Author's response to reviews

Title: MiR-216b inhibits cell proliferation by targeting FOXM1 in cervical cancer cells and is associated with better prognosis

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Version: 1 Date: 07 Aug 2017

Author’s response to reviews:

Dear Dr. Danielle Talbot, Editor of BMC Cancer,

Thank you very much for your e-mail and the valuable comments from the reviewers about our paper titled “MiR-216b inhibits cell proliferation by targeting FOXM1 in cervical cancer cells and is associated with better prognosis” (BCAN-D-16-02659). Based on you and the reviewer’s comments and request, we have addressed the concerns and revised the manuscript carefully.

All the technique, description and figure problems mentioned by the reviewers were carefully revised. Specific addresses were listed as follows:

Leslie Randall (Reviewer 1): Please include all comments for the authors in this box rather than uploading your report as an attachment. Please only upload as attachments annotated versions of manuscripts, graphs, supporting materials or other aspects of your report which cannot be included in a text format.

Please overwrite this text when adding your comments to the authors.
The authors present a well-written, well-executed investigation of FOXM1 and miR-216b as prognostic markers in cervical cancer. These data, and their preceding data, presented in the current manuscript are well supported by the experiments performed. However, these pathways were not identified as relevant in a systematic -omic evaluation of cervical cancer by the Cancer Genome Atlas Project (TCGA). I would ask the authors to comment in the discussion on their hypothesis on why the TCGA did not identify FOXM1 and/or miR-216b as key biomarkers in cervical cancer. The reference is provided for your convenience: Cancer Genome Atlas Research Network. Integrated genomic and molecular characterization of cervical cancer. Nature. 2017 Mar 16;543(7645):378-384. doi: 10.1038/nature21386. Epub 2017 Jan 23. PubMed PMID: 28112728; PubMed Central PMCID: PMC5354998.

Response: We thank the reviewer for the positive comments, and the reviewer’s suggestion is well taken. The interaction of microRNAs and transcription factors in tumors contains very complicated networks, and the relationship of miR-216b-FOXM1 is only been reported recently. It is a pity that miR-216b-FOXM1 modulation was not included in the recent published work of systematic -omic evaluation of cervical cancer by the Cancer Genome Atlas Project (TCGA). The reason may lies in different control and hierarchical clustering. In our study, the relative T/ANT (cancer tissues/ adjacent non-cancer tissues) ratio of FOXM1 and miR-216b expression was examined in cervical cancer tissues, whereas in TCGA studies, cancer tissues/normal controls were compared. The change of p21, myc expression and Wnt/ β-catenin signal pathway in cervical cancer were also revealed in TCGA study, using squamous/adenocarcinomas and HPV positive/negative hierarchy, whereas in our study, most of the cancer tissues were squamous and HPV positive. We believe our study will further enrich and helps to understand the molecular modulation mechanism of tumor associated genes and factors in cervical cancer. We included this reference and the above discussion has been added into the revised manuscript in line 9-23. page16.

Gwo Fuang Ho (Reviewer 2): This is a well researched article with convincing science, logical deduction of the molecular steps implicated, and interesting findings of significance to the community.

One observation, however, is the equal distribution of the patients in each group: 150 patients, 75 were miR-216b high level and 75 were miR-216b low level - were samples selected randomly (or deliberately balanced for the two groups) or the proportion of high and low miR-216b expression is 50% in the population? Far more patients in the mir-216b high group were early stage, which was reflected in the finding of better survival. If samples were not randomly selected, rather, were deliberately hand-picked, this introduced a huge bias in the analysis and affected the validity of the conclusion.

Response: We thank the reviewer for the positive comments, and the reviewer’s concerns are well appreciated. The patients were randomly collected from January 2009 to December 2012 in the Department of Obstetrics and Gynecology, the First Affiliated Hospital, Sun Yat-sen University. All the patients that fulfilled our criteria (cervical cancer with pathological biopsy confirmation and clinical follow-up data) were enrolled, irrespective of the stage. Initially we have collected 163 patients, but 13 were deleted because their samples were ruined, degraded or
too few. The remaining 150 samples were analyzed for miR-216b expression by qRT-PCR, and the 150 samples were divided into miR-216b high and low groups according to the miR-216b median level. The results showed that 75 were miR-216b relatively high and 75 were relatively low. We believe the equal distribution of the two groups is only a coincidence because we did not deliberately hand-picked these cases. Because our sample number is small and coming from only one hospital, we think this 50% may not represent miR-216b expression in the population of cervical cancer patients. We have revised the description in line 2-4 in page 6 in “Materials and Methods”.

