table>
<thead>
<tr>
<th>Gene</th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDRG1</td>
<td>-0.253</td>
<td>0.282</td>
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<td>0.064</td>
<td>0.788</td>
</tr>
<tr>
<td>MAPK14</td>
<td>-0.229</td>
<td>0.332</td>
</tr>
</tbody>
</table>

3. The references should cite the first valid description, e.g. of prognostic miRNAs in HCC. As I see it, this is not the case.

**Reply:** Thanks for your comments. We had revised the references.

4. The source of the cell line is not provided.

**Reply:** Thanks for your comments. The cell line, Hep 3B, was obtained from American Type Culture Collection (ATCC®, Manassas, VA). We had revised the manuscript based on your suggestion, as shown in yellow highlight.

(Original manuscript, Page 11, line 9)

Cell Line Culture
Human HCC cell lines Hep 3B were obtained from American Type Culture Collection (ATCC®, Manassas, VA) and had been validated in 2014, and were cultured in MEM
medium (Invitrogen, Carlsbad, CA) plus 10% newly born calf serum.

5. Did the authors use a control-anti-miR in the transfection experiments?

Reply: Thanks for your comments. In the transfection experiments, we transfected either miR-19b inhibitor or its control into Hep3B. After transfection, the differential mRNA expression profile between Hep3B transfected with miR-19b inhibitor or its control were evaluated using mRNA microarray. The results were shown in supplementary table 2 and 3. We had revised the manuscript based on your suggestion, as shown in yellow highlight.

(Original manuscript, Page 11, line 12)

Transfection □ A quantity of approximately $2 \times 10^5$ Hep 3B cells were seeded and cultured in □ 6-well plates. For each well, 90 pmol of miR-19b inhibitor or control were added to 300 µL □ Opti-MEM medium and 10 µL of Lipofectamine® 2000 (all Applied Biosystems®). □ The mixture was added to the cells and incubated for 6 hours before replacing the □ medium. Cells were collected for RNA extraction 24 hours after transfection. □

6. The criteria applied in the selection of the tumor/normal liver pairs are not provided.

Reply: Thanks for your comments. HCC tissues were collected from surgical resected samples presenting with tumorous features macroscopically. Adjacent non-tumor tissues were collected > 2cm away from the edge of the tumors. In all patients, the diagnosis of HCC was confirmed histologically by pathologists. We had included the criteria in the method and revised the manuscript following your advise.

(Original manuscript, Page 7, line 8)

Snap-fresh HCC tissues and paired adjacent non-tumorous liver tissues were obtained from each patient during surgery. Tissues were stored in liquid nitrogen after surgical resection until use. HCC tissues were collected from surgical resected samples presenting with tumorous features macroscopically. Adjacent non-tumor tissues were collected > 2cm away from the edge of the tumors.

Reviewer: Binkui Li

- Major Compulsory Revisions

1. Although the authors emphasized that their study objects were HCC with multifocal diseases or vascular invasion, they did not describe the details of the patients (5 patients in microarray and 81 patients in qPCR) in the study, data showed in Table 1 were not clear enough to clarify. For example, how many patients were multifocal diseases with or without vascular invasion, and how many patients were diseases with vascular invasion and single tumor or multiple tumors?
**Reply:** Thanks for your comments. In our cohort, there were 48 patients presenting with solitary tumor and vascular invasion; 14 patients were multiple tumors with vascular invasion; 10 patients with solitary tumor and no vascular invasion; and 9 patients with multiple tumors and no vascular invasion. We had added the clinical information in supplementary table 1.

**Supplementary Table 1** Demographics of selected patients

<table>
<thead>
<tr>
<th>Vascular invasion</th>
<th>Solitary</th>
<th>Multiple</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence</td>
<td>48</td>
<td>14</td>
<td>62</td>
</tr>
<tr>
<td>Absence</td>
<td>10</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>23</td>
<td>81</td>
</tr>
</tbody>
</table>

2. The authors concluded miR-19b was overexpressed in HCC and high miR-19b level was significantly correlated with better disease-free and overall survival in patients with HCC presenting with vascular invasion or multifocal diseases after curative surgery. However, this conclusion was not so rigorous, because the authors could not rule out the possibility of that miR-19b can also predict prognosis in early stage HCC patients. A better way to discover prognostic miRNA marker for HCC presenting with vascular invasion or multifocal diseases should be by comparing the difference of miRNA expression profile between patients with good prognosis and those with poor prognosis.

