

ANSWERING REVIEWERS

February 14, 2015



Dear Dr. Ma,

Please find enclosed the edited manuscript in Word format (file name: 16198--revised by author.doc).

Title: Up-regulation of Nemo-like kinase is an independent prognostic factor in colorectal cancer

Author: Wei Zhang, Jian He, Yan Du, Xian Hua Gao, Yan Liu, Qi Zhi Liu, Wenjun Chang, Guangwen Cao, Chuan Gang Fu

Name of Journal: *World Journal of Gastroenterology*

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The manuscript has been improved according to the suggestions of reviewers:

1. Format has been updated.
2. Revision has been made according to the suggestions of the reviewer.

Reviewer #1

NLK is an important regulator of several signal transduction pathways including Wnt and Notch signaling pathways, both of which play critical roles in tumorigenesis. Deregulation of NLK is closely associated with progression of several cancers. In this study, the authors found that NLK is overexpressed in colorectal cancer, and proposed that upregulation of NLK is an independent prognostic factor for colorectal cancer. Findings from this study are important for both cancer biology and translational cancer research.

Response: We thank the reviewer for the positive remark.

- 1) The title may be modified as “Up-regulation of Nemo-like kinase is an independent prognostic factor in colorectal cancer”.

Response: We thank the reviewer for the constructive suggestion and revised it accordingly.

- 2) Abstract: The transition from the background to the purpose of the study should be smoother. Write the aim, not the procedure, of the study, e.g. “The aim of the present study was to.....”, or “The present study was performed to”

Response: We thank the reviewer for the constructive suggestion and revised it accordingly.

- 3) What is the rationale that triggered you to assess NLK expression as a potential biomarker to predict colorectal cancer? This part needs extensive words, sentences, and paragraphs reorganization. Introduction: The background and rational reasons to perform this study are well introduced, but the transition from the background to the purpose of the study should be smoother. The transition sentence “Towards this end, we focused on nemo-like

kinase (NLK),....” is unusual. The authors should first described the current knowledge of NLK in tumorigenesis, and then raise a question or hypothesis to assess the excessive NLK expression as a potential biomarker to predict colorectal cancer.

Response: We fully understand the reviewer’s concern. The reason for us to investigate this particular gene as a biomarker was because NLK is an important regulator of several signal transduction pathways including Wnt and Notch signaling pathways, both of which play critical roles in colorectal tumorigenesis; thus we initialed the current study. According to your suggestions, we tried to improve our writing.

4) Results: The authors stated that “Furthermore, overexpression of NLK protein in the tumor tissues was further verified using western blot, and overexpression of NLK mRNA was verified using qRT-PCR with 10 cases of colorectal cancer and paired normal mucosae (Fig. 1). The level of NLK mRNA in the tumor tissues was significantly higher than that in the normal tissues ($p < 0.05$).”, but I did not find these data, please provided the Western blots and qRT-PCR data.

Response: We thank the reviewer for the carefulness. We fixed the typo (Western blots and qRT-PCR data were shown in supplemental Figure 1, not in Figure 1).

5) Overall, the manuscript is fairly written and organized. However, there are a few grammatical and language expression problems throughout the manuscript.

Response: This manuscript has been edited and proofread by Medjaden Bioscience Limited (Hong Kong, China).

Reviewer #2

This is an interesting paper assessing the role of nemo-like kinase in colorectal cancer cells. The methods and set of experiments is up-to- date and well designed and the conclusions are supported by the findings and a large clinical dataset.

Response: We thank the reviewer for the positive remark.

1) Authors should re-do all analysis separately for colon and rectal cancers since prognosis and therapy of these cancers are different.

Response: We thank the reviewer for this constructive suggestion and have re-done all analyses accordingly. The results of separate analyses are similar. The tables and figures of these separate analyses are shown as supplemental tables and figures.

2) If possible right-sided cancers should be also analyzed separately for the same purpose.

Response: We fully agree and performed the analyses accordingly, but the data are similar; thus, I chose didn’t show them since there are too many figures and tables.

Reviewer #3

The manuscript by Zhang et al., deals with the gene/protein NLK and its role in CRC. The authors analyse a large series of clinical cases using TMA and correlate the expression findings with clinical parameters. As an add-on, some functional in vitro studies are conducted which shall confirm the hypothesis basing on the biomarker analysis: NLK is a relevant molecule predicting CRC aggressiveness and prognosis. The language is acceptable and with the exceptions named in the following, the methods are suited to address the questions raised. The results are not always as convincing as wished by the authors. Thus, there are a number of major concerns which have to

be ruled out by the authors.

Response: We thank the reviewer for the positive comments and thoughtful suggestion! We have revised the manuscript accordingly.

