**Author’s response to reviews**

**Title:** Epithelial-mesenchymal transition markers screened in a cell-based model and validated in lung adenocarcinoma

**Authors:**

Jing Song (songjingdlmu@sina.com)

Wenqing Wang (wenqingwang@ablifeco.cc)

Yingyan Wang (yingyan_wang2009@163.com)

Yongxin Qin (qyx0072002@sina.com)

Yingzi Wang (Wangyz0119@163.com)

Jian Zhou (jianzhou@ablifeco.cc)

Xuelian Wang (xuelianwang@ablifeco.cc)

Yi Zhang (yizhang@ablifeco.cc)

Qi Wang (wqdlmu@163.com)

**Version:** 1 **Date:** 07 Jun 2019

**Author’s response to reviews:**

Dear Editor Hodges and Gummlich,

On behalf of my co-authors, I am very grateful for the opportunity to revise our manuscript entitled "Epithelial-mesenchymal transition markers screened in a cell-based model and validated in lung adenocarcinoma" (manuscript ID BCAN-D-18-02688) in BMC cancer. After one month revision by performing additional experiments and modifying the manuscript, I am pleased to send you the revised manuscript, which my co-authors and I wish to be considered to be published in BMC Cancer. All authors have contributed significantly and are in agreement with the content of the revised manuscript.
We are also very appreciative of the two reviewers who provided valuable and professional comments and suggestions to our manuscript. Based on these comments, we have earnestly revised our manuscript by performing requested experiments, analyzing miRNA-seq and TCGA RNA-seq data, modifying the figures and tables (below).

- On the comments of the in vitro cell model mimicking early EMT development provided by both reviewers, we have clarified our description in background, results and discussion and focused our cell model on the early EMT progression and markers.
- We also redid the morphological change and enhanced invasive ability experiments in Fig. 1 and checked the ZEB1/ZEB2 expression level by RT-qPCR experiment in Fig. 2.
- For the miRNA expression profiling, we provided more detail information on the global and DEmiRNAs results. Specially, we performed miR-3613 mimic experiments in A549 cells and validated its repressive function on EGFR signaling pathway by RT-qPCR experiment.
- To provide a more available link between our cell model and clinical observations, we reanalyzed the integrated relationship between LUAD RNA-seq from TCGA and DEGs from in vitro cell model. We detected a module with 69 genes that showed increased expression level in early stages of LUAD (Fig. 7D), which is consistent with our main findings.
- Finally, we revised the abstract to be more intensive and adequate according to the suggestions of these two reviewers. We also provided a model figure to illustrate our strategy and main findings of the manuscript.

The point-for-point responses to the comments of these two reviewers were provided. You can find these responses in the “Response to Reviewer Comments” file. To indicate the changes in the revised manuscript and supplemental files, we used another text color (light blue) to highlight them. After this revision, we hope that the quality of our manuscript merit the standard for publication in BMC Cancer.

The following information is the responses to Reviewers

I am looking forward to your kind response.

Best regards,
Reviewer reports:

Rintaro Noro (Reviewer 1): The authors used a cell-based model mimicking the tumor microenvironment, which successfully stimulated the vimentin expression and repressed E-cadherin expression for proving EMT. This strategy would be very interesting, however there are many deficiencies,

Response: Thanks for the reviewer’s kind approval of our study and the critical comments. We have revised our manuscript according to the comments accordingly as detailed below.

1) These strategy was only one way for inducing EMT. Why did you decide this strategy?
Response: We have reorganized the introduction in which the reason of the strategy for inducing EMT is further clarified in the last and last second paragraphs (Lines 78-79, 87-91).

2) They discovered that several new transcription factors were among the earliest genes to respond to cancer micro-environmental cues which could play critical roles in triggering further EMT signals.
They should clarify the meaning of "earliest".
Response: We apologize for the confusion, and have changed “earliest genes” to “early genes (3h)” (Line 52).

3) Why the expression of early EMT hallmark genes, GALNT6, SPARC and HES7 were decreasing in stage III and IV? You have to do the mechanical study in advanced stage using in vitro or in vivo study.
Response: As we have described in the manuscript, these genes were considered as early EMT hallmark genes (Lines 366-368). The clinical samples from stage III and IV from TCGA database should represent the late or advanced stages of EMT (Lines 365-366). We will be very interesting to study the molecular mechanisms for the dynamic expression of these early EMT hallmark genes during cancer progress in the future, as suggested by the reviewer.
4) In this study, you had better include the clinical application in discussion. For example, predicting staging? or recur?

Response: We have added the potential clinical applications in discussion part (Lines 435-437).

5) Can you demonstrate the miR- will affect EGFR signaling using cell lines using EGFR mutation and A549?

Response: During this revision, we have transfected miR-3613 mimic in A549 cells to check its influence on the expression of EGFR signaling genes. The results showed that miR-3613 can significantly reduce the expression of genes in EGFR signaling pathway (Lines 349-355, Fig. S4C, Fig. 6E)

Reviewer 2 (Reviewer 2): PEER REVIEWER ASSESSMENTS:

OBJECTIVE - Full research articles: is there a clear objective that addresses a testable research question(s) (brief or other article types: is there a clear objective)?

Yes - there is a clear objective

DESIGN - Is the current approach (including controls and analysis protocols) appropriate for the objective?

No - there are minor issues

EXECUTION - Are the experiments and analyses performed with technical rigor to allow confidence in the results?

No - there are minor issues

STATISTICS - Is the use of statistics in the manuscript appropriate?
Yes - appropriate statistical analyses have been used in the study

INTERPRETATION - Is the current interpretation/discussion of the results reasonable and not overstated?

