Author's response to reviews

Title: microRNA-145 promotes differentiation in human urothelial carcinoma through down-regulation of syndecan-1

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Author's response to reviews: see over
August 18, 2015
Dr. Paul Lambert
Editorial Board Manager,
BMC cancer

Dear Dr. Lambert:

Thank you for your efficient reviews of our manuscript MS: 3662479731747172, entitled "microRNA-145 promotes differentiation in human urothelial carcinoma through down-regulation of syndecan-1 ".

We have revised the manuscript according to the reviewer’s comments and resubmit it for consideration for publication in BMC Cancer.

Referee 1
Major Revision

1. It will be convincing if the authors could elaborate on their choice of miR-145 over other micro-RNAs for this study.

Thank you for the important points for us. Our previous studies have focused on molecular mechanisms concerning syndecan-1 - microRNAs (miRNAs) axis in progression of prostate cancer (Fujii T, Shimada K, Tatsumi Y, Fujimoto K, Konishi N. Syndecan-1 responsive microRNA-126 and 149 regulate cell proliferation in prostate cancer. Biochem Biophys Res Commun. 2015;456:183-9.). The miRNA array analysis using prostate cancer cells revealed that there are some candidate miRNAs such as miR-126, 149, 331-3p, 345, 30d, 23a and 145. We have considered whether these candidate miRNAs were related to urinary bladder carcinogenesis. As the results, we found that miR-145 played an important role in cell proliferation and differentiation through the expression of EMT, differentiation and stem cell markers in the bladder cancer.

2. In Figure 2, is the control, the cells with no treatment or were they also transfected with non-scrambled RNA. Since all the groups should be subjected to similar conditions experimentally, the control taken here should also be transfected
with a missense RNA.

We examined RT-PCR method using Pre-miR miRNA Precursor Molecules Negative control #2 (Life technologies) as control molecules. In the current study, each mRNA was no significant difference by transfection of Negative control. All mRNAs are normalized by GAPDH mRNA.

(-); only lipofectamine,  control: Negative control

*p<0.05, (-) vs miR-145 or Negative control (Negative control: no significant difference)
3. All the analysis in Figure 2 should be with the controls subjected to similar transfection conditions with a non scrambled sequence to see that the effects are due to miRNA overexpression and not due to procedural changes.

We examined RT-PCR method using Pre-miR miRNA Precursor Molecules Negative control #2 (Life technologies) as control molecules. In the current study, each mRNA was no significant difference by transfection of Negative control. (please see “Major revision 2”)

4. In Results, line 151 it states that the cells changed morphologically after additional transfection. Also, Figure 2A shows image with additional transfection. Please provide an image of how the cells looked like after initial transfection and complement them with control after initial and additional transfection for proper comparison.

Thank you for the reviewers’ comments. We observed cellular morphology at both initial and additional transfection. Although, each cellular image changed, the cellular morphology was more changed in the additional transfection than in the initial transfection.

We have added the picture of the cellular morphology at initial transfection for comparison. (Figure 2A)

5. The authors have also mentioned in the methods section, siRNA against sydecan-1. If they have not used it in any experimental analysis, please remove it.

We are sorry for incorrect sentences. We performed quantitative RT-PCR for the expression of NANOG, MUC-1, TP63, NSE and E-cadherin under transfection of
syndecan-1 siRNA (Figure 3C). But we described in “Figure 2B and data not shown”. We have rewritten that points as follows:
“Similarly, silencing of syndecan-1 by siRNA up-regulated some stem cell and differentiation markers (Figure 3C). “
(p9, line 182).

6. Mention the staining used in Figure 4A in methods section, line 123.

Thank you for the reviewers’ comments. We have added in methods section as follows:
“All tissue samples were fixed in 10% formalin for 48 hours and processed through graded alcohols to paraffin. Paraffin blocks were sectioned at 3-µm intervals and stained with hematoxylin and eosin (HE) for histological diagnosis. For each HE stained sample, corresponding sections, to include cancer foci of interest, were cut at 8-µm intervals for extraction of total RNA. “
(page 6-7, line 125-129)

Minor Revision

1. In the Methods section, mention from where the cell lines were received (purchased or from other source).

We are sorry for not to describe the source. We have described the following sentence in the methods.
“The human urothelial carcinoma cell lines, T24 was purchased from American Type Culture Collection (Manassas, VA), and KU7 was derived from human papillary bladder cancer. (Tazaki H, Tachibana M. Studies on KU-1 and KU-7 cells as an in vitro model of human transitional cell carcinoma of urinary bladder. *Hum Cell* 1988; 1: 78–83.)”
(page 5, line 87-89)

2. Line 416. There is mention of prostate cancer, I believe it is a typo error.
Please correct it appropriately.

We are sorry for the mistyping. We have changed it to the following sentence.
“Urothelial carcinoma of the bladder tissues.” (page 21, line 430-431)
3. Incorporate Mention of Figure 4A in the Results section.

