Author's response to reviews

Title: miR-320b suppresses cell proliferation by targeting c-Myc in human colorectal cancer cells

Authors:

Hantao Wang (hantaowang@126.com)
Fuao Cao (fuaocao2003@163.com)
Xu Li (xuli.ch@163.com)
Hua Miao (13567330917@139.com)
Jifu E (jefershon@sohu.com)
Junjie Xing (xij2118@163.com)
Chuan-gang Fu (fugang416@126.com)

Version: 3  Date: 11 March 2015

Author's response to reviews: see over
Dear Editor Mr. Ryan Relox,

We are submitting our revised manuscript entitled “miR-320b suppresses cell proliferation by targeting c-Myc in human colorectal cancer cells” (MS: 2079497320154697) to BMC Cancer for consideration of publication.

We have studied editor and reviewer’s comments carefully and have made revision which marked in yellow in the paper. According to the comments, we have performed additional experiments and included the new data to make the revisions accordingly. We have provided the point-by-point responses below, and we think we have addressed all the concerns raised by the reviewers in our revised manuscript.

We would like to express our great appreciation to you for comments on our paper. Looking forward to hearing from you.

Thank you very much for your consideration.

Yours sincerely,

Chuan-gang Fu, M.D.

Professor and Director, Department of Colorectal Surgery, Changhai Hospital, Shanghai 200433, China

Email: fugang416@126.com
Point-to-point responses

Responses to Reviewer# 1

The authors analyze the role of miR-320b in CRC tumor growth and confirmed that it is down-regulated and inversely expressed respect to c-myc. Based on CLUSH prediction results, they suggest that miR-320b promotes c-myc regulation.

Response: Many thanks for the positive comments. According to the comments and suggestions, we performed additional experiments and revised our manuscript with new data. We have provided the point-to-point responses below, and hope we can address your concerns in our revised manuscript.

Question 1. The authors state to have all clinic-pathological and biological data for the tumors analyzed, but table 1 reports only age, gender and tumor size. Either add other information or change the sentence stating what they have available. Investigate a possible link of miR-320b expression with the data they have available for these tumors and to the others that will be eventually added.

Response: Following the comments, we have corrected the description in our manuscript. We added the related description in the manuscript (Page 5, Line 7-9).

Question 2. In vivo experiments are sound; however results are not sufficiently robust, since they have been conducted only on 3 mice/experimental condition. Either demonstrate that all the 6 tumors/condition tested reflect the figure shown, or repeat the experiment with at least 6 mice/condition.

Response: Following the comments, we have corrected the description in our manuscript. We added the related description in the manuscript (Page 10, Line 11-12).

Question 3. The predicted binding of miR-320b to the coding region of c-myc retrieved by the CLASH database has been functionally validated in the cell lines where it has been identified (i.e. HEK293 cells that origin from kidney). This is a quite
new and uncommon way of gene regulation by a miRNA, to be sure that this interaction occurs even in colon cancer, authors should repeat its functional validation in a CRC cell line. The fact that the introduction of miR-320b mimics in HCT-116 and SW-480 cells results in decreased expression of c-myc does not indicate a direct interaction among them also in CRC. This miRNA could regulate other genes which in turn regulate (directly or indirectly) c-myc.

**Response:** Following the comments, we performed luciferase reporter assays in SW-480 cells again. As shown in Figure 4 B, transfection of miR-320b caused a significant decrease in luciferase activity in SW-480 cells transfected with the reporter plasmid with wild type targeting sequence of c-Myc mRNA but not reporter plasmid with mutant sequence of c-Myc, which was similar to that in HEK293 cells. We added the related description in the manuscript (Page 6, Line 7-8, Page 11, Line 8-9, Page 21, Line 3-4).

**Question 4.** English needs a strong revision, especially the abstract.

**Response:** Thanks for the suggestion. We have corrected grammar and spelling mistakes in the revised manuscript.

**Question 5.** For variables, also report data as mean+/- standard deviation.

**Response:** Thanks for the comments, we have added the description in figure legend of Figure 5. We added the related description in the manuscript (Page 22, Line 6).

