Author's response to reviews

Title: Suppression of microRNA-31 increases sensitivity to 5-FU at an early stage, and affects cell migration and invasion in HCT-116 colon cancer cells

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Version: 2 Date: 3 September 2010

Author's response to reviews: see over
Dear Miss Judith Gorton and Dr. Evi Lianidou,

Thank you for your email, along with reviewer’s comments. We have carefully revised the manuscript according to the reviewers’ comments, and would resubmit a revised manuscript (the changes highlighted). The detail responses to the comments are following:

**Reviewer: George Yousef**

*Reviewer's report:*

*This is an interesting study that explores the role of miR-31 on different aspects of colon cancer cell line pathogenesis, including cell proliferation, apoptosis, cell cycle, colony formation, migration and invasion. The study results show also that miR31 increases the sensitivity of the colon cancer cell lines to 5-FU chemotherapy. The results are valid and of clinical and therapeutic significance since it pave the road for more therapeutic applications of microRNAs. The experimental design is solid and the conclusions are justified based on the results.*

**Major Criticism:**

1) The 5-FU dose given is not indicated or justified. There is only a vague statement about a “clinical dose” that was used without further explanation. It would add significantly to the value of the manuscript if the authors have tried different doses of 5-FU in order to obtain the optimal dose that can illustrate the added effect of miR-31 on cell proliferation, apoptosis, etc.

   Regarding the 5-FU dose selection, we first reviewed others’ studies, and found 1) plasma concentration of 5-FU was more than 5µM in patients subjected to continuous drug infusion at a constant rate of 450-966 mg/m²/day (Petit E, et al. *Cancer Res* 1988, 48:1676-1679; Rossi L, et al. *Pharmacol Res* 2007, 56:248-253), and 2) Borralho et al. used 8µM 5-FU to treat HCT-116, and regarded it as a clinically relevant concentration (Borralho PM, et al. *FEBS J* 2009, 276:6689-7600). Then based on these results, we tried the different doses of 5-FU on both HCT-116 cell lines. As shown in a new figure (Figure 1) in the revised version, 5-FU inhibited the cell
proliferation in a dose-dependent manner. Thus we used 8µM 5-FU for our further experiments.

We have added more information about this point on the text (page 7, lines 15-20; page 11, lines 2-10).

2) **In many of the experiments, there is no indication of the reproducibility. I assume that the authors did the experiments in duplicates or triplicates to ensure reproducibility. That has to be indicated in the materials section.**

   In our original manuscript, we have mentioned the reproducibility in the “Statistical analysis” of the Methods section, i.e., “All data were expressed as the mean ± SEM from at least three independent experiments” (page 10, lines 10-11). In order to present the reproducibility clearly, we have also mentioned it on page 7, line 12; page 8, lines 7, and 18-19; page 9, lines 3 and 15; and page 10, lines 7 and 8, in this revised version.

3) **It would add significantly to the value of the study if the same experiments are performed on other colon cancer cell lines so that the results are more generalizable and are not unique for one particular cell line.**

   This is a very good suggestion; unfortunately, we did not perform the experiment on other colon cancer cell lines in this project, and would like to do it in the future project.

4) **The statement in page 16 of the results section (page 16, first paragraph), is not justified. This is a mere speculation that is not based on strong experimental support or rationale. It is difficult to believe that miR-31 will simultaneously turn on pro and anti-invasion forces.**

   According to the reviewer’s comments, we have omitted the sentences “2) migration and invasion are complex processes, and various cytoplasmic proteins and transcription factors have been identified to mediate these processes. So the final effect of miR-31 on cell invasion was depended on the balance of pro- and anti-invasion forces.” in the revised manuscript (page 15).

5) **In the discussion section (page 15), there is a mix up of the discussion about the role of miR-31 in cancer. The authors are citing two different examples, one from head and neck cancer and the other from breast cancer. According to the introduction section, the biological rule of miR-31 in these two cancers is opposing (overexpression verses underexpression). It is difficult to mix up the functions from these two cancers into one working hypothesis.**

   The expression of miR-31 is different in the two types of cancers; it is over-expressed in head and neck cancer, and under-expressed in breast cancer. According the reviewer’s comments, we have just mentioned the results in head and neck cancer to support our working hypothesis, and deleted the sentence “As for the invasion assay, it was in line with the previous study showed miR-31 expression was specifically attenuated in metastatic breast cancer cell lines and ectopic miR-31 did
not affect proliferation in vitro, but did reduce invasion by 20-fold." (page 15).

