Author's response to reviews

Title: YKL-40 Regulated Epithelial-Mesenchymal Transition and Migration/Invasion Enhancement in non-small cell lung cancer

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Author's response to reviews: see over
First of all, we appreciate the opportunity to revise our manuscript on our study entitled “YKL-40 regulate Epithelial-Mesenchymal Transition and enhance migration/invasion in non-small cell lung cancer”. We had taken your suggestions as well as those pointed out by the two reviewers. Taken together, based on the reviewers’ and your suggestions we had made positive changes. Based on your suggestions, we:

- Clarified the ethical point in our methodology
- Added new clinical data supporting our findings
- Have sent our manuscript to be copyedited

Reviewer 1

1. **Comment:** The major concern is lacking in vivo functional validation of the role YKL-40 in metastasis. An in vivo model would help support authors claim to analyze the impact that the microenvironment has EMT compared to cell culture.

   **Response:** Thank you for your suggestion. We have conducted in vivo analysis of the metastatic ability of YKL-40 transfected CL1-1 cell line and includes this result in figure 3.

2. **Comment:** The statement “We believe that YKL-40 may serve as therapeutic targets for NSCLC patients in the future.” May be an overstatement if only “diagnosis, 39% to 91% of patients with metastatic disease had elevated serum levels of YKL-40.” This is a very large deviation and may not prove meaningful for clinical testing. This large deviation is evidenced in the authors own western blot data in which patient 10 has tumor tissue with equal protein levels of YLK-40 than subject 1 non-tumor tissue. Additionally while some studies have found that YLK-40 increases, it only does so for 24 hours and decreases thereafter leading to the elevated levels are only detectible for a short period of time as evidenced by Salvatore, V., Teti, G., Bolzani, S., Focaroli, S., Durante, S., Mazzotti, M. C., & Falconi, M. (2014). Simulating tumor microenvironment: changes in protein expression in an in vitro co-culture system. Cancer Cell International, 14(1), 40.

   **Response:** Thank you for your comment on our study. In the first part of our result, we have include more supporting evidence, by providing YKL-40 IHC staining as well as analysis of YKL-40 gene expression from TCGA database which shows the upregulation of YKL-40 in all lung cancer patients. Moreover, previous study have proven that YKL-40 targeting antibodies does attenuates cancer progressions as reported by Faibish M, Francescone R, Bentley B, Yan W, Shao R: A YKL-40-neutralizing antibody blocks tumor angiogenesis and progression: a potential therapeutic agent in cancers. Molecular cancer therapeutics 2011, 10(5):742-751. Furthermore, the cited study is concordance with our result. In the study, it was shown that the expression of YKL-40 is significantly upregulated when it was cocultured with HF which simulate the effect of Tumor microenvironment. The elevated expression of YKL-40 was decreased after 24 hours, however the expression of YKL-40 remained detectable up to 96 hours (end point). Even so, the study was conducted in vitro, which cannot completely reflect the actual expression in cancer patients. But, lots of studies have reported the high expression of YKL-40 in cancer patients, especially those with poor prognosis as evidenced by following studies (statement added into manuscript lines 268-270)


3. **Comment:** It has also been shown that YKL40 is known to be regulated by PI3K/AKT rather than YKL-40 may play a role in the regulation of PI3K/AKT/mTOR cascade as claimed in the article as evidenced by Jeet, V., Tevz, G., Lehman, M.,

Response: Jeet, et al’s discussion of “YKL40 is known to be regulated by PI3K/AKT” in the paper was based on a study by Recklies et al, which was published in 2002. However, the study conducted by Recklies et al actually provides evidence of YKL-40 role in regulating PI3K/AKT, which is in concordance with our report. There are lots of study that proves the role of YKL-40 in regulation of PI3K/AKT in subsequent years as well. Following are two of the studies. (cited and stated in manuscript lines 293-298)


4. Comment: It will be interested to whether modulation of YKL-40 impacts on cell proliferation.

Response: Thank you for pointing out the potential correlation of YKL-40 and lung cancer cell proliferation. We have done the related experiment on this, and shows that YKL-40 indeed promotes the cancer cell proliferation significantly, as shown in the following graphs.

