Author's response to reviews

Title: Identification of a 3-microRNA signature associated with prognosis of patients with intrahepatic cholangiocarcinoma

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Version: 9
Date: 4 December 2014

Author's response to reviews: see over
Rebuttal Letter

Reviewer: Laura Gramantieri

Reviewer's report:

The aim of the study is to identify miRNAs signatures associated either with diagnosis and prognosis of ICC. Indeed, a 30 miRNAs signature is identified as a diagnostic tool, while a 3-miRNAs signature is associated with prognosis in patients undergoing surgery for ICC.

The study design is not original and no mechanistic insights are provided. In addition previous studies performed by using the same approach and on the same malignancy were published, reporting miRNA signatures different from those reported here. My major compulsory revisions are the following:

Comment 1:

The choice of normal intrahepatic bile ducts as a control tissue when trying to identify a signature with a diagnostic purpose should be argumented. I suppose that a diagnostic aid might be sought in the case of nodular liver lesions difficult to be characterised with the traditional histopathological tools. Thus I think of poorly differentiated HCCs or metastatic liver tumors, etc. Conversely, we don’t need to know which miRNAs differentiate ICC from normal bile ducts, because the differential diagnosis between these two entities does not represent a clinical need in which molecular tools are likely to be helpful. Different is the case of signatures identified with the aim of unraveling the transcriptomic aberrations and molecular mechanisms associated with the development of ICC.

Response to comment 1:

Thanks for your comments! We agree that our manuscript is not the first report on miRNA profiling of ICC. However, there are only three miRNA profiling studies on ICC until now as we state in Background section, and most importantly, the 3 studies only employed 27, 23 and 15 ICC samples, respectively. In contrast, there are 63 ICC
samples in our study, two times more than the previous studies. Thus, the result from our study might be more reliable and robust than that from other studies.

As to the 30-miRNA signature, we agree with your opinions: This signature for distinguishing ICC from NIBD will not help in clinical diagnosis of ICC. Actually, the main purpose of identification of this miRNA signature is to discover the miRNAs that may play important roles in ICC development and progression, which will provide evidences for further study on molecular mechanism. Therefore, we have removed the word “diagnosis” from the title and other paragraphs of this manuscript and made corresponding changes (see line 3, 79, 99, 104, 144, 252-254, 367-369 on the paper with track change).

**Comment 2:**
The study was performed in patients resected for ICC and a miRNA signature associated with prognosis is identified. I wonder whether patients received any treatment following surgery. Also, I wonder if cirrhotic patients were treated in the same way and with the same schedules as non cirrhotic patients. It would be surprising to know that either patients did not receive any adjuvant treatment or they received the same treatments irrespective of the presence or absence of cirrhosis. Were all the death cancer-related? In cirrhotic patients also? This last question derives from the observation that in table 1 AFP increase is one factor associated with overall survival.

**Response to comment 2:**
Thanks for your comments! I am sorry for not accounting for the treatment following surgery in our previous manuscript. In general, the patients who received hepatectomy would not be given any other therapies after surgery. If patients had HBV infection, serum SALT elevation (>40 U/L) and serum positive for HBsAg, HBeAg and HBV DNA, they would undergo antiviral therapy.

The liver function of all the patients in our study were Child A classification before surgery. There were 20 cirrhotic patients, all of who were mild cirrhosis.
Therefore, we determined the treatment plan according to the tumor situation (such as location, size) and the patient’s general condition but not the presence or absence of cirrhosis before the surgery. All the patients were given the regular liver protection treatment after hepatectomy.

All the death were cancer-related, including that in cirrhotic patients. With univariate Cox regression analysis, AFP concentration was found to be associated with overall survival because all of 4 patients with higher AFP concentration died within 18 months after surgery. Kaplan-Meier curve analysis also shows that patients with low-level AFP have much better survival than those with high-level AFP (P <0.05). However, we found that high-level AFP was correlated with overall survival in Univariate Cox regression analysis, we speculate that this significant correlation might be caused by the statistical bias because of the limited sample size with high-level AFP (only 4 patients). We added the explanation in the revised manuscript (see line 309-312 on the manuscript with track change).
**Overall Comparisons**

<table>
<thead>
<tr>
<th></th>
<th>Chi-Square</th>
<th>df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Rank (Mantel-Cox)</td>
<td>4.453</td>
<td>1</td>
<td>.035</td>
</tr>
</tbody>
</table>

