

Dear Professors:

Thank you very much for your suggestions about our manuscript titled “miR-145 Exerts Tumor-Suppressing and Chemo-Resistance Lowering Effects by Targeting CD44 in Gastric Cancer”, all of your suggestions are very important on my paper writing and research work. Now, we would like to answer the questions point-by-point as follows:

I refer to the page number of the revised manuscript.

**For Reviewer (code: 03310535)**

This is an interesting study describing a novel mechanism by which miR-145 modulates gastric cancer cell growth and chemo-resistance through direct inhibition of CD44 expression. The aim is clearly stated, the findings are well described and the data are convincing. The paper is generally well written, but there are some minor typographical and grammatical errors throughout (in addition to those noted below). Specific comments: ?

Q: The method used to measure the level of CD44 mRNA should be stated in the abstract. ?

A: “Quantitative real-time PCR was used to measure to the level of CD44 mRNA” was added in Abstract Section (**P2, line13**).

Q: There is unnecessary repetition of ‘improved’ and ‘gastric cancer’ in the first sentence of the introduction. ?

A: The sentence has changed to “Despite advances in medical technology to improve gastric cancer outcome, gastric cancer is still the fourth most common cancer worldwide” (**P3, line 22**).

Q: According to GLOBOCAN 2012 data (and the cited paper), gastric cancer is the 5th most common cancer worldwide and the 3rd leading cause of cancer-related death. ?

A: The sentence has changed to “The 5-year survival rate among gastric cancer patients is still less than 35%, and remains the third leading cause of cancer-related deaths” (**P3, line 23**).

Q: The reason for the specific reference to China in the third sentence of the introduction is not clear. There is a higher incidence of gastric cancer in China, but the low survival rate is applicable worldwide ?

A: The sentence has changed to “Seventy percent of gastric cancer-related deaths occurred in developing country, with China having ~ 40% of them. In China, this low survival rate is mainly due to the disappointing early detection rate, tumor recurrence, and high chemotherapy resistance”. (**P3, line 25**)

Q: A reference is required to support the statement that CD44 expression is upregulated in advanced gastric lesions. ?

A: The paper (Oncotarget, 2016 7[9]:9815-9831)) was cited to support the statement (**P4, line 15**).

Q: Each sub figure should be referenced in the text body when the relevant data is discussed. ?

A: Yes, we have done this in the text body (**P8, line 19/23/26; P9, line**

**2/7/8/12/16/17/19/29; P10, line 1/3/4/6/19/21/22; P11, line 6/8/23/25; P12, line 29).**

Q: A brief description of ABCG2 would be helpful. ?

A: Yes, this is a good suggestion. The sentence of “Drug resistance is closely related to increased drug efflux mediated by an energy-dependent mechanism involving the ABC (ATP binding cassette) transporters, mainly ABCB1 (ATP binding cassette subfamily B member 1), ABCC1 (ATP binding cassette subfamily C member 1) and ABCG2 (ATP binding cassette subfamily G member 2)” was added in **P10 line 25**.

Q: In graphs showing mRNA expression levels, ‘calculated’ and ‘calibrated’ should be replaced by ‘normalised’ in the y axis label. ?

A: Yes, this have done in figures (**P20, Figure 1B/C/D/E; P21, Figure 2D/E; P23, Figure 4C**).

Q:The X axis labels are missing in Figures 1D, 2D & 2E ?

A: Yes, this have done in figures (**P20, Figures 1D; P21, Figure 2D/E**)

Q: The X axis legend for Figures 2B & 2C appears to be incorrect. I assume that the solid bar represents the miR-145 mimics on the left & the miR-145 inhibitor on the right in both graphs. ?

A: This had changed in Figures 2B & 2C (**P 21, Figure 2B/C**).

Q: While CD44 is associated with cancer progression and treatment resistance, it is not in itself an ‘oncogene’. ?

A: On the one hand, the cell-surface glycoprotein CD44 has several important physiological functions in cell–cell and cell–matrix interactions including proliferation, adhesion, migration, hematopoiesis, and lymphocyte activation, homing, and extravasation. On the another hand, CD44 has been implicated in a number of diseases such as cancer, arthritis, interstitial lung disease (ILD), vascular disease, wound healing, and infections by pathogens. CD44 has been accepted as a CSCs marker, and its expression is up-regulated in different types of cancer. As far as we know, it is not in itself an ‘oncogene’.

