Author's response to reviews

Title: A microRNA profile associated with Opisthorchis viverrini-induced cholangiocarcinoma in tissue and plasma.

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Author's response to reviews: see over
Dear Dr Budczies:

Thank you for efforts in regard to our manuscript, “A microRNA profile associated with Opisthorchis viverrini-induced cholangiocarcinoma in tissue and plasma” (MS: 2032896589150592). We also thank the reviewers for their valuable comments and would like to submit a revised manuscript for your consideration. We have provided a point-by-point response to the reviewers’ comments as follows and have uploaded the original manuscript marked with our changes in bold.

Reviewer Federica Ganci:

1. "no references were included on the previous works regarding the association of circulating miRNAs in cholangiocarcinoma"
   We have included a sentence describing previous works to the introduction (beginning Line 71 of the new manuscript) and cited the suggested paper along with one other describing a miRNA associated with Opisthorchis viverrini-induced CCA and a citation to a recent review of the topic. We have also included a table (Table 1 in the revised manuscript) with a comparison of our results to the literature.

2. "half (49%) of the 143 million reads obtained after the application of filters mapped to the human genome (line 120, pag.5); why only 49%?"
   The reviewer is correct that this section is not explained well. The low mapping figure is because mapping, as performed by mirDeep, was done after compression of the reads, i.e. removal of duplicates, which results in a single read for every miRNA plus all singletons. When all filtered reads are mapped to the human genome using typical Bowtie parameters (-n 3 -l 28) alignment values ranged between 82-97% with an average of 85%. This has been noted in the manuscript (beginning line 122 of the revised manuscript).

3. "In other words, were all 67 miRNAs included into the 316 ones? Do they share the same direction of deregulation?"
   All but 8 of the miRNAs disregulated in the CTT v. D-NT were also disregulated in the CTT v. N-NT sample and all of these had the same direction of deregulation. This is noted on line 139 of the revised manuscript.

4. "the authors analyzed only the deregulation of miRNAs in ICC versus D-NT according to the histological differentiation, but they did not include information on the same analysis using the other normal control (N-NT) which it is shown in figure 3. For instance, how many miRs are deregulated in ICC vs N-NT? And how many ones are common between the two subgroups?"
   See previous question, moreover we have added further text to the results (starting line 170) and discussion (starting line 290) sections discussing results in the context of the N-NT control. We have also provided additional data in Suppl. Table 1 providing specific miRNA expression levels for all comparisons discussed in the text.
   "In addition, maybe a mistake is present at line 163, pag 7 where the authors wrote “(N-NT)” instead to “D-NT”"
   We have corrected this typo, on line 166 of the revised manuscript.

5. "an addition of a table summarizing the results obtained from the use of the two different platforms is suggested in order to help the reader in the understanding of the paragraph. For instance, how many deregulated miRs are common between the two analyses considering the comparison respect the two controls?"
   We take the reviewers point and have included further comparisons between the NGS and microarray platforms. We have added further text to the Results (beginning Line 177) and Discussion (beginning line 309), provided a Venn diagram comparing significantly dysregulated miRNAs called using the two platforms (Suppl. Fig. 1A) and provided further data in Suppl. Table 1.
"Instead, the authors did not specify the control used in the supplementary fig.1. In addition"

We have amended this oversight in the caption to Suppl. Fig. 1 to provide the specific comparison used (CTT vs. D-NT).

"the authors did not find any miRs deregulated in well differentiated ICC versus D-NT, but in the cited previous work, they found 12 ones performing the same comparison; why this difference?"

As we now point out in the revised manuscript, despite the differences in the specific miRNAs called as significantly dysregulated, the FC values obtained for miRNAs using the two platforms are similar. This is likely due to differences in the statistical models employed or by the cross-hybridization of closely related miRNA species on the microarray. We have previously reported this in a study directly comparing NGS to microarray analysis (Plieskatt, J.L. et al. J Transl Med 12(1),3, 2014) and others have shown similar results (Git, A. et al RNA 16(5), 991–1006, 2010).