Noor Hasima Nagoor (Reviewer 3): Please include all comments for the authors in this box rather than uploading your report as an attachment. Please only upload as attachments annotated versions of manuscripts, graphs, supporting materials or other aspects of your report which cannot be included in a text format.

Please overwrite this text when adding your comments to the authors.

1) The direct binding of miR-216b to FOXM1 has been demonstrated previously (Zheng et al., Eur Rev Med Pharmacol Sci. 2016;20(12):2541-50 and Sun et al., Cell Biol Int. 2017. doi: 10.1002/cbin.10754). The authors should take note of this.

Response: The reviewer’s point is well taken. We submitted our original manuscript in Oct. 2016, when these reports could not been looked up online yet. We have revised our manuscript and included these references.

2) The effects of miR-216b on cervical cancer cells need to be evaluated on more than one cell line.

Response: We thank the reviewer for this comment, and we are sorry for not clarifying this in the originally submitted manuscript. We tried 5 cervical cancer cell lines including Hela, SiHa, Caski, C33A and HCC94 in preliminary tests. The results showed that miR-216b had similar effects on the five cell lines, but Hela cells exhibited better reaction to miR-216b mimics and inhibitors, and cell proliferation inhibition effect was the most obvious. We deduce that is because Hela cells had moderate miR-216b and FOXM1 expression. We have revised the description in line 14-16 in page 10.

3) The authors should include the concentration of miR-216b mimics and inhibitors used in each assays.

Response: We thank the reviewer for the comment, and we are sorry for not clarifying this in the originally submitted manuscript. The concentration of miR-216b mimics and inhibitors (20nM, 2μl/well) were used as recommended by the manufacture. We have revised the manuscript and added the concentration in line 25-26, page 6 in the revised manuscript.

4) Page 6, Line 19: Has the expression of FOXM1 in HCC94 (low) and SiHa (high) cells been reported previously? If so, please cite the relevant studies.
Response: We thank the reviewer for the comment, and we are sorry for not clarifying this in the originally submitted manuscript. The low expression of FOXM1 in HCC94 and high in SiHa cells been reported in our previous study (Gynecol. Oncol. 2012; 127(3):601-610). We have revised and cited the relevant study in the revised manuscript in line 14-15 in page 6.

5) How did the authors arrived at the conclusion that FOXM1 expression was higher in HeLa, C33A and SiHa cells, and lower in Ca Ski and HCC94 cells (Figure 1A)? In comparison to?

Response: We apologize for this error. In fact, FOXM1 expression was higher in Ca Ski, C33A and SiHa cells, and lower in HeLa and HCC94 cells (Fig. 1A), and we have changed the description in the revised manuscript in line 10-11 in page 10.

6) Page 10, Line 11-15: This statement did not correspond to the results found in Figure 1B. Instead, Figure 1B appeared to be more relevant to the statement found in Line 17-23 (same page). Authors should include another figure for the statement mentioned in Line 11-15.

Response: We apologize for this error. We have changed the description into “Quantitative RT-PCR showed that in Ca Ski, C33A, and SiHa cells, the miR-216b relative ratio was lower and in HeLa and HCC94 cells, miR-216b level was relatively higher (Fig. 1B).” in line 12-14, and included Fig.1B in the statement of line 17-19.

7) The authors should clarify on what was the "control" used in Figure 1A and 1B and why.

Response: We thank the reviewer for the comment, and we are sorry for not clarifying this in the originally submitted manuscript. The control used in Fig.1A and 1B was normal cervical tissues, because it is hard to find a real “normal” cervical cell line. Normal cervical tissue was also used as negative control in other research of cervical cancer markers (e.g. Ann Oncol. 2012; 23(3):638-646). We have added this control explanation in Figure 1 legends and revised the “Materials and Methods” accordingly in line 22-25, page 5 in the revised manuscript.