Q: A weakness of the study is that only in vitro data using one cell line are presented. It remains unknown whether the mechanism identified is more widely applicable or is relevant in vivo. This should be acknowledged in the discussion.

A: The sentence of “this needs to be further verified using more gastric cell lines and *in vivo* assay” was added to acknowledge the weakness of present study. (**P13, line 15**)

Q: A specific comment on the potential clinical relevance of the findings would also add to the discussion.?

A: Yes, this is a good suggestion. The sentence of “probably, miR-145 targeting CD44 could make it a potential target for preventing recurrence and chemo-resistance in patient with gastric cancer” was added to indicate the potential clinical relevance of the findings. (**P13, line 13**)

Q: Abbreviations, including miRNA, MRE, WT, MT, RLU, 5-FU and MTT should be written in full on first use. ?

A: miRNA (microRNA, **P4 line 22**); MRE (miRNA-recognition elements, **P8 last line**); WT, MT (WT, wild type; MT, mutant type, **P7 line 1**); RLU (relative luciferase activity, **P21 line 2**); 5-FU (5-Fluorouracil, **P7 line 24**); MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, **P2 line 16**)

Q: ‘Tumorspere’ should read ‘tumorsphere’; ‘Turkey’ should read ‘Tukey’; ‘westerin’ should read ‘western’.

A: Those had been changed [Tumor sphere (**P6, line 23**); Tukey (**P8, line 6**); western (**P22, line 4**)].

### **For Reviewer (code: 03440494)**

The manuscript is interesting, the design and methodology appear adequate but not overall well written (correct English mistakes), especially in the discussion part.

**Q:** -The results are not extensively discussed.

**A:** According to the reviewer’s excellent suggestion, the significance of CSC marker gene expression (**P11, line 17**) and ABCG2 was discussed in the discussion part (**P13, line 3**).

**Q:** -There is much detail in the discussion part: ”The latter sported by the following observations. (a) miR-145 was down-regulated, whereas CD44 was up-regulated in gastric tumor spheres, highly enriched in GCSCs; (b) forced expression of miR-145 through transfection of miR-145 mimics in MGC-803 cells decreased the expression of CD44; (c) knock-down of miR-145 by miR-145 inhibitor in MGC-803 cells increased the expression of CD44; (d) forced expression of miR-145 through transfection of miR-145 mimics in MGC-803 cells decreased the activity of CD44-3’UTR; (e) knock-down of miR-145 by miR-145 inhibitor in MGC-803 cells increased the activity of CD44-3’UTR; (f) mutation of the MRE for miR-145 on CD44 3’UTR abrogated the regulatory effects by the miR-145 mimics or miR-145 inhibitor. These results indicate that miR-145 negatively regulates the expression of CD44 in gastric cancer cells”. Please reduce and present clearly your findings.

**A:** These sentences were reduced to” The latter was confirmed by the following observations; (a) there is the inverse correlation between miR-145 and CD44 expression in gastric tumor sphere; (b) miR-145 regulates CD44 protein expression in MGC-803; (c) CD44 3’UTR is regulated by miR-145.” (**P 12, line 8**)

**Q:**-The authors say “Our result demonstrated that enforced expression stimulates ABCG2 mRNA expression”. In fact the expression of what?

**A:** The sentence was changed to “Our result demonstrated that enforced CD44 expression stimulates ABCG2 mRNA expression”. (**P 12, line 28**)

Q:-Please read and add this references in the discussion part : ? “ABCG2 regulates self-renewal and stem cell marker expression but not tumorigenicity or radiation resistance of glioma cells BoyoungWee\*, Alexander Pietras\*, TatsuyaOzawa, Elena

Bazzoli, Ondrej Podlaha, ChristopheAntczak,? , BengtWestermarck, Sven Nelander, Lene Uhrbom, Karin Forsberg-Nilsson, Hakim Djaballah, Franziska Michor & EricC. Holland. Scientific Reports | 6:25956 | DOI: 10.1038/srep25956 ? “New trends for overcoming ABCG2/BCRP-mediated resistance to cancer therapies. David Westover and Fengzhi Li ?. J Exp Clin Cancer Res. 2015; 34: 159. ? Andre R. Jordan,1,? Ronny R. Racine,2,? Martin J. P. Hennig,2,3 and Vinata B. Lokeshwar2,4,5,\* The Role of CD44 in Disease Pathophysiology and Targeted Treatment. Front Immunol. 2015; 6: 182.