"authors could add additional references regarding the deregulated miRNAs identified in this work already known in literature (for instance miR-141 or miR-200b etc..) in order to support and to demonstrate the reproducibility and the importance of data obtained."

We have included a table (Table 1) summarising the literature on cholangiocarcinoma-associated miRNAs and text to the discussion (beginning line 325).

Reviewer Bin Teh:

1. "authors showed the distinct miRNA patterns which were associated with increasing histological differentiation. However, these distinct patterns have been previously found in Ov-related ICC by the same group (Plieskatt JL. et. al. (2014)). The only difference is the analysis platform (targeted miRNA vs small RNA-seq). To distinguish between the two studies, the authors really need to describe more and focus on the differences between the two findings e.g. what are the novel miRNAs discovered by small RNA-seq etc etc ?"

The reviewer is quite right that we re-analysed the same samples using a different platform from our previous study, but a major focus in this work was to extend the work to circulating miRNAs and determine which dysregulated miRNAs would be suitable for use as circulating markers of disease using qPCR. Moreover, in this new comparison FFPE samples were directly paired (same patient) to the plasma samples, allowing a direct comparison between tissue and circulating matrices. This is the first time that such a high-throughput approach has been used to identify circulating miRNA markers for cholangiocarcinoma. Nonetheless, we agree that the differences between the two platforms were not adequately described and we have expanded our analysis. This has been described in the Results (beginning Line 177) and Discussion (beginning line 309), a Venn diagram comparing significantly dysregulated miRNAs called using the two platforms has been provided (Suppl. Fig. 1A) as well as further supplementary data in Suppl. Table 1. Novel miRNAs are an important aspect of NGS and although some novel miRNAs were identified we are currently validating these potential novel miRNAs using PCR etc and such findings may be reported separately after the novel miRNAs are fully validated.

2. "As described in the discussion, comparing with the normal liver tissues which are mostly hepatocytes, may be the confounding factor in this study. Then, dysregulated miRNAs that were found are from two distinct cell types. The authors should carefully consider the interpretation. To clearly describe this point, the authors need to emphasize and specify the type of miRNAs that were dysregulated in ICC when compared with both D-NT and N-NT."

We certainly agree that control tissue is a problem for any study of cholangiocarcinoma. Accordingly, as per the reviewers suggestion, we have expanded our discussion of the difference between using D-NT and N-NT as controls. We have added text to the results (starting line 170) and discussion (starting line 290) sections discussing results in the context of the N-NT control. We have also provided additional data in Suppl. Table 1
providing specific miRNA expression levels for all comparisons discussed in the text. We have also provided a table comparing the miRNA findings in this paper to that in the literature (Table 1 in the revised manuscript) along with an explanation in the discussion (beginning line 325).

3. "The authors should ensure consistency in their use of abbreviation e.g. Ov or OV"

We have checked the manuscript and corrected all occurrences of OV to Ov.

4. "Revise the reference no. 9"

We have revised the reference as suggested by the reviewer (now reference 12).

5. "Authors should explain the symbol “???” as mentioned in Ref. no. 43"

This typo has now been removed (now reference 46).

6. "I don’t quite agree to use the term “for the diagnostic” in the title because the supporting data is not enough or did not clearly described."

As per the reviewers suggestion the title of the paper is now “A microRNA profile associated with Opisthorchis viverrini-induced cholangiocarcinoma in tissue and plasma.”

7. "The authors should try to correlate the dysregulated miRNA(s) with patient survival? This may support the association with histological grading."

We agree with the reviewer that associating the dysregulated miRNA(s) with patient survival would be beneficial and is a step towards our objective of prognostic and diagnostic biomarkers. However, given the limited sample number in the manuscript, we are unsure of its significance and feel it would be premature. We plan to defer this to later studies and manuscripts as we narrow our candidate signature and expand our sampling number. In these studies, the sampling point (diagnosis, treatment, recovery, etc) will be explored and followed for patients along with survival analyses based on these miRNA signatures and patient outcomes.

Once again, we thank the reviewers for their helpful comments and would appreciate your consideration of the revised manuscript.

Kind regards,

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