1) Please indicate the number of disagreements (between X.H.G. and J.H.) for the IHC staining procedure.

Response: We fully understand the reviewer's concern. It is true that there was a number of disagreement, but the overall concordance rate was very high (>94%). Disagreements were then resolved by consensus. A description has been added to the revised manuscript.

2) Contrary to what is stated by the authors, there is no Figure showing the data of qRT-PCR of CRC and paired normal mucosa.

Response: We thank the reviewer for the carefulness. We fixed the typo (Western blots and qRT-PCR data were shown in supplemental Figure 1, not in Figure 1).

3) If you find that NLK expression is significantly higher in rectal than in colon cancer, then you must consequently perform the complete statistical analyses (shown in Table 1 and 2) separately for rectal and colon cancer - either in addition or instead of the "CRC" analysis.

Response: We thank the reviewer for this constructive suggestion and have re-done all analyses accordingly. The results of separate analyses are similar. The tables and figures of these separate analyses are shown as supplemental tables and figures.

4) My biggest problem lies in the in vitro data - they are overall not at all convincing and even the authors state in their discussion part that there are contrary effects described for DLD-1 as those found by the authors for HT29. First of all, please analyse more cell lines concerning then level of NLK expression; at least 5 cell lines of each molecular subgroup of CRC may give a better picture. Then, all functional analyses have to be done at least for one additional cell line.

Response: We fully understand the reviewer's concern. It is true that more cell lines could help us to make meaningful conclusion of these in vitro data. However, our current study was more to emphasize on the ex vivo data and the in vitro data are just confirmative. To this end, our data are consistent to Dieter's study (PMID: 21982235) and Chen's study (PMID: 25371216). Surely, we will continuously work on the mechanism and function of this molecule in vitro. Due to the funding and manpower issues, we couldn't perform additional experiments at this moment. Hopefully, the reviewer understand us. Thank you!

5) Additionally, I missed an explanation why MMP-2 was analysed as a functional target of NLK.

Response: In this study, we have selected several metastasis-related molecules (including MMP-2), but we only obtained positive result for MMP-2.

6) Finally, the data of the influence of NLK on the cell cycle - do you really consider a difference of maybe 4% between G1 and S phase as relevant? First, I doubt that these experiments have been properly performed. With all respect, but I've never seen such tiny SDs for real biological replicates of cell cycle analyses! And then, even if you may find it statistically sound - please have a unprejudiced look on Figure 6b - would this be convincing to you when reading it in a manuscript of a competitor?

Response: We fully understand the reviewer's concern and carefulness. These experiments have been properly performed according to the instruction manual of Flow cytometry cell cycle assay. We then double-checked our original data again, and found there was a difference of 9.03% in G0/G1 stage between the Lv-shNLK group and the Lv-shCon group. Also, there was a difference of 8.21% in S stage between the Lv-shNLK group and

the Lv-shCon group. And the original data of cell cycle assay were also shown in supplemental Table 5. We hope the original data would be convincing to the reviewer.

7) What about the clinical cases and their attribution towards the different molecular subclasses of CRC? These data should be generated and correlated with the NLK expression in order to get a clear picture. Here, I would agree that it may be too much work for "just one" manuscript. But, if you continue this work, consider this additional analyses.

Response: We thank the reviewer for the insightful suggestion. It is really clinically significant to clarify the role of NLK in different molecular subclasses of CRC and will be our next project to study.

8) I miss Lynch or HNPCC clinical cases to complete the picture.

Response: We didn't collect enough HNPCC cases. So it was not included in this study.

9) Please discuss the results of PMID: 25371216 and 24972723 which describe similar findings.

Response: We added discussion on them accordingly.

10) Did you really use 95% O₂ in the incubators?

Response: We used "5% CO₂ and 95% air" in the incubators. We fixed the typo accordingly.

11) Introduce "CRC" as an abbreviation.

Response: We added it accordingly in the revised manuscript.

12) HEK293T is not a CRC cell line.

Response: It is true that HEK293T is an embryonic Kidney cell line. We revised it accordingly.

13) Which lentivirus kit was used?

Response: We used 2 μ l of Polybrene (at a stock of 4 μ g/ μ l) to the 1 mL of virus/media at a final concentration of 8 μ g/mL.

14) It is not of interest to the reader, why you did not use 742 cases but 712 cases. It may be of interest, why you did not replace the "missing" 30 ones.

Response: We fully understand the reviewer's concern and it is the nature of tissue microarray. In our case, the missed case were relatively small and better than those from literature. The reason for us not to add these missed case was because i). the staining was difficult to control with the others and ii). We have "sufficient" cases and 30 case addition couldn't affect the overall data.

3 References and typesetting were corrected.

Sincerely,



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