8) The authors should include correlation analysis for miR-216b and FOXM1 expression in cervical cancer cells in Figure 1.

Response: We thank the reviewer for this comment. We have done the correlation analysis for miR-216b and FOXM1 expression and included the result in Fig. 5A, for the convenience of discussing the relevance of miR-216b and its target gene.

9) Figure 1C mentioned in the figure legend has been left out from the label in the corresponding figure.

Response: We thank the reviewer for pointing out this error. Appropriate correction has been made in Fig. 1C.

10) The results for normalized band intensity (to β-actin) in western blot should be presented (Figure 1A, 3A, 4B, 5A and 5B). If not, it will be very hard to interpret the results, as the band
intensity for β-actin was not exactly the same across samples. Statistical analyses should also be carried out to determine its significance.

Response: We thank the reviewer for this comment, and the reviewer’s suggestion is well taken. Normalized band intensity to β-actin with statistical analysis results have been added to all the western blotting results included in Figure 1A, 3A, 4C, 5A and 5B. For better appearance and layout of figures, we changed the order of Fig. 4B and 4C, and in Fig.5A, relative ratio values were shown right above the β-actin bands. We have changed the figures and the corresponding legends.

11) It was mentioned in the Materials and Methods that cell proliferation was detected on day 1, 3, 5 and 7 using MTT, however results shown in Figure 2B contained results for day 1 to 6 and Figure 5C contained results for day 1 to 5. Can the authors please explain this?

Response: We apologize for this confusing expression. Cell proliferation was detected on day 0 to day 5 by MTT method. We have changed the description in Materials and Methods into “Cell proliferation was detected on day 0-5 by MTT method” (see line 9, page 9) and revised the Fig.2B accordingly.

12) The lines used in the graph for Figure 2B can be differently coloured (like Figure 2A) for better presentation.

Response: We thank the reviewer for the suggestion, and the reviewer’s suggestion is well taken. Fig. 2B and Fig.5C has been revised for concordance.

13) The western blot results in Figure 3A should include results for LEF1.

Response: We thank the reviewer for this suggestion, and the reviewer’s suggestion is well taken. We have revised Fig.3A and included LEF1 WB results, the legend and description in line 21-28, page 11 in the revised manuscript.

14) Since p21 is negatively regulated by FOXM1, why was p21 excluded from the results in Figure 3B?

Response: We thank the reviewer for this suggestion. We have revised Fig.3B and included p21 real-time PCR results, the legend and the description in line 3-4, page 12 in the revised manuscript.

15) The authors should specify what is "NC" in Figure 4C (is it cells transfected with mimics negative control?). Were the results compared to cells transfected with mimics negative control or vector control? Did the authors also include cells transfected with negative control inhibitors?

Response: We apologize for this error. The red bar was not vector control, it was NC, HeLa cells transfected with microRNA negative control (mimics control), and the results of relative luciferase activity were compared to NC. The green bar was cells transfected with negative control inhibitors (NC-in), and it was the control of miR-216b inhibitor group. We have
16) Can the authors comment on why luciferase activity was significantly increased in miR-216b-in cells in Figure 4C?

Response: We thank the reviewer for this comment. In our study, the 3’-UTR of FOXM1 were cloned after hRluc, and the 3’-UTR-PsiCHECK2 vector was transfected into HeLa cells. HeLa cells express moderate FOXM1 and miR-216b. Endogenous miR-216b could bind the 3’-UTR of FOXM1 and inhibit its expression, thus the relative luciferase activity of NC was lower. When miR-216b mimics were added, the 3’-UTR expression was further inhibited. When miR-216b inhibitors were added, endogenous miR-216b was blocked and the 3’-UTR expression elevated, and as a consequence, the luciferase activity was significantly increased. Similar results can be found in other microRNA function studies. (e. g. Li H, Zheng D, Zhang B et al. Mir-208 promotes cell proliferation by repressing SOX6 expression in human esophageal squamous cell carcinoma. J Transl Med, 2014; 12:196. Sarah Jurmeister, Marek Baumann, Aleksandra Balwierz et al. MicroRNA-200c Represses Migration and Invasion of Breast Cancer Cells by Targeting Actin-Regulatory Proteins FHOD1 and PPM1F. Mol Cell Biol. 2012 Feb; 32(3): 633–651)

17) The authors should explain and elaborate on the results shown in Figure 5B.

