Author's response to reviews

Title: microRNA-99a acts as a tumor suppressor and is down-regulated in bladder cancer

Authors:

Yongming Kang (yongming_kang@126.com)
Yougang Feng (uroygfeng@sina.com)
Yue He (yueyue0826@163.com)
Jun Liu (tj620080@163.com)
Bo Liang (liangbo_16@163.com)
Ping Yang (yo1104@sohu.com)
Zhou Yu (6988344@qq.com)

Version: 3
Date: 18 April 2014

Author's response to reviews: see over
Dear Dr. Ampalaya,

Thank you for your and the reviewers’ comments and suggestions on our manuscript titled “microRNA-99a acts as a tumor suppressor and is down-regulated in bladder cancer” (manuscript ID 1343897201117672).

We have revised the manuscript according to the comments and suggestions. The revisions in the text are marked with red colour. The concrete revisions and explanations are described in the “Response to reviewers”. In addition, we ensured that the revised manuscript conforms to the journal style.

With the best regards,

Sincerely,

Yongming Kang
Department of Urology
Suining Central Hospital
127 Deshengxi Road, Chuanshan District
Suining 629000, China
Email: Kangyongming1979@163.com; yongming_kang@126.com
Tel: 86-0825-2292603
Response to reviewer 1

Reviewer's report

Title: microRNA-99a acts as a tumor suppressor and is down-regulated in bladder cancer

Version: 2 Date: 27 January 2014

Reviewer: Takahiro Ochiya

Reviewer's report:

In this manuscript, Feng et al. showed that miR-99a was down-regulated in bladder cancer patients and could be used as diagnostic marker. The overall concept of this study was correct and the experiments in this manuscript were well considered. The experimental method was pertinent and supported the conclusions. However, the work was too descriptive at this stage and major modification appeared necessary for the publication of this manuscript in “BMC Urology”.

Major Compulsory Revisions

1. The authors performed statistical analysis by using T-test. The authors should use another statistical analysis method because T-test is improper in these experiments. (e.g. Table 2 as multivariate statistics, Figure 1C and 2 as Mann–Whitney U test or Pearson’s chi-square)

   We appreciated the suggestions from the reviewer and used Bonferroni multiple-comparison test for statistical analysis in Figure 1C and 2. The method and the statistical analysis results have been revised in the revised manuscript.

2. In Figure 3B and 3D, the authors performed cell growth assay by using CCK-8 kit. The authors should check the reason why cell growth is inhibited in bladder cancer cells over-expressing miR-99a (e.g. cell cycle arrest).

   According to the suggestions from the reviewer, we checked the cell cycle distribution in HT1376 and J82 cells transfected with miR-99a mimics. The results showed that miR-99a could increase the fraction of cells in G1 phases
in HT1376 and J82 cells which suggested that miR-99a might inhibit bladder cancer cell growth through arresting the G1/S transition (Fig 3C, 3F).

![Fig 3C](image)

**Fig.3C** Cell cycle distribution of HT1376 cells transfected with miR-99a mimics.

![Fig 3F](image)

**Fig.3F** Cell cycle distribution of J82 cells transfected with miR-99a mimics.

3. The authors should provide detailed clinical data regarding bladder cancer patients.

We appreciated the suggestions from the reviewer, however, it is too much to provide detailed clinical data about every bladder cancer patient. Referring to the clinical data presented in the published papers about bladder cancer (Catto JW et al, Cancer Res 2009, 69:8472), we have supplied more detailed clinical data including smoker, recurrence, progression in Table2.
4. In Figure 1B, it was unclear why the authors decided the cutoff at 1.5. The authors should explain that point. The cutoff was just a threshold for judging the different expression of miR-99a in bladder cancer tissues (C) and non-neoplastic tissues (N). When the logarithm of the relative expression of miR-99a in C/N was greater than 1.5, it was considered to have increased levels of miR-99a in bladder cancer tissues compared with the corresponding non-neoplastic tissues and vice-versa. According to the published similar work (Gong J et al, Carcinogenesis. 2014, 35:497), we set the cutoff as 1.5. This point has been explained in the revised manuscript.

5. It was already reported that miR-99a is down-regulated in low-grade bladder cancer patients (Catto et al., Cancer Research. 2009). The difference of this manuscript with this previously published paper is unclear for the reader. It would be valuable to discuss the differences between both studies in the introduction section.

