Author's response to reviews

Title: MiR-133b is frequently decreased in gastric cancer and its overexpression reduces the metastatic potential of gastric cancer cells

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Version: 3 Date: 23 August 2013

Author's response to reviews: see over
Dear Ms. Cherry Battad:

We resubmit our manuscript entitled "MiR-133b is frequently decreased in gastric cancer and its overexpression reduces the metastatic potential of gastric cancer cells" to BMC Cancer for publication.

The old manuscript ID: 1908295334100373.

In this report, we found that expression of miR-133b was significantly down-regulated in 70% (98/140) of the gastric cancer tissues compared with adjacent non-tumor tissues. Expression of miR-133b negatively correlated with lymph node metastasis of gastric cancer in patients. Similarly, the expression of miR-133b was much less in seven tested gastric cancer cell lines than in a immortalized gastric epithelial cell line GES-1. Overexpression of miR-133b significantly inhibits migration and invasion of gastric cancer cells in vitro. In a mouse gastric cancer model, overexpression of miR-133b suppressed peritoneal spreading of metastasis. Moreover, the transcriptional factor Gli1 was identified as a direct target for miR-133b. Subsequently, Gli1 target genes OPN and Zeb2 were also inhibited by miR133b. These results suggested that miR-133b plays an important role in gastric cancer metastasis.

The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have seen the manuscript and approved to submit to your journal.

The authors have declared that no competing interests exist.

Thank you very much for your attention and consideration.

Thank you for your kind review of our manuscript. According to your suggestions, the revised manuscript has been submitted. Details changes made in response to reviewers' comments are addressed point by point as follows:

Responses to Reviewers:
Reviewer: Gennaro Colella

Reviewer's report:

Minor Essential Revisions

1) Please carefully read the manuscripts and correct typing errors such as lack of space and/or double space between two words (e.g. in Background section at lane 14 where a space between “tissues” and “[6] must be introduced)”

Answer: We are sorry for having made those errors. We have corrected those typing errors.

2) Please carefully read the manuscript and replace “mimics” with “mimic” when you are talking about a single miRNA mimic (e.g. miR-133b mimic, NC mimic);

Answer: Changes have been made as indicated by the reviewer.

3) Please carefully read the manuscript and replace all “P<0.XXXX ” with “p<0.XXXX ” to uniform them;

Answer: Done.

4) In my opinion the TITLE is misleading because it seems that in gastric cancer the repression of cell metastasis is positively correlated to a lower expression ofmiR-133b. I think that a title like “Mir-133b is frequently decreased in gastric cancer and its overexpression reduces the metastatic potential of gastric cancer cells” sounds better;

Answer: Many thanks for the suggestion. The title of the article has been changed as the reviewer indicates.

5) “Background” section, lane 24: replace the phrase “…that miR-133b could repress…” with “…that miR-133b overexpression could repress…”;

Answer: Done.

6) “Methods/Cell lines and culture” section, lane 3: replace “MKN45 and MKN28” with “MKN-45 and MKN-28”;

Answer: Done.

7) “Methods/RNA Isolation and…” section, lane 1: add the “CA, ” after “Carlsbad,”;

Answer: Done.

8) “Methods/RNA Isolation and…” section, lane 4: replace “Ferments” with
“Fermentas”;

Answer: Done.

9) “Methods/RNA Isolation and….” section, lane 6: add City, State and Country where Applied Biosystems is;

Answer: We have added the City, State and Country of Applied Biosystems to the Methods.

10) “Methods/RNA Isolation and….” section, lane 7 and 17: in “DDCt” replace DD with greek characters;

Answer: We have replaced “DDCt” with “∆∆Ct”.

11) “Methods/Transient transfection….” section, lane 2: add space between“(NC)” and “(Sense::)”;

Answer: Done.

12) “Methods/Transient transfection….” section, lane 5: replace“Lipofectamine2000TM” with “LipofectamineTM 2000” and delete “Carlsbad, CA,USA”;

Answer: Done.

13) “Methods/Cell migration and….” section, lane 2/3: if it is not necessary to indicate the Catalog number of the product, please delete it. Add the City and the State where BD Discovery Labware is;

Answer: We have added the City and the State where BD Discovery Labware is to Methods.

14) “Methods/Cell migration and….” section, lane 8: add “, USA” after “MA”;

Answer: Done.