Response: We thank the reviewer for this comment, and the reviewer’s suggestion is well taken. We have added the description and explanation of the results of Fig. 5B in the revised manuscript in line 8-15 in page 13.

18) Since results showed that miR-216b affects cell cycle related factors, did the authors carried out cell cycle analysis to examine the effects of miR-216b on cell cycle?

Response: We thank the reviewer for raising this important point. The cell cycle analysis results were not included in the originally submitted manuscript, but we did carry out cell cycle analysis. The results showed that miR-216b could decrease the ratio of cells in S period and miR-216b-in had the opposite effect, therefore we further carried out western blotting analysis of related cell cycle factors. We added a supplemental figure showing the cell cycle analysis results and revised the results and discussion in line 15-19 in page 11 in the revised manuscript.

19) Others: Page 4, Line 11: cervical caner should be writen as cervical cancer. Caski should be written as Ca Ski. Hela should be written as HeLa

Response: We thank the reviewer for pointing out the typos. Corrections have been made in the revised manuscript.

Wa Xian (Reviewer 4): He et al described detailed in vitro studies using Hela cells to study the role of MiR-216b in cervical cancer cell lines and its link with FOXM1. Furthermore, the authors attempted to prove that MiR-216 is a prognosis marker for cervical cancer. It should be noted that Sun M et al published in Cell Bio Int on 2017, Feb 22 and they have reported that
microRNA-216b inhibits cell proliferation and migration in human melanoma by targeting FOXM1 in vitro and in vivo. The highlight of the He et al. is the correlation between increased expression of microRNA-216b and patients' survival. However, it is unclear how miR-216b was measured in these patients. In the method, the authors claimed that qPCR was done on 150 patients and divided them to two groups (high and low). The qPCR data should be presented in the main figure. In addition, the expression of FOXM1 in these patients should be studied to examine the correlation. The authors should focus on further strengthening the findings in Figure 6 in order to improve the novelty and significance of current manuscript. In addition in Figure 1, it is unclear what is negative control that the authors referred to.

Response: We thank the reviewer for raising these important points, and the reviewer’s suggestion is well taken. We submitted our original manuscript in Oct. 2016, when these reports could not been looked up online yet. We have revised our manuscript and included the references of Zheng et al. and Sun et al. in line 12 in page 16. The level of miR-216b was measured by qRT-PCR and normalized using U6 expression. And the 150 samples were divided into miR-216b high and low groups according to the miR-216b median level. We added a supplemental figure 2 to exhibit the qRT-PCR results. Because many of the clinical samples were not enough to do the western blot analysis, we did the correlation analysis for miR-216b and FOXM1 expression in 8 of the patients. The correlation analysis of the expression of FOXM1 and miR-216b in cervical cancer patients was included in Fig 5A. We have revised the discussion of Fig. 6 in line 11-14 of page 17 in the revised manuscript, to improve the novelty and significance of our study. The negative control in Fig. 1 was normal cervical tissues obtained from patients underwent hysterectomy because of uterine leiomyomata, for the reason that it is hard to find a real “normal” cervical cell line. Normal cervical tissue was also used as negative control in other research of cervical cancer markers (e.g. Ann Oncol. 2012; 23(3):638-646). We have added this control explanation in Figure 1 legends and revised the “Materials and Methods” accordingly in line 22-25, page 5 in the revised manuscript.

The above related description and discussion was enclosed in the revised manuscript. A revised manuscript with the correction marked as tracked changes was attached. Revised figures and supplemental materials were also attached. Should you have any questions, please contact us without hesitate. Thank you very much.

Best Regards,

Yours sincerely

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