We apologized for the neglect of referring Catto’s paper in the introduction section. Consulting from the suggestion of the reviewer, we described the difference of this manuscript with Catto’s paper in the revised introduction and discussion section. Catto et al. reported that the expression of miR-99a is down-regulated in low-grade bladder cancer patients and also identified a target of miR-99a in bladder cancer progression. The differences of our study with Catto’s work are as follows: 1) We investigated miR-99a expression in tumor tissues and the adjacent non-neoplastic tissues which were derived from a common patient instead of different individuals to exclude the differences of miR-99a expression in different individuals. 2) In our study, the expression of miR-99a was investigated in more clinical samples. We detected miR-99a expression in 100 pairs of bladder cancer tissues and the adjacent non-neoplastic tissues. Catto et al. detected miR-99a expression in 52 bladder cancer samples and 20 normal urothelial samples. 3) In addition, we also measured the expression of miR-99a in the plasma of bladder cancer patients.
which will promote the study of circulating miRNAs in bladder cancer.

6. In figure 3B and 3D, the authors performed cell growth assay using two cell lines, HT1376 and J82. It was unclear why the authors did not use T24 cell line, which showed down-regulation of miR-99a expression. The authors should explain that point.

We performed cell growth assay in HT1376 and J82 cells because there was lower expression of miR-99a in this two cell lines than in T24 cells. The results in the two cell lines were consistent and showed that overexpression of miR-99a could reduce cell proliferation of bladder cancer cells, so we did not repeat the cell growth assay in T24 cells. According to the suggestion from the reviewer, we have explained this point in the revised manuscript.

7. The authors should provide histologic examination of normal and bladder cancer patients stained with HE.

We appreciated the suggestions from the reviewer and provided the HE staining of the normal and bladder cancer tissues in Fig 1B.

8. The authors should shorten the manuscripts in the discussion section.

We have deleted some sentences which are repetitive with the results or the introduction section to shorten the discussion.
Response to reviewer 2

Reviewer's report

Title: microRNA-99a acts as a tumor suppressor and is down-regulated in bladder cancer

Version: 2 Date: 21 March 2014

Reviewer: Ross M Drayton

Reviewer's report:
This article uses appropriate and well-defined methods to answer a clearly defined question, namely whether microRNA-99a is downregulated in bladder cancer tissue, and whether this alteration is detectable in plasma. The data generated is sound and in the main supports the conclusion. Justification for the study of microRNA-99a in bladder cancer is given by reference to a single paper (Han et al 2011), however, there are papers published before this that have identified this dysregulation (for example Catto et al, Cancer Research 2009). The manuscript adheres to the relevant standards for reporting. The authors understand the limitations of their data and the conclusions drawn are appropriate. The paper would benefit from some discussion of the potential limitations of detecting microRNA-99a downregulation in the blood. For example, presumably the rest of the (healthy) bladder (and many other tissues and organs) continue to deposit high/background levels of microRNA-99a into the plasma. How likely is it that a modest downregulation in a relatively small number of cells (ie, the tumour) is going to have a measurable effect on plasma microRNA-99a levels? Other possible explanations could be offered and should be discussed. The writing on the borderline of reaching an acceptable standard. There are several spelling and grammatical errors that do not detract from the overall meaning.

Minor Essential Revisions

1. Re-write Background section to acknowledge work on microRNA-99a in
bladder cancer that pre-dates this study.

We apologized for the neglect of referring Catto’s paper in the manuscript. Consulting from the reminding of the reviewer, we have described the difference of this manuscript with Catto’s paper in the revised introduction section.

2. Add further discussion on limitations of plasma-based microRNA biomarkers for detecting downregulated molecules in tumour cells as mentioned above.

We appreciated the suggestions from the reviewer and discussed the possible explanations for the down-regulation of miR-99a in plasma. The origin of tumor-associated miRNAs in circulation remains unanswered. Current opinion deems that cancer-associated miRNAs in the circulation may be secreted by cancer tissues, also can originate from immunocytes in the tumor microenvironment (Ma R et al, J Exp Clin Cancer Res. 2012, 31:38). As the reviewer speculated, it was difficult to understand that down-regulation of miR-99a in a relatively small number of tumor cells can have a measurable effect on plasma miR-99a levels. We think there are two possible explanations for the down-regulation of miR-99a in plasma of bladder cancer patients: 1) The down-regulation of miR-99a in the cancer tissue was significant to be able to have effect on plasma miR-99a levels, meanwhile, the Taqman probe stem-loop real-time PCR was sensitive enough to detect the faint change of miR-99a levels in plasma. 2) The down-regulation of miR-99a in the plasma not only origin from the tumor cells but also from the immunocytes in the tumor microenvironment which needs to be improved further.

3. Further additional Proof-read

We appreciate the nice suggestion from the reviewer. We have tried our best to correct the grammatical errors and the unclear sentences in the revised manuscript.