15) “Methods/Construction of the reporter….” section, lane 12: delete “, USA” If the Promega distributor of the Dual-Glo Luciferase assay and pRL-TK vector is the same;

Answer: Done.

16) “Methods/Western blot analysis” section: please indicate City, State and Country of the distributors of the antibodies used;

Answer: Done.
17) “Methods/Retrovirus production” section: please re-edit the text of the section for a more clear comprehension;

Answer: We have re-write the section:

“Retroviral transfection for stable cell lines

As previously described [8], retrovirus containing miR-133b or on insert (NC, negative control) were produced. After the infections of MKN-28 cells, positive cells were selected and named RV-miR-133b and RV-miR-NC. MiR-133b expression was confirmed by qRT-PCR.”

18) “Methods/Statistical analysis….” section, lane 5: replace “Means±SDs” with “Mean±SD”;

Answer: Done.

19) “Methods/Statistical analysis….” section, lane 6: add City and State where IBM is;

Answer: Done.

20) “Results/Overexpression of … in vitro” section, lane 11: replace “metrix gel” with “matrix gel”;

Answer: Done.

21) “Results/Gli1 is…” section, lane 2: replace “algorithms software” with “algorithm softwares”;

Answer: Done.

22) “Results/Gli1 is…” section, lane 8: replace “was” with “were”;

Answer: Done.

23) “Results/Gli1 is…” section, lanes 9 to 12: please re-edit the text for a more clear comprehension;

Answer: We have re-edited the text in our revised manuscript:

“Luciferase reporters were constructed containing the full-length of either a wild-type Gli1 3’UTR (pMIR/Gli1) or a mutated Gli1 3’UTR (sequence of the
putative miR-202-3p binding site was mutated, pMIR/Gli1/mut) (Fig.4A).”

24) “Results/Gli1 is…. ” section, lane 21: add “mimic” after miR-133b;

Answer: Done.

25) “Figures/Figure1” section, lane 1/2: replace “its” with “their” and check for character dimension uniformity;

Answer: Done.

26) “Legend to Figures/Figure1” section, lane 7: replace “means” with “mean”;

Answer: Done.

27) “Legend to Figures Figures/Figure2” section, lane 4: replace “means” with “mean”;

Answer: Done.

28) “Legend to Figures Figures/Figure3 (B)” section: in my opinion it should be better to put a phrase like “Quantification of the peritoneal nodules is shown in the bar graph. The results are the mean of 6-10 mice ± SD” rather than a description of the results;

Answer: Point is well taken and change has been made as indicated by the reviewer.

29) “Legend to Figures Figures/Figure4 (B), (C), (D)” section: it should be better to describe what graphs and/or blots show rather than comment the results and/or shortly describe the method…;

Answer: We have re-write the legend of Figure4 (B), (C), (D) in our revised manuscript:

“(B) Luciferase activities of reporter containing wild-type Gli1 3’UTR or mutant Gli1 3’UTR are shown in the bar graph. (C) Representative Western blotting images of indicated protein in MKN-28 cells. Relative mRNA levels of Gli1(D), Zeb2(E) and OPN(F) in indicated cells analyzed by qRT-PCR is shown in the bar graph. The results are means of three independent experiments ± S.D. *, p <0.05”

30) “Table1” section: it should be useful to have an exhaustive legend for theTable1;
Answer: We have added the legend for the Table1:

"140 cases were stratified into 3 groups based on relative miR-133b expression:
miR-133b low expression (tumor/non-tumor ratio <0.5), miR-133b moderate
expression (tumor/non-tumor ratio 0.5-2.0) and miR-133b high expression
(tumor/non-tumor ratio >2). Then relationship between the miR-133b expression
levels and clinicopathologic parameters was analyzed using the Pearson Chi-square
test."

31) “Additional files/additional file1 Legend” section: the title of the legend
cannot be part of the legend text. Please correct it.

Answer: We have re-edit the legend of additional file1:

“MiR-133b mimic significantly enhanced miR-133b level in
MKN-28 and SGC-7901 cells

Relative levels of miR-133b in MKN-28 (A) and SGC-7901 cells (B) were analyzed
by qRT-PCR is shown in the bar graph. The results are means of three independent
experiments ± S.D. ***, p < 0.001.”