Test of equality of survival distributions for the different levels of afp.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Response to quality of written English:**
Thanks for your comment! Now we have very carefully proofread this manuscript.
Review: Kwang-Huei Lin

Reviewer's report:

MicroRNA signatures associated with diagnosis and prognosis of patients with intrahepatic cholangiocarcinoma

Detailed comments to the author

The manuscript by Zhang et al. showed that a 30-miRNA signature and a 3-miRNA signature was established, which might be potential biomarkers for diagnosis and prognosis of ICC. Further, a 30-miRNA signature consisting of 10 up-regulated and 20 down-regulated miRNAs in ICC was established for distinguishing ICC from NIBD with 100% accuracy. Another 3-miRNA signature was identified for predicting prognosis in ICC and associated with high-risk overall survival and disease-free survival than those with low-risk. Further studies focusing on these miRNAs may shed light on the mechanisms associated with ICC pathogenesis and progression. Overall, this manuscript is not sufficiently to address 3-miRNA signature identified for predicting prognosis in ICC. Some suggestions for this paper are as follows:

Major Compulsory Revisions:

Comment 1:

The diagnostic accuracy of a test is the first thing we concern. Sensitivity and specificity (ROC curve) are the most widely used to indicate the accuracy of the markers. The authors should perform the ROC analysis for individual miRNA as well as various combinations. The authors are suggested to perfume ROC analysis compared with known markers or published miRNAs. Also, why authors selected those three miRNAs but not miRNA-566, -423-5p or -143-3p, -451a? Although the authors studied on 30-miRNA signature the diagnosis role in ICC, there is no strong evidence to indicate that. The author should provide more experimental evidence to
show the 30-miRNA signature affect diagnosis.

**Response to comment 1:**

Yes, we agree that ROC is usually used to determine the efficiency of a biomarker. After screened miRNAs with univariate Cox regression analysis, we found that only 3 miRNAs (miR-675-5p, miR-652-3p and miR-338-3p) were associated with survival (all $P < 0.05$) of ICC in this study. Therefore, we only could use these 3 miRNAs for further analysis. According to your comments, we performed ROC analysis on single miRNAs and different combinations of the 3 miRNAs. The result showed that the performance of 3-miRNA signature is the best in comparison with any single miRNAs or other combinations (see below ROC analysis). If other miRNAs that were not associated with survival or that were reported to be correlated with prognosis in other studies but not in our study, are constructed into a new miRNA signature, the new signature definitely will has a low predictive power for survival or completely lose the power. Therefore, other miRNAs cannot be used to construct a prognostic signature in this study. Pertaining to the 30-miRNA signature, as the other reviewer pointed out, this 30-miRNA signature actually would not help in clinical diagnosis of ICC. Thus, we have removed “diagnosis” from the statements for the 30-miRNA signature.
<table>
<thead>
<tr>
<th>Test Result Variable(s): miRNA</th>
<th>Area</th>
<th>Std. Error</th>
<th>Asymptotic Sig.</th>
<th>Asymptotic 95% Confidence Interval</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td>Lower Bound</td>
</tr>
<tr>
<td>miR-675-5p</td>
<td>0.686</td>
<td>0.074</td>
<td>0.021</td>
<td>0.541</td>
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<tr>
<td>miR-652-3p</td>
<td>0.622</td>
<td>0.078</td>
<td>0.13</td>
<td>0.469</td>
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<tr>
<td>miR-338-3p</td>
<td>0.622</td>
<td>0.078</td>
<td>0.13</td>
<td>0.469</td>
</tr>
<tr>
<td>2-miRNA</td>
<td>0.686</td>
<td>0.074</td>
<td>0.021</td>
<td>0.541</td>
</tr>
<tr>
<td>(miR-675-5p, miR-652-3p)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-miRNA</td>
<td>0.686</td>
<td>0.074</td>
<td>0.021</td>
<td>0.541</td>
</tr>
<tr>
<td>(miR-675-5p, miR-338-3p)</td>
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<tr>
<td>2-miRNA</td>
<td>0.587</td>
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<td>0.436</td>
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<td>(miR-652-3p, miR-338-3p)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>3-miRNA</td>
<td>0.747</td>
<td>0.066</td>
<td>0.002</td>
<td>0.618</td>
</tr>
<tr>
<td>(miR-675-5p, miR-652-3p, miR-338-3p)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Comment 2:**

Since 3-miRNAs (miR-675-5p, miR-652-3p and miR-338-3p) signature has been reported as a prognostic factor of other cancer type (eg. breast, gastric, liver, neuroblastoma, cervical and colon cancer), the authors should combine the published miRNAs (miRNA-204, 31, 376c, 124, 200...) for a panel of ICC signature to increase the specificity of ICC.