![YKL-40 and NSCLC Proliferation](image)

This real time cell proliferation experiment was performed using the RTCA DP instrument (Roche Diagnostics GmbH). However, since we have another investigation for the tumorigenesis role of YKL-40, therefore we did not include this part in our report.

5. Comment: Please justify the inclusion of the 5 cell lines measured in the invasion and migration assays and why they were placed in the respective categories.

Response: The inclusion of the 5 cell lines are to show the correlation of endogenous YKL-40 expression level of each cell lines with the migration and invasion ability of each cell lines. Furthermore, since each cell lines possess different characteristics, it’s necessary for us to do classification of low/high migrating cells before deciding which cells lines are suitable for the following experiments. Moreover, similar classification has been done on our previous study, reported in Chen WL, Kuo KT, Chou TY, Chen CL, Wang CH, Wei YH, Wang LS: The role of cytochrome c oxidase subunit Va in non-small cell lung carcinoma cells: association with migration, invasion and prediction of distant metastasis. BMC cancer 2012, 12:273.
1. **Comment:** No mention is made that any of the 10 patient samples were screened for any of the inflammatory conditions such as “asthma and chronic obstructive pulmonary disease” which could confound results as these conditions increase YKL-40.

**Response:** Thank you for mentioning this essential point. We have written additional information in line 107-108 clarifying that patients in this study are free from any inflammatory conditions.

2. **Comment:** The statement “It is believed that inflammatory mediators, such as pro-inflammatory cytokines of …IL-6,” is not correct. IL-6 has been shown to have anti-inflammatory properties as recently described by Reilly, S. M., & Saltiel, A. R. (2014). Countering inflammatory signals in obesity. [News and Views]. Nat Immunol, 15(5), 410-411. doi: 10.1038/ni.2874

**Response:** Thank you for your comment on our statement regarding IL-6 as pro-inflammatory cytokine. However, the study cited on the comment has not been proven completely. It is rather theoretical report. Moreover, the potential role of IL-6 as anti-inflammatory cytokine are limited to metabolic studies. IL-6 has been studied extensively for the past decades. It was widely accepted that IL-6 is a pro-inflammatory cytokine and numerous study, including the study cited in the comment and the following studies


3. **Comment:** The statement “high expression of YKL-40 correlated with poor prognosis in NSCLC patients.” should be elaborated on. Did the patients die of lung cancer or another illness? This is not clear in from the article.

**Response:** In according to the Kaplan Meier analysis, the K-M curve will drop only if the NSCLC patient were died of lung cancer, otherwise it will be marked by + symbol if the patients left the study or died of other illness, as reported in Rich JT, Neely JG, Paniello RC, Voelker CC, Nussenbaum B, Wang EW: A practical guide to understanding Kaplan-Meier curves. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 2010, 143(3):331-336.

4. **Comment:** Consider changing “further YKL-40 knockdown of the same cell” to “YKL-40 knockdown”; adding “cells” after “tumor than in non-tumor.” line 169; “knockdowned-cell” to “knockdown cells” line 187; “after overexpression or knockdown YLK-40 gene in NSCLC cell lines.” to “after YLK-40 overexpression or knock down in NSCLC cell lines.” Line 36-37; “gene was found” to “was” line 72; “in this study focus on YKL-40 correlated with” this study explored the relationship between YLK-40 and” line 75; “in a vertical slab gel unit” to “by vertical gel electrophoresis” line 136; delete “and instantly” line 139; “Following three times wash” to “After washing three times” line 143; “investigate” to “investigated” line 168; “in sustaining” to “sustained” line 220; delete “gene” or change to “genes” line 222; change “short survival in a number of poorly prognoses” to poor survival in” line 223;
**Response:** Thank you for your suggestions in our manuscript. We have made revision on our manuscript according to every points mentioned and further sent to be copyedited.