32) Figure 1B: the graduation of the y axis of the graph is not clear. Why 0.3
is located in the top segment and not in the bottom segment? I imagine that 0.3 is the
last (higher) value of the bottom segment whereas 1 can be the first (lower)value of
the top segment. If so, please correct it;

Answer: We are sorry that we didn’t make it clear in our former version. Both
the last (higher) value of the bottom segment and the first (lower)value of the top
segment are 0.3. So we located “0.3” in the middle of the segments in our revised
version.

33) Figure2: in all the panels (both pics and graphs) please replace “NC”
and“miR-133b” with “NC mimic” and “miR-133b mimic”;
Answer: Done.

34) Figure 3B: to uniform labels of panels A and B of the figure replace “NC” and “miR-133b” with “RV-miR-NC” and “RV-miR-133b” respectively in the x axis of the graph in panel B;

Answer: Done.

35) Figure 4: in all the panels (both pics and graphs) please replace “NC” and “miR-133b” with “NC mimic” and “miR-133b mimic”;

Answer: Done.

36) Supplementary Figure S1 and S2: in all the panels (both pics and graphs) please replace “NC” and “miR-133b” with “NC mimic” and “miR-133b mimic”;

Answer: Done.

Major Compulsory Revisions

1) “Methods/Transient transfection of miRNA mimics” section: the negative control mimics are two single stranded oligonucleotide or, as I imagine, the two strands of a double stranded oligonucleotide which is generated after an annealing procedure? If the latter is the case I was not able to find complementarity between sense and antisense strand. Could you clarify this point?

Answer: The negative control mimics are two strands of a double stranded oligonucleotide which is generated after an annealing procedure. The duplexes were modified with 3’-TT on both strands. Sense: 5’-UUC UCC GAA CGU GUC ACG U-3’ is complementary with antisense: 5’-ACG UGA CAC GUU CGG AGA A-3’.

2) “Results/The expression of…” section, lanes 7 and 8: here the authors say that “expression of miR-133b was much less in all eight GC cell lines tested” but on
the basis of the data reported in figure 1C at least one cell line, BGC-823, shows miR-133b levels quite similar to those found in GES-1. Could the authors comment it?

**Answer:** We are extremely sorry for having made this mistake. We have corrected it:

“Cellular experiments found similar results that expression of miR-133b was much less in seven GC cell lines tested than in the immortalized normal gastric mucosal epithelial cell line GES-1 (Fig.1C).”

3) “Table1” section: in the expression category “Low” (n = 67) the sum of patients with > 60 years (36) and ≤ 60 years (29) is 65 and not 67: why?

**Answer:** We are extremely sorry for having made this mistake. The number of patients “Low” and age > 60 years is “38”. We have corrected it in Table 1 in our revised manuscript.

4) “Table1” section: in all the expression categories the sum of patients with Distal/Middle/Proximal third Location is different form 67, 55 and 18: why?

**Answer:** We are extremely sorry for having made this mistake. We have corrected it in Table 1 in our revised manuscript.

5) The authors suggest that “miR-133b-Gli1-Zeb2/OPN” pathway seems to be important in reducing the metastatic potential of gastric cancer cells. Like Gli1 also Zeb2 and OPN are important effectors of this pathway thus, in my opinion, in order to have a sort of double check and a more complete picture of the molecular status of the three protein players of the pathway it should be useful to confirm the Gli1-mediated downregulation of Zeb2 and OPN protein expression also at mRNA level by performing a qRT-PCR assay;

**Answer:** Thanks for the excellent suggestion. We have examined the expression of Zeb2 and OPN at mRNA level by qRT-PCR (Fig.4E and Fig.4F). Results showed that the mRNA levels of Zeb2 and OPN both were markedly reduced in MKN-28 cells transfected with miR-133b mimic.
**Reviewer: Manuela Garibaldi**

**Reviewer's report:**

This work shows that miR-133b, which is downregulated in gastric cancers with lymph node metastases, can inhibit metastases both in vitro and in vivo. This mechanism probably involves Gli1, a transcription factor directly correlated with lymph node metastasis in gastric cancer that the authors demonstrated to be a direct target of miR-133b.

