Dear Editor,

Thank you very much for your letter and advice. We have revised the paper, according to reviewers. We responded point by point to the comments as listed below and had made the changes in the paper. We hope that the revised manuscript is acceptable for publication.

Thank you.
With best wishes,
Sincerely yours,
Linling Ju

Response to Reviewers
We would like to thank the reviews for the constructive comments.

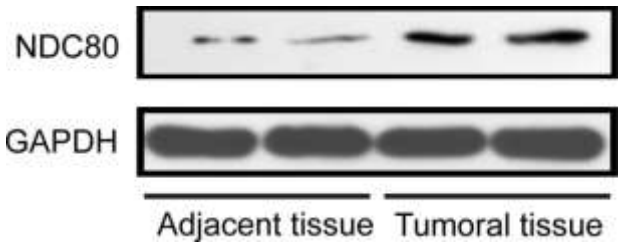
Response to Reviewers 1

The paper provides an interesting information on as aspect of the kinetochore protein, NDC80 and hepatocellular carcinoma. This study shows NDC80 gene levels are significantly up-regulated in hepatocellular cancer tissue, that indicates NDC80 has critical role for hepatocellular cancer progression. Then, the paper reveals NDC80 function in SMMC-7721 cells using lentivirus mediated siRNA methods. The paper clearly shows NDC80 is essential for proliferation and colony formation in SMMC-7721 cells. In addition, the authors also found NDC80 reduction in SMMC-7721 induced S-phase arrest. The authors need to be addressed following few concerns before publication.

Major concern

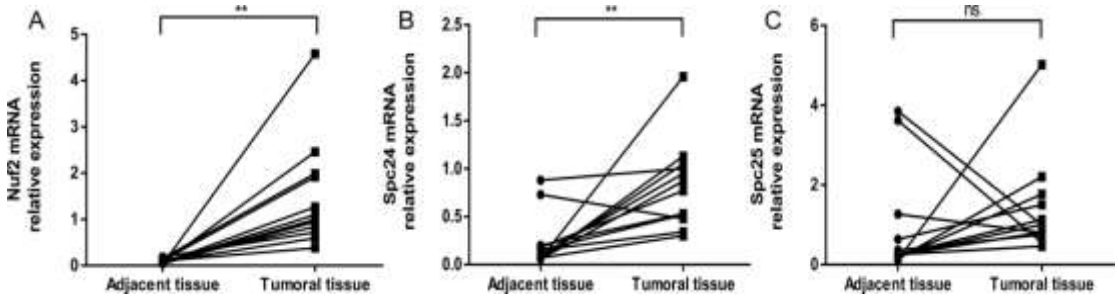
1) The authors tested only NDC80 gene expression levels in Figure 1A using cancer tissue whereas the manuscript keep mentioning NDC80 is overexpressed. Why the authors do not test NDC80 proteins levels using same tissue the authors tested mRNA levels? This experiments make strengthen of your conclusion and data quality.

Thanks for your suggestion and we agree with your opinion strongly. We have detected the level of NDC80 protein expression in HCC and adjacent tissues by western blot analysis. As showed in figure 1B, the similar trend on NDC80 protein levels was observed as on its mRNA levels by western blot analysis.



2) The authors mentioned in the introduction, NDC80 is a part of highly conserved Ndoc80 complex. NDC80 tightly binds Nuf2, Spc24 and Spc25. The paper needs to test whether only NDC80 is up-regulated in hepatocellular carcinoma or