**Question 6.** The legends for the tumors in figure 3A are reversed.

**Response:** Thanks for the comments, we are very sorry for our mistake in Figure 3A. We have corrected the legends in Figure 3A.

**Question 7.** In figure 5B change c-Myc si with miR-320b si

**Response:** Thanks for the comments, we are very sorry for our mistake in Figure 5B. We have corrected the legend “c-Myc si” to “miR-320b” in Figure 5B.
Responses to Reviewer# 2

Increasing numbers of reports implicate an aberrant expression of certain miRNAs, including miR-320b, in tumor growth, carcinogenesis or response to chemotherapy in different malignancies. The down-regulation of miR-320b appears to negatively regulate the expression of different genes, including c-Myc, a factor involved in many cellular processes, specifically tumor cell growth and proliferation. The present study shows that the expression of miR-320b suppresses cell proliferation by targeting c-Myc in human colorectal cancer cells.

Response: Many thanks for the positive comments. According to the comments and suggestions, we performed additional experiments and revised our manuscript with new data. We have provided the point-to-point responses below, and hope we can address your concerns in our revised manuscript.

Question 1: The quality of written English is not acceptable in the present form. Language correction is required for the whole manuscript. Please, pay attention not only to mis-spell but especially to grammatical and syntax errors and how sentences are arranged.

Response: Thanks for the suggestion. We have corrected grammar and spelling mistakes in the revised manuscript.

Question 2: This study has an evident weakness related to the data presented in Western blot analysis reported in Figure 4D and 5A. In particular, the description of results obtained for the expression of c-Myc protein appears to be discordant between the two figures, for both cell lines. The down-regulation of c-Myc protein levels in cells transfected with mir-320b mimics as compared to negative-control transfected cells is evident in Figure 4D but not in Figure 5A. Densitometric analysis must be provided. Besides, the original image files with the whole nitrocellulose membrane are needed in order to check the equal loading.

Response: Thanks for the comments. We believed that the discordance in Figure 4D
and Figure 5A was due to the different transfection efficiency in cell at different time. Following the comments, we performed the experiment in SW-480 cells again, as shown in the following figure, miR-320b down-regulated the expression of c-Myc in SW-480 cells significantly. We also had showed the quantification on the western blot in Figure 4D and Figure 5A. Besids, as shown in the following image files with the whole nitrocellulose membrane, protein were loaded equally.

![Image of Western Blot](image.png)

**Question 3:** With respect to Figure 5A, besides protein-extract from negative-control transfected cells, it is needed that Authors provide samples deriving from:

- empty vector pGL3 alone transfected cells
- empty vector pGL3 plus negative control oligonucleotide transfected cells
- vector pGL3-c-Myc alone transfected cells

**Response:** Following for the comments, SW-480 cells were transfected with negative control oligonucleotides (NC), empty vector pGL3, empty vector pGL3 plus negative control oligonucleotides (pGL3+NC) and vector pGL3-c-Myc, 48h later, the expression levels of c-Myc were analyzed by Western blot. As shown in the following figure, vector pGL3 and negative control oligonucleotides did not effected the expression of c-Myc in SW-480 cell, while, vector pGL3-c-Myc significantly up-regulated the expression of c-Myc in SW-480 cells.
Figure pGL3 which contain c-Myc cDNA up-regulated the expression of c-Myc in SW-480 cells.

SW-480 cells were transfected with negative control (NC), empty vector pGL3, empty vector pGL3 plus negative control oligonucleotide (pGL3+NC), vector pGL3-c-Myc, 48h later, the expression levels of c-Myc were analyzed by Western blot.

**Question 4:** With respect to Figure 5B, please provide explanation for the reported “c-Myc si” bars. The relative legend doesn’t comply with the Figure 5B.

**Response:** Thanks for the comments, we are very sorry for our mistake in Figure 5B. We have corrected the legends “c-Myc si” to “miR-320b” in Figure 5B.