Author's response to reviews

Title: Expression and role of oncogenic miRNA-224 in esophageal squamous cell carcinoma

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Author's response to reviews: see over
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Ms. Annie Lyn Bravo
BMC Cancer

RE: Resubmission of our manuscript (#1812497771146365)

Dear Ms. Bravo,

Thank you very much for your email with encouraging news regarding our manuscript. We also thank the reviewers for their positive/constructive comments and suggestions, which truly helped us to improve our manuscript. After incorporating their comments into the revised manuscript, I would like to re-submit it for your consideration for publishing in BMC Cancer. The amendments are highlighted in red in the revised manuscript, and our point-by-point answers to the reviewers’ comments are attached below. This manuscript has been edited and proofread by Medjaden Bioscience Limited (Hong Kong, China).

Thank you again, and I hope that the revision is acceptable. I am looking forward to hearing from you soon.

Sincerely,

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Our responses to the reviewers’ comments:

Reviewer #1

Major points
1. The qRT-PCR methodology needs to be better described and/or corrected. Primer sequences for the RT primers and PCR primers for miRNA 224 and U6 need to be provided. Also, the methods state that expression was calculated as $2^{-\Delta\Delta CT}$ but this is not what is shown in Figure 1A and 1C. Instead, the $\Delta\Delta Ct$
appears to be plotted or perhaps the -ΔΔCt. This is confusing and harder to interpret than simply plotting the actual relative expression as described in the methods. This should be corrected.

We fully understand the reviewer’s concern and agree. We also appreciate the constructive suggestions. We have revised them accordingly. However, the primer sets of miR-224 and U6 were designed by RiboBio (Guangzhou, China) and the sequences were not disclosed. We have contacted the company staff again for asking the sequences, but still no gains. Thus, it is a pity that we can’t provide them in the revised manuscript (previous publications in Ref 1 and 2 also didn’t disclose them).

The relative miR-224 expression levels were calculated using $2^{-\Delta\Delta Ct} = 2^{-[(\text{Ct of miR-224}) - (\text{Ct of U6})]}$. The increasing folds of miR-224 were calculated using $2^{-\Delta\Delta CT} (\Delta\Delta CT = \Delta CT_{\text{IEU/ESCC}} - \Delta CT_{\text{Normal}})$. The data we obtained according to the above two formulas were skewed distribution, which can not be directly used for statistical analysis. Thus, according to statistical principle, all data were applied for the statistical analysis after logarithm transition(y=LnX) and then shown in results. A statement have been added in the Methods of the revised manuscript(Page 8, Line 9-10) to address this issue.

2. Page 12, lines 10-15. This section is very confusing. ESCC cases were split based on miRNA 224 expression levels and this was used for calculations in Table 1 but is this necessary? Why not use expression as a continuous variable and compare the clinicopathologic features (as appears to be the case in Figures 1B and 1C)? Also, the difference between the two panels in figure 1C is unclear. What is the difference between TNM and pathologic stage?

Thank you for raising this critical issue. To investigate clinicopathological significance of miR-224 expression in ESCC patients, several statistical methods including mono factor analysis of variance have been used. Whether miR-224 expression was analysed as quantitative or qualitative data, we obtained similar result. However, when miR-224 expression was analysed as continuous variable, results showed that standard deviation was too large, sometimes approached the mean. Thus, we chose Chi-square test, which is also used by Liao WT[3] in his report, to analyse these data and reduce the noise (Page 26, Lines 3-6).

To further investigate the relationship between miR-224 expression and TNM stages or pathological grades in ESCC patients, additional statistical analysis were done and results were shown in column diagram. The trend of TNM group is similar with that of pathological group. Thus, we obtained these two similar column diagram. This is just an interesting coincidence.

3. Figure 6. Data should be shown from all 12 cases.

We fully appreciate the reviewer’s suggestion. Indeed, it is true that we showed data on all 12 cases in sFig. 3.