No - there are major issues

OVERALL MANUSCRIPT POTENTIAL - Is the current version of this work technically sound? If not, can revisions be made to make the work technically sound?

Maybe - with major revisions

Response: Thanks for your careful reading and evaluation of our manuscript. We have revised the manuscript according to your comments, which is detailed below.

PEER REVIEWER COMMENTS:

GENERAL COMMENTS: The problem identified in this work is real. It is indeed a challenge that the patient-derived observations are not exactly replicated in laboratory model systems. EMT is indeed important for cancer progression but we are still not any closer to exploit this in clinics.

Response: We are grateful to your recognition of the importance of our work.

Introduction is little too long and not very focused. It wanders from one topic to another and does not appear very coherent.

Response: We have seriously shortened the introduction, and make it more focused in the revised version.

My one concern is the model system itself. Conditioned medium from A549 cells was used to generate CAFs and then the conditioned medium from CAFs was used to induce EMT in A549 cells. Are the results reproducible in other lung cancer cells?
Response: The results are highly reproducible in A549, although we have not tried other lung cancer cells yet. We have recently published our success in CAF-induced EMT in PC9 cells (DOI: 10.1016/j.actbio.2019.04.053), which was added in the revised ms (Line 384).

Clearly, authors are trying to present A549-CAF system as the best system to study EMT - but this is not even mentioned in Abstract?

Methods section of Abstract is too inadequate. Did authors only conduct RNA seq and nothing else?

Response: We thank the reviewer for the critical comment, and have revised the abstract method to include other experiments applied in this study (Lines 44-50).

Conclusions described in Abstract are too over-reaching and do not truly reflect on results described in manuscript.

Response: Thanks for your comments! We have revised the conclusion in the abstract (Lines 61-64).

Figure 1A - while at 24 h, I can see elongation of cells in CAF group, there does not seem to be much difference at 72 h? Further, there seem to be more cells in Control group - does EMT inhibit proliferation? Also, Figure 1B needs a quantitative data. Finally, authors mention 3 and 6 hours while describing results, but only show 3 hours in the Figure.

Response: We have re-performed the experiment to improve the figure quality. In the revised ms, we replaced Fig. 1A-B with new experimental results. In the revised Fig. 1A, CAF-induced cell elongation can be found from 3h to 72h. The revised Fig. 1A also showed similar cell number between the control and CAF samples. We have also provided the quantitative results with statistical significance analysis (Fig. 1C) for Fig. 1B. We have removed the text description for 6 hours (Lines 207-212).

Figure 2 - my major concern is why EMT is not evident at 72 hours? EMT markers are not statistically significantly different at 72 hours. Does that mean there is no EMT at 72 hours? This does not go well with authors own description of Fig 1 results where they claim EMT at 72 hours (even though I do not see it - see my comments above). Is CAF-mediated EMT not sustained? In such a case, is the system even reliable?
Response: We concur with the reviewer’s observation. We suppose that the presented CAF-induced EMT cell model can effectively reflect early EMT stages, but not necessarily the later. From the RNA-seq and miRNA-seq data, we found the number of DEGs and DEmiRNAs were decreased by time points, as well as the detected marker genes. We thusly focused on identification of the early EMT hallmark genes. Results from the in vitro model has been well supported by those from analyzing the LUAD RNA-seq data from TCGA, i.e. these marker genes also showing elevated and then decreased expression pattern in the early and advanced stages of LUAD samples, respectively.

Authors have tested several EMT markers. I would suggest including ZEBs as well.

Response: Thanks for your suggestion! We have performed the suggested experiment (Fig. 2B, Lines 223-228).

miR-3613 effects on EGFR pathway are not adequately described. Were miR-3613 levels manipulated in cells with appropriate transfections before looking at EGFR pathway genes? In fact, even though authors describe in Results that miR-3613 was the most differentially expressed, they do not show any such data where a number of top miRNAs are presented for direct comparison! Is DEmiRNA same as miR-3613 Or is it some measure of all the differentially expressed miRNAs? In summary, the miRNA results are not very convincing, mechanistically speaking.

Response: Thanks for your comments! We have performed miR-3613 mimic experiment in A549 cells to check its impact on EGFR signaling. We have now provided the DEmiRNAs list in Table S6. In fact more than 50 miRNAs were identified as differentially expressed miRNAs at each time point. However, we only found that miR-3613 were consistently down-regulated from 3h to 24h upon CAF induction (Figure 6B, Fig. S4). We have improved this section by adding the new results from transfection of miR-3613 mimic which was followed by checking the expression level of its predicted targets in EGFR signaling pathway (Fig. S4, Fig. 6E, Lines 349-355)

The bioinformatics data is interesting but somehow there is no clear message. IS it possible to summarize main findings in a cartoon figure i.e. what novel information did we learn - what are the early vs late genes in EMT induction?
Response: Thanks for your suggestion! We have provided a model figure of our main conclusions (Fig. S5).

Finally, the study started with a premise that there is gap between in vitro systems and the clinical observations. It is not clear how this work bridged that gap. Isn't this yet another in vitro model which might still have discrepancies with clinical data? More robust correlation with TCGA data needs to be shown.

Response: We have shown that the elevated expression of early EMT hallmark genes in our in vitro model is well recaptured by the LUAD clinical data. We have better clarified this conclusion in the revised version. We have also provided the correlation analysis between upregulated genes from 3 to 24 hours with TCGA data (Fig. 7D, Lines 372-378).