**Response to comment 2:**

We agree with you that some miRNAs of the 3 miRNAs have been reported in some other cancers. However, as an integrated signature, the 3-miRNA signature has not been reported in any other cancers. As the above mentioned, after screened the
miRNAs, the 3-miRNA combination turned out to be the best predictor for survival in patients with ICC in our study. Although other single miRNAs are associated with prognosis in other cancers, they are not correlated with survival in our study. If these miRNAs are used to build a new signature with the 3 miRNAs, as mentioned in Answer 1, the new signature definitely will lose the predictive power for survival in patients with ICC. Therefore, the other miRNAs cannot be combined with the 3-miRNA signature.

**Comment 3:**
It is interesting that 3-miRNAs (miR-675-5p, miR-652-3p and miR-338-3p) signature are so dramatically dysregulated in ICC as well as other cancers. Do the authors investigate which factors that may account for the down or over-expression of the 3-miRNAs signature?

**Response to comment 3:**
At this moment, we are conducting a further study on biological function and expression regulation of the important miRNA. Since there is a number of studies on miR-652-3p and miR-338-3p but no report on miR-675-5p, we are mainly focused on investigation of miR-675-5p.

**Minor Essential Revisions**

**Comment 4:**
Among 3-miRNAs (miR-675-5p, miR-652-3p and miR-338-3p) signature, however, miR-652-3p didn’t appear in 30-miRNA signature (Table 2). Could you also discuss this?

**Response to comment 4:**
To establish the signature for distinguishing ICC from NIBD, we selected the miRNAs with more than 2-fold expression change between ICC and NIBD. While screening miRNAs for identifying the prognostic signature, we included the miRNAs with more than 1.5-fold expression change. miR-652-3p expression change between
ICC and NIBD is 1.52-fold. Consequently, miR-652-3p is not presented in the 30-miRNA signature.

**Comment 5:**
In the Figure 2, the author's use patients were divided into a high-risk group and a low-risk group by the median signature risk score as the cut-off point. But the patient numbers vs OS (Figure 2A) and patient numbers vs DFS (Figure 2B) Plot are not corrected matching with number shown below each panel. The authors should explain and correct them.

**Response to comment 5:**
Sorry for the mistake that the patient number of the two groups in the paragraph of “Identification of 3 miRNA signature associated with survival in ICC” is not consistent with number in Figure 2! Now this error has been corrected in the paragraph. We divided patients by the median signature risk score as the cut-off point, and thus patient number in each group usually should be equal. However, there are 5 patients with same median risk score in the low-risk group, and consequently low-risk group has 34 patients and high-risk group has 29 patients. We have explained this in the manuscript (see line 271-273 on the manuscript with track change).

**Comment 6:**
There are several similar studies published. The authors should discuss more detail about the similarity or difference.

**Response to comment 6:**
We have searched publications for ICC in PubMed, and only can find 5 papers on miRNA profiles in ICC, two of which report on ICC cell lines. Thus, there are only 3 studies on miRNA expression profiles in ICC tissues. In the previous version of our manuscript, we discussed two of the 3 studies. One of the 3 studies was published on October of this year, which now has been added the discussion of the newest paper in
Discussion section (see line 131-133 and 346-351 on the manuscript with track change).

Comment 7:
The authors report 30-miRNAs dysregulated in ICC without further study. Whether those 30-miRNAs associated any diagnosis or prognosis value are still questionable.

Response to comment 7:
As we mentioned above, since modern image technology and traditional pathological tool have sufficient power to diagnose ICC, our 30-miRNA may not be necessary to be used in the clinical diagnosis of ICC, and actually the purpose of this study is not aimed at the diagnosis of ICC with miRNA signature. Therefore, we have removed the word “diagnosis” for 30-miRNA signature from this manuscript.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests: 'I declare that I have no competing interests'