A: Good suggestion. These references were added. The following sentence was added in the discussion part: recently, one report highlights the value of changing the perspective of CD44 expression from that of a simple marker to a signaling molecule (**P12, line 20**); In addition to the well-known effects of ABCG2 on cytotoxics and targeted agents, ABCG2 is also increasingly linked with failure of PDT (photodynamic therapy) and CSCs marker (**P13, line 3**); Interestingly, ABCG2 is reported to regulate self-renewal and stem cell mark expression but not tumorigenicity or radiation resistance in glioma cells, while the role of ABCG2 in resistance to radiation therapy remains to be further investigated (**P13, line 5**).

Q: -There are many human gastric carcinoma cell lines “GES-1, BGC-823, SGC-7901, HEK293T cells...). Why you chose to work with MGC-803 cell?

A: GES-1 is a normal cell (Chinese Journal of Oncology, 1994 16, 1:7-10); HEK293T is a human embryonic kidney cells (J Gen Virol, 1977 36, 1:59-74). BGC-823, SGC-7901 and MGC-803 are gastric cancer cell lines. This is a good question, as another reviewer’s comments above. This is a weakness of the study with only one cell line presented. We acknowledged this in the discussion (**P13, line 15**). We’ll design the experiments (*in vitro* or *in vivo*) to determine whether the mechanism identified is more widely applicable.

Q: -The authors investigate several gastric cancers stem cell marker expression in the tumor spheres and monolayer cells. Why they chose “Sox2, OCT-4 and Nanog mRNA expression” and what they play in the gastric tumor environment? They increase malignancy or affect tumorigenicity or what? Please clarify and discuss you findings in correlations with your results.

A: The following sentences were added in discussion parts (**P11, line 17**): SOX2, OCT4 and Nanog make up the core transcriptional network responsible for the regulation of stem cell self-renewal and pluripotency; Several groups demonstrated that Sox2, OCT-4 and Nanog are enriched in gastric CSCs. Gastric CSCs identified using the CD44 surface marker in MKN-45 gastric carcinoma cells had elevated levels of Nanog, Sox2 and Oct4; The results showed the tumorspheres expressed much higher levels of Sox2, OCT-4 and Nanog in our experimental system (Fig 1E); It demonstrated that the spheres enrich the cancer gastric CSCs population. At the same time, miR-145 expression was repressed in the spheres (Fig 1B); We speculated that miR-145 paly an inhibitory role in stemness properties of gastric cancer cells.

Q: -Please indicate the abbreviation of MREs (a Putative miRNA regulatory element).

A: According to the paper (Genes Dev. 2004; 18[10]:1165-1178), it indicated that the abbreviation of MREs is miRNA-recognition elements (**P 8 last line**).

Q: -Why the Luciferase activity was measured at 36 h after transfection?

A: miR-145 mimics and inhibitor are purchased from RiboBio (Guangzhou). According to the product instruction, the functional effect is detected by 24 ~ 72 hours after transfection. The detection time is dependent on the different experiment. In our experiment, the Luciferase activity was measured at, western blot analysis time was at 72 h after transfection. These times are suitable for the experiments.

Q: -What do you authors mean by this sentence:” We used the TargetScan database (<http://www.targetscan.org>) to identify miR-145 predicted to target CD44”. Please clarify?

A: The sentence had changed to “The prediction of MRE (miRNA-recognition elements) site for miR-145 on the CD44-3’UTR was performed with the TargetScan (<http://www.targetscan.org>) algorithms” (**P 9, line 1**).

**Thank you very much again for your suggestions, we would like to learn more from you!**

Kind regards, happy new year!

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