4. The conclusion that the oncogenic activity of miRNA 224 is a result of targeting PHLPP1 and 2 and the subsequent AKT activation is likely but not proven. There is no direct proof provided that the invasion, migration etc.
phenotypes are driven by PHLPP1 and 2 or by AKT activation. The authors should either attempt to make this connection or change their conclusions accordingly.

Thank you for your insightful suggestion. Our existing results indicated that miR-224 acts as an oncogenic miRNA in ESCC by targeting PHLPP1 and PHLPP2. No direct proof showed that PHLPP1/2 activation and the subsequent AKT activation would change phenotypes of ESCC cell. The conclusion have been changed in the revised manuscript(Page 3, Line 17-21; Page 16, Line 14) according to our results.

**Minor revisions**

1. **Is IEN the same as dysplasia?** If so, which terminology is more commonly used for ESCC? This should be clarified and if appropriate, changed throughout the text.

   We fully understand the reviewer’s concern and agree. According to the 2010 WHO classification, the term ‘intraepithelial neoplasia’ was introduced as an inclusive term for dysplasia and squamous cell carcinoma in situ[4]. Now, IEN is more commonly used in ESCC.

2. **Page 12, line 7. Please remove the term “significantly overexpressed”.** As far as I can tell this is simply based on the arbitrary >2-fold increase and not a statistical test of significance.

   We fully understand the reviewer’s concern and agree by revised it accordingly(Page 13, Line 9-10).

3. **Page 7, line 17 and 18. How many mir-224 mimics were used?** It appears to be two but with completely different sequences. Please explain. It may be more helpful if all oligonucleotide sequences were provided in a table rather than in the text.

   We fully understand the reviewer’s concern and agree. In this study, we just used one double stranded miR-224 mimic, which was incomplete complementary. According to Genepharma company, it is more stable than the single strand sequences. We have clarified it in the methods section in the revised manuscript and used Table 1 to list the sequences(Page 8, Line 20-22; Page 9, Line 1-2; Page 26, Line 1-3).

4. **Page 15, line 12. This should read “increased AKT signaling.”**

   We thank the reviewer for the carefulness and corrected the typo(Page 16, Line 14).

5. **In general, the figure legends could do a better job of describing the actual data presented with less emphasis on the methodology.**

   We thank the reviewer for the suggestion and modified them accordingly(Page 22-23, Line 1-22).

Reviewer #2
Major points
1. The authors indicate that the sampled if low and high grade intraepithelial neoplasia were matched with normal tissue 5 cms away for the study tissue. They should clarify whether some of these different grades of squamous dysplasia or cancer specimens were obtained from the same patients i.e did some patients. If some of the dysplasia samples were obtained from patients who had already developed squamous carcinoma this would constitute a weakness of the study and should be mentioned.
We fully understand the reviewer’s concern and agree. After biopsies were obtained, we would check postoperative pathological results in time. Patients (only three cases) with a postoperative pathological results including both intra-epithelial neoplasia (IEN) and ESCC were included into ESCC group. And only those patients who had IEN only were included in the IEN group. When we did subgroup analysis, patients (only two cases) with a postoperative pathological results including both low-grade IEN (LG-IEN) and HG-IEN were included into HG-IEN group. A statement have been added in the Methods of the revised manuscript (Page 7, Line 10-12) to address this issue.

2. The justification for a distance of 5 cm between the study dysplasia or carcinoma tissue and normal tissue should be mentioned. Is this an arbitrary number?
We fully understand the reviewer’s concern and agree. The number (5 cm away from tumor lesion) is very much frequently used in esophagectomy and recommended by NCCN guideline[5] of esophageal cancer. We added such information in the revised manuscript accordingly (Page 7, Line 9-10).

Minor
1. Page 5 line 4 “studied” should be “studies”.
We thank the reviewer for the carefulness and corrected the typo.

Editor’s notes

1. Contact information - Please include email addresses for each author on the title page.
2. Ethics - Please revise the Methods section of your manuscript to include the name of the ethics committee that approved your study.
We either added or corrected them accordingly in the revised manuscript.

References