5. **Comment:** Thank you for your suggestions in our manuscript. We have made revision on our manuscript accordingly and further sent to be copyedited.

**Response:** Thank you for your suggestions in our manuscript. We have made revision on our manuscript accordingly further sent to be copyedited.

6. **Comment:** This sentence is a fragment “To investigate the YKL-40 expression level effect tumor migration and invasion in NSCLC.” Line 175

**Response:** Thank you for your suggestion. Revision made accordingly and further sent to be copyedited.

7. **Comment:** Clarify “shRNA to re-knockdown in CL1-1 YKL-40 overexpressed-cell.” Line 188, “YKL-40 knockdown-reoverexpression CL1-5 was reversely processing EMT (Figure 4B).” line 216

**Response:** Thank you for your suggestion. Revision has been made accordingly as follows

- Clarify “shRNA to re-knockdown in CL1-1 YKL-40 overexpressed-cell.”: Clarification was made as described in revised manuscript in Line 219-221
- “YKL-40 knockdown-reoverexpression CL1-5 was reversely processing EMT (Figure 4B).”: Clarification was made as described in revised manuscript line 255-257

8. **Comment:** A comma is needed after markers line 215, “Mesenchymal” shouldn’t be capitalized

**Response:** Thank you for your suggestion. The revision on this point has been made on every mesenchymal word throughout the manuscript.

9. **Comment:** The paragraph between lines 202 and 216 needs to be reworked due to poor use of the English language.

**Response:** Thank you for your suggestion. Revision has been made and further sent to be copyedited.

Reviewer 2

**Minor essential revisions:**

1. **Comment:** In the conclusion part, “We believe that YKL-40 is a major key factor on cancer metastasis in NSCLC.” Don’t use “believe” word. It is not scientific and professional word. You should change it to “all of our data suggest that YKL-40 is a major key factor in NSCLC metastasis.”

**Response:** Thank you for your suggestion. Revision has been made and further sent to be copyedited.

2. **Comment:** In the introduction part, Line 47-48: “The most frequently reported cases among lung carcinoma subtype” is not a complete sentence. Please revise it.(line 47-48)

**Response:** Thank you for your suggestion. Revision has been made in line 68-69 and further sent to be copyedited.

3. **Comment:** In the result part, Line 185-186: “To verify whether YKL-40 effect tumor migration/invasion, we constructed shRNA to knockdown its mRNA level in CL1-5 cells and YKL-40 gene to overexpress its mRNA level in CL1-1 cells” need to be revised. I suggest that: “to investigate the function of YKL-40 on cancer cell migration/invasion, we both knockdown YKL-40 in CL1-5 cells with its shRNA and overexpressed YKL-40 in CL1-1 cells with YKL-40 gene expression construct “. (line 186-188)

**Response:** Thank you for your suggestion. Revision has been made and further sent to be copyedited.
4. **Comment**: I would suggest the author to change the label of those cell lines in Figure 3. Like CL1-1.wt, CL1-1.Vec, CL1-1.YKL-40, CL1-1.YKL-40.KD. All of the labels need to be revised

**Response**: Thank you for your suggestion. Revision has been made in figure 3

5. **Comment**: Statistics need to be performed on those RT-PCR results and migration or invasion assay. P-value needs to be added to show the significance.

**Response**: Thank you for your suggestion. We had added the p-value indicator into migration and invasion results in figure 3 as well as PCR analysis result in figure 4.

6. **Comment**: English writing need to be significantly improved.

**Response**: As suggested by BMC cancer editor, we have sent our manuscript to be copyedited.

7. **Comment**: For determining the expression level of YKL-40 in lung cancer patients compared to non-tumor tissues. If possible, please provide representative images of YKL-40 IHC staining in tumor and non-tumor tissues.

**Response**: Thank you for your suggestion. We had included the comparison of YKL-40 IHC staining in lung cancer tissue and non-tumor lung tissue in Figure 1B.