6) Although there is what seems like increasing sensitivity of the cell lines to 5-FU with inhibition of miR-31, the effect is minimal (as seen in figure 2), although statistically significant. This should be highlighted in the discussion section and the author should indicate the need of more experiments on different cell lines to support this phenomenon and further validate it.

  According to the reviewer’s suggestion, we have highlighted the limitation and re-written the text (page 14, lines 11-14).

**Minor Criticism:**

1) The author should expand more on discussing the potential targets of miR-31, possibly through bioinformatics target prediction analysis.

  It is a good suggestion; we have discussed the potential targets of miR-31 through bioinformatics target prediction analysis, and also mentioned the experimentally verified miR-31 targets in other cancers in the revised manuscript (page 16, lines 11-25).

2) Few typographical and spelling mistakes have to be fixed. E.g., page 5, line 2: should read: “underexpressed in serous ovarian carcinomas.”

  We have corrected it (page 5, line 2), and other misspellings in the revised manuscript, by the authors and Dr. David Hinsewood (who is a native English speaker, and, as a postdoctor, works at our laboratory).

3) Certain paragraphs are redundant and mentioned repeatedly throughout the text, E.g., the first paragraph of the results section.

  The redundant sentence in the first paragraph of the results section i.e., “The anti-miR-31 is a sequence-specific and chemically modified oligonucleotide to specifically target and knockdown miR-31 molecule.” has been omitted in the revised manuscript (page 11).

4) The results (page 11, line 7) should read: “confirming that miR-31 was.”

  We have corrected it in the revised manuscript (page 11, line 14).

5) The discussion has very lengthy paragraphs that are sometimes more than a full page in length. This has to be reorganized.

  According to the reviewer’s point, we have divided the lengthy paragraphs into two separately paragraphs, with a modification of the text (pages 14 and 15).

6) The statement in the conclusion (page 17), “not only the expression but also the function of miR-31 showed a cancer specific manner” is not justified. Since the authors are dealing only with one type of cancer, that is colorectal cancer.

  According the reviewer’s suggestion, we have deleted this sentence in the revised manuscript (page 17).
Reviewer: Georgia Sotiropoulou

Reviewer's report:

The manuscript by Wang et al. entitled: "Suppression of microRNA-31 increased the sensitivity of HCT-116 cells to 5-FU at early stage, and affected cell migration and invasion ability in a p53 independent manner in colon cancer cells" aimed to investigate the functional roles of miR-31 in colorectal cancer (CRC).

There are a few major concerns regarding this manuscript:

(A) The novelty and value of the results presented here are rather limited. The study is limited to in vitro assays only to describe a phenotype, while no attempt was made to touch on the underlying mechanisms. The authors do not comment on the significance and potential implications of their observations, especially in the light of other important published studies, as for example those by Creighton CJ, et al. Cancer Res 2010, 70:1906-1915 and Valastyan S, et al. Cell 2009, 137:1032-1046.

Our previous study showed that miR-31 expression was up-regulated in human CRC compared to normal mucosa (Wang CJ, et al. Dis Markers 2009, 26:27-34), and the similar evidence has also been reported by others (Bandrés E, et al. Mol Cancer 2006, 5:29; Motoyama K, et al. Int J Oncol 2009, 34:1069-1075). We designed the present project as a continuous study to see the effect of miR-31 on the cellular response to 5-FU (which is widely used in the treatment of a range of cancers), and try to understand the functional role(s) of miR-31 in colon cancer. According to the reviewer’s comments, we have commented the significance and potential implications of our and their findings (Creighton CJ, et al. Cancer Res 2010, 70:1906-1915; Valastyan S, et al. Cell 2009, 137:1032-1046) in the revised Discussion section (page 15, lines 12-20).

(B) More importantly, the conclusions drawn are not supported by the experimental data. The conclusion presented in the title that "Suppression of microRNA-31 affected cell migration and invasion ability in a p53 independent manner" is not justified, since the authors found that cell invasion was increased by 8-fold in HCT-116p53+/+ cells and by 2-fold in HCT-116p53-/- cells. Similarly, the increase in the sensitivity of HCT-116 cells to 5-FU is quite modest.

It is different response of the two cell lines to suppression of microRNA-31 as the reviewer comments, we have therefore addressed this point and changed related text such as the title and conclusion etc. (page 1, lines 1-3; page 3, lines 5-6; page 5, lines 18-20; page 16, lines 1-5; page 17, lines 2 and 3).