- **Major Compulsory Revisions**

1. *In vitro and in vivo data regarding the role of miR-133b in inhibiting metastases are new. It is not clear the reason for they decided to investigate miR-133b among the 15 miRNAs they selected in the paper they cite (ref 6).*

   **Answer:**
   
   We are sorry that we didn’t make it clear in our former version. We have given more detailed reason in “Background” of our revised manuscript (page 4, paragraph 1).

1. *The association between reduced miR-133b expression and presence of lymphnode metastases has already been found by Wu WY et al., (Wu WY, Xue XY, Chen ZJ, Han SL, Huang YP, Zhang LF, Zhu GB, Shen X. Potentially predictive microRNAs of gastric cancer with metastasis to lymph node. World J Gastroenterol. 2011; 17:3645-51. PMID: 21987613).*

   **Answer:**
   
   Point is well taken and we have cited the reference and discussed it in the part of Discussion in our revised manuscript (page 15, paragraph 1).
The criteria used for dividing cases into three groups according to expression of miR-133b are not indicated. Was the choice of the cut-off values based on previous knowledge? If the authors chose the cut-off based on the observed association between the expression of the miR and the outcome, the reported results would not be valid (overestimation of the real association). A better categorization should be obtained by determining cut-off values using tertiles. Alternatively, the association between the expression of the miRNA and the clinical characteristics of cases must be done using miRNA levels as continuous variable. This type of analysis would guarantee a better statistical power compared to the analysis that uses the categorized values of the miR.

Answer:

We are sorry that we didn’t make it clear in our former version. The values we used for dividing cases are the ratios of expression of miR-133b of tumor tissues to their adjacent non-tumor tissues. When the ratio is equal to 1, it means that the expression of miR-133b in the tumor is the same as in non-tumor tissue. In Table 1, group of miR-133b low expression means expressions of miR-133b are lower in tumor tissue than their adjacent non-tumor tissues in the same case.

We have added an exhaustive legend for “Table 1”:

“140 cases were stratified into 3 groups based on relative miR-133b expression: miR-133b low expression (tumor/non-tumor ratio <0.5), miR-133b moderate expression (tumor/non-tumor ratio 0.5-2.0) and miR-133b high expression (tumor/non-tumor ratio >2). Then relationship between the miR-133b expression levels and clinicopathologic parameters was analyzed using the Pearson Chi-square test.”

In the version of miRanda available at www.microrna.org (Aug 2010) no prediction for miR-133b targeting GLI1 is present. Authors should explain how they identified the putative interaction between miR-133b and GLI1. Was it retrieved in an
older version of the program? If so, specify which.

Add references for the two prediction databases.

Answer:
Yes, it retrieved in an older version of the program (2007). According to the reviewer’s suggestion, we have given more detailed information of prediction databases and add references in our revised manuscript (page 13, paragraph 3).

1 In the section of “In vivo metastasis peritoneal spreading assay” indicate the cell line used for the experiment.

Were cells tested and authenticated?
Point is well taken and we have indicated the cell line used for the experiment in the part of section of “In vivo metastasis peritoneal spreading assay” in our revised manuscript (page 10, paragraph 1).
Cells were tested and authenticated by qRT-PCR analyzing expression of miR-133b (Additional file 3).

1 In fig 4C reduction of GLI1 expression after introduction of miR-133b is detectable, I have some concerns about reduction of the two genes that are target of GLI1 (ZEB2 and OPN).

Answer:
In order to have a sort of double check and a more complete picture of the molecular status of the three protein players GLI1 (ZEB2 and OPN) of the pathway, we confirmed the Gli1-mediated downregulation of Zeb2 and OPN protein expression also at mRNA level by performing a qRT-PCR assay (Fig.4E and Fig.4F). Results showed that the mRNA levels of Zeb2 and OPN both were markedly reduced in MKN-28 cells transfected with miR-133b mimic.

1 The discussion section is very poor. Authors should describe the reason for they selected GLI1 among the putative targets of miR-133b. There are other miR-133b targets that are involved in metastasis development and spread, for example one of them is the MET oncogene. A description of the known functions of GLI1 and of the
possible related effects on metastasis development should be also added.

**Answer:**
Point is well taken and we have rewritten the part of **Discussion** in our revised manuscript.

1. *The English is poor, the manuscript needs a strong revision.*

**Answer:**
English editing has been made as advised. We now have corrected the typos and a number of grammar inconsistencies in the revised manuscript.

Sincerely yours,

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