**Reply:** Thanks for your comment. We sincerely appreciated your comment and it will be very helpful to our future studies on the prognosis of more advanced HCC. In the present study, we investigated the miRNA deregulation in patients with HCC. Therefore, we chose miRNA microarray comparing tumor and non-tumor liver tissue as our initial approach. It was an interesting finding that miR-19b correlated with better survival in patients with more advance HCC after surgery. We had further analyzed another cohort, which included only patients with BCLC stage A who received curative surgery. The patient number was 42. Using miR-19b median expression level as a cut-off value, we divided patients into miR-19b high-expression group and miR-19b low expression. Log-rank test was used to analyze disease-free survival and overall survival. However, we found that miR-19b expression level did not correlate with either DFS or overall survival in BCLC stage A patients (Log-rank test, \( p = 0.285 \) and \( 0.841 \), respectively). Therefore, we believed that miR-19b had prognostic value only in patients with more advanced HCC, but not early HCC.

3. Generally, a miRNA overexpressed in cancer is an oncogene and often predicts poor prognosis. However, high miR-19b level predicted good prognosis in this study. The authors should explain the possible reasons in discussion and also list some other similar reports to support their findings.
Reply: Thanks for the comment. We had revised the manuscript following your advice. The revised content was highlighted in yellow color.

(Original manuscript, Page 17, Line 10)

Although it is a general concept that genes overexpressed in tumor may be oncogenic, there are exceptions. Huynh. H, et al had demonstrated that Retinoblastoma 2 protein (pRb/130), a tumor suppressor gene that is commonly down-regulated in cancer, was overexpressed in HCC[33]. In this study, the author showed that pRb/130 was elevated in majority of HCC samples, but still functioned as a tumor suppressor. Another examples is p16^{ink4a}, a tumor suppressor but was found to be overexpressed in Human papilloma virus (HPV)-related cancer[34, 35]. The overexpression of p16^{ink4a} was correlated with better treatment response and prognosis[36-38]. Similar to pRb/130 and p16^{ink4a}, we showed that miR-19b was overexpressed in HCC compared with non-tumorous liver tissue, and higher level of miR-19b was correlated with better survival after curative surgery. Overexpression of miR-19b might be an attempt to stop cell proliferation. MiR-19b might slow down cancer progression, and therefore, its overexpression was correlated with better survival. However, detailed mechanisms will have to be revealed and validated in future studies.

4. In many previous reports, high AFP level was associated with poor prognosis in HCC. In the present study, the authors also found that AFP correlated with worse disease-free survival. However, they found high level of miR-19b expression was correlated with an elevated serum AFP level, and high level of miR-19b expression predicted good prognosis. It seems that these data were in many ways self-contradictory.

Reply: Thanks for your comments. In order to further analyze the association between AFP level and miR-19b expression, we first compared the AFP level between patients with high and low miR-19b expression. There was no statistical difference (Student t-test, p=0.408). Next, we evaluated the correlation between AFP level and miR-19b expression level, and there was not significant correlation (Pearson correlation coefficient, r = -0.032, p = 0.778). Based on these results, we believed that there may not be an association between the expression level of miR-19b and AFP level, despite the result of Chi square test. We had revised the results and discussion of our manuscript based on your advice.

(Original manuscript, Page 13, line 18)

The results revealed that a high level of miR-19b expression was correlated with an elevated serum α-fetoprotein (AFP) level (P = 0.017). However, there were no significant correlations of miR-19b expression with other clinical features such as gender, age, vascular invasion, TNM stage, liver cirrhosis, tumor differentiations and tumor numbers (all P > 0.05). The serum AFP level of miR-19b low-expression group and high-expression group was compared. There was no significant difference (Student's t-test, P = 0.408). There was no correlation between miR-19b expression level and serum...
AFP level (*Pearson* correlation coefficient, $r = -0.032, p = 0.778$).

(Original manuscript, Page 16, line 18)

AFP was previously reported to be a prognostic factor of HCC [28]. In the present study, we found that AFP correlated with worse disease-free survival in the multivariate analysis. This finding was consistent with previous reports. However, AFP did not predict overall survival in the present study. Compared with AFP, miR-19b may be more powerful as a prognostic factor. In Table 1, more patients in high-expression of miR-19b had elevated serum AFP. However, when we compared the AFP serum level of patients with high-expression miR-19b with their counterpart, there was no significant difference. Besides, there was no significant correlation between miR-19b expression level and serum AFP level. Therefore, it was unlikely that there was an association between miR-19b expression and serum AFP level.

- **Minor Essential Revisions**

The part “Potential Targets of miR-19b” seems not necessary for this study.

**Reply**: Thanks for your comments. We further tested the expression level of the potential targets in 20 resected HCC. Among the 5 potential targets revealed by our cell line microarray, we found that there were trends toward negative correlation between the expression level of miR-19b with HIF1A and MAPK14 (*Pearson’s* correlation, $r = -0.219$ and -0.229; $P = 0.352$ and 0.332, respectively). The insignificant $P$ value may result from small sample number. Such finding implied that MAPK14 and HIF1A might be the potential targets of miR-19b. MAPK14 and HIF1A are known oncogenes that promote carcinogenesis. These findings further supported our conclusion that high expression of miR-19b predicts better survival.

**Supplementary Figure 1**

**Supplementary Figure 2**