We agree with the reviewer that the increase in the sensitivity of HCT-116 cells to 5-FU is quite modest; we have highlighted the limitation in this revised manuscript (page14, lines 11-14).

(C) Unfortunately, there are several problems pertaining to the experimental part.
For example:

(1) It is not described whether the cell lines used came from another lab as a gift or these were established by the authors. It is not shown that p53 is expressed and functional in HCT-116p53+/+ cells as compared to control HCT-116p53/-/-cells.

The HCT-116p53+/+ and HCT-116p53/-/-cell lines were a kind gift from Dr. Vogelstein B (Johns Hopkins University, Baltimore, MD), which has been mentioned not only in the Acknowledgments section but also in the Methods section in this revised manuscript (page 6, lines 3-5).

The two wild type p53 in HCT-116 p53-/- have been targeted by homologous recombination, resulting in a mutated p53 with a 40 amino acid truncation, the HCT-116 p53-/- cells do not express detectable wild type p53 (Bunz F, et al. Science 1998, 282:1497-1501; Murray-Zmijewski F, et al. Cell Death Differ 2006, 13:962-972). The two cell lines are commonly used at our laboratory and Karolinska Institute, Sweden (Karimi M, et al. Carcinogenesis 2010, 31:1045-1053; Vilborg A, et al. Proc Natl Acad Sci U S A 2009; 106:15756-15761), and the p53 expression was tested shortly before by one of my colleagues at our laboratory through Western blot, the result showed that no full size p53 was detected in HCT-116p53-/-cell line (Pfeifer D, Linköping University Medical Dissertations No. 1108, 2009, paper V).

(2) It is not possible to assess the reproducibility of the data presented here, as it is not described in the manuscript how many times each experiment has been repeated, whether each point was done in duplicate, triplicate, etc. This is important, as it is widely known that there is variability inherent in this kind of in vitro assay.

In our original manuscript, we have mentioned the reproducibility in the “Statistical analysis” of the Methods section, i.e., “All data were expressed as the mean ± SEM from at least three independent experiments” (page 10, lines 10-11). In order to present the reproducibility clearly, we have also mentioned it on page 7, line 12; page 8, lines 7 and 18-19; page 9, lines 3 and 15; and page 10, lines 7 and 8, in this revised version.

(3) The conclusion that "Suppression of miR-31 did not change the cell cycle distribution" based on data presented in Figure 4 is subject to skepticism as the cells were not synchronized.

It seems whether cells should be synchronized or not is still a debatable issue, mainly depending on the aim of the study. We reviewed others’ papers related cell cycle analysis, cells were synchronized in some studies, especially those studies focused on the cell cycle checkpoint functions and mechanisms (Deckbar D, et al. Cancer Res 2010, 70:4412-4421; Shibata A, et al. Mol Cell Biol 2010, 30:3371-3383; Bu Y, et al. Biochem Biophys Res Commun 2010, 397:157-162). However some papers did not mention the cell synchronization, especially those papers focused on the effect of a new molecular or drug (Sasak T, et al. BMC Cancer 2010, 10:370; Zhang et al. BMC Cancer 2010, 10:367; Chen R, et al. Cancer Res 2010, 70:6587-6597).

In the present study, we are interested in whether the anti-miR-31 could change
the cell cycle distribution, so we think that it is reasonable not to synchronize the cell. However, this is an interesting suggestion, and we may consider comparing the cell cycle difference with or without cell synchronization in the future.

(D) The manuscript is poorly written and should be extensively edited. This is one of the largest problems of this manuscript; there are too many grammatical and syntax errors to address in a review. The authors need to communicate with an English editor before resubmission of this manuscript.

We have carefully checked the manuscript and corrected errors, and finally we have asked a native English speaker, Dr. David Hinselwood, to revise the manuscript (an acknowledgment to him on page 17, line 15).

(E) The Discussion section is too lengthy. There is redundancy throughout the manuscript.

In the Discussion of this revised manuscript, we have omitted many words/sentences, meanwhile, according to the two reviewers’ comments, we have added some text (page 15, lines 12-20, for the comments “the significance and potential implications of our and other important published studies by you, question A), (page 16, lines 11-25 for the comment “the targets of miR-31” by the reviewer 1, Minor Criticism, question 1). Although the length is not significantly reduced when comparing with previous one, we believe that the content is more reasonable/valuable. If you would like us to shorten the discussion we could do it without any problem.

We would appreciate if this revised manuscript could be reconsidered for publication in the BMC Cancer.

Yours Sincerely,

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