We are sorry for not providing mention of Figure 4A in the sentence. We provided in the Results section as follows:

“Urothelial carcinoma was histologically classified into low grade, high grade and high grade with squamous, glandular or neuroendocrine differentiation (Figure 4A).”

(page 9-10, line 189-191)

4. Figure 4 legends. Please mention the statistical analysis used for comparison in the legends.

We are sorry for not providing mention of the statistical analysis. We have provided in the Figure 4 legends as follows:

“ *p < 0.05, low grade v.s. high grade ”

Referee 2

Major Compulsory Revisions

1. Authors did not mention any previous report or experiment in present manuscript that validate the down expression of mir145 in human urothelial carcinoma and cell lines (T24, KU7). Further, rationale of overexpressing or using precursor mir145 in experiments in human urothelial carcinoma is needed in the manuscript.

Thank you for your critical comments. As we examined expression of miR-145 in bladder cancer cell lines using T24, KU7 and UMUC6, miR-145 expression was suppressed in these cell lines. Moreover, we have examined mRNA expression of MUC-1, E-cadherin, NSE, TP63 and NANOG under transfection of miR-145 inhibitor, these mRNA expression were not changed by suppression of miR-145. Our results show that it keeps miR-145 low level and functions as cancer suppressing miRNA in the bladder cancer.

| MUC-1 | E-cadherin | NSE | TP63 | NANOG |
2. As reported in other cancers, authors failed to show if top targets of mir145 such as EGFR, FSCN1, N-cadherin, and IGF are decreased following adding precursor mir145 in cell lines.

Thank you for your suggestion. We have added to describe in detail about reports in other cancers as follows:

“For example, miR-145 has been found to inhibit cancer cell growth, invasion, and metastasis by suppressing EGFR and NUDT1 in lung adenocarcinoma, FSCN-1 in esophageal squamous cell carcinoma, N-cadherin in gastric carcinoma, and IGF in hepatocellular carcinoma [37-40].”

(page 11, lane 225-228)

3. Interestingly, authors noticed that miR-145 induces expression of stem cell markers such as SOX2, OCT4, NANOG, and E2F3. However, expression of stem cell markers favors invasiveness due to epithelial to mesenchymal transition. In addition, expression of E-cadherin was increased. Authors should evaluate the expression of N-cadherin to see if invasiveness has decreased following mir145 expression.

Thank you for the important points. We have added the experiment on mRNA expression of N-cadherin under overexpression of miR-145. As a result, the expression of N-cadherin decreased by transfection of miR-145 precursor, leading to the decreased invasiveness. On the other hand, our results showed that miR-145 induces expression of stem cell markers such as SOX2, OCT4, NANOG, and E2F3. These stem cell markers induced by miR-145 may contribute to another capacity such as cell differentiation, except for invasiveness, in bladder cancer cells.
Minor Essential Revision

There is inconsistency of statement of mir145 expression in low grade vs high grade carcinoma in last paragraph of introduction and results. Introduction (lines 82-83) reflects mir145 is up-regulated in high-grade urothelial carcinoma, but not in low grade tumors. However, result showed opposite (lines 183-184).

We are sorry for some errors. We have changed it to the following sentence.
“Moreover, miR-145 and syndecan-1 were found to be up-regulated in low-grade urothelial carcinoma, but not in high-grade tumors.”
(page 4, line 81-83)

Referee 3

Major Points

1) Briefly describe the cell culture condition or provide the references (Line 86).

We have added the following sentences on the cell culture condition.
“T24 and KU7 were cultured in RPMI1640 media supplemented with 10% fetal bovine serum and 50 units/mL penicillin-streptomycin at 37°C in 5% CO2.”
(page 5, line 89-90)

2) Table 2 is not provided (Line 121)

We have omitted Table 2 on patient data for confusing. We described about fifteen patient data in materials as follows:
“We examined fifteen trans-urethral resection of bladder tumor specimens without undergoing chemotherapy or Bacillus Calmette-Guerin treatment (age: 51-84 years,
grade: low grade 5 cases; high grade 10 cases.”

(3) Provide scale as well as magnification power on the Figure 1B

We are sorry for not providing magnification power. We have provided in the Figure 2(A) “x200”.

(4) The study published in Oncogene (2010) 29, 1073-1084 (Ostenfeld MS), suggested that “miR145 induces caspase-dependent and independent cell death” but author has seen senescence instead of cell death even in both study used same cell (T24) lines. Author should comments on that.

Thank you for the important suggestion. In our current study, the overexpression of miR-145 induced cell senescence, whereas no induced apoptosis in TUNEL assay. Furthermore, the expression of apoptosis related protein such as bcl2, BAX and bclXL did not change significantly in mRNA level. Thus we cannot mention the consistency with previous data, because we did not verify in detail by caspase assay in this study. At least these findings from our senescence, TANEL and RT-PCR assay show that miR-145 is important in regulation of cell proliferation through senescence mechanism in bladder cancer cells.

5) In Figure 2B, Y-Axis legend make self explanatory, mentioned the name of base line
housekeeping gene against that author has calculated the “Fold Changes” in mRNA expression.

Thank you for the reviewers’ comments. We have added the following sentence in Figure legends.

“Y-axis in B,C,D was indicative of relative mRNA expression compared with control. All mRNAs data were normalized by GAPDH.”
(Figure 2, 3, S1)

6) Why “Standard Error” is very high in cytokeratin 5 mRNA expressions?

Thank you for your comments. As cytokeratin 5 mRNA expression level is low in T24 cells, we were not able to recognize a significant difference. When we could obtain enough mRNA by using more quantity (2µg of total RNA) for reverse transcription, it was shown that the quantity of cytokeratin 5 mRNA was significantly higher than a control.

We replaced this graph in Figure 2A.

7) Order of Figure No·3 is not correctly display/explained in text. Revised the text from line-162 to 177.

We are sorry for some errors in text. We performed quantitative RT-PCR for the expression of NANOG, MUC-1, TP63, NSE and E-cadherin under transfection of syndecan-1 siRNA (Figure 3C). As we described in “silencing of syndecan-1 by siRNA up-regulated some stem cell and differentiation markers (Figure 2B and data not shown)”. We have rewritten as follows:
“Similarly, silencing of syndecan-1 by siRNA up-regulated some stem cell and differentiation markers (Figure 3C). “
(page 9, line 181-182).

8) In Figure S1-B, add the name CD44 in the legend.

Thank you for your comments. CD44 was not changed under overexpression of miR-145 in KU-7 and T24 cells.
We have added the description in figure legends as follows:
“mRNA expression of stem cell markers (SOX2, NANOG, Oct4, and E2F3) was increased, but not CD44 under conditions of overexpression of miR-145.”
(Figure 3 and S1 legends)

9) Figure S1-D result is contradictory the as the statement used by author in legend as well as in text. (Figure has shown high Syndecan 1 expression after miR145 transfection): Line 430

Thank you for the reviewers’ comments. We performed this experiment again. The final result was obtained as decreased syndecan-1 expression after miR-145 transfection, which was the same result in T24 cells. We replaced Figure S1 (D) with new data.

10) Author has shown the experiment when cells are transfected with miR-145, Syndecan-1 mRNA expression is down regulated. Could author have conducted the
vice-versa experiment i.e. What is the effect on miR-145 expression after inhibiting the Syndecan-1 expression.

Thank you for your suggestion. We conducted the vice-versa experiment. As a result of suppression of syndecan-1, miR-145 expression was slightly increased without significant difference. These results indicate that miR-145 and syndecan-1 may interact each other in bladder cancer cells.

Minor points

1. There are many typographical errors in manuscript e.g. Syndecan-1.
   Corrected: Syndecan-1. In Figure 3B: mIR-145 pre

   We are sorry for our mistakes. We checked the text and Figures and corrected them.

2. Word “that” is repeated in line no-77.

   We are sorry for our mistakes. We corrected it.

3. Provide full IUPAC name of ‘MTS’ (Line-108)

   Thank you for the reviewers’ comments. Full IUPAC name of ‘MTS’ is “3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt”. We used the commercial kit “CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega)”. We replaced this description as follows: “CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) was used for MTS assay as previously described [11] to measure cell proliferation.”
We hope these extensive changes will now suffice for acceptance of our manuscript for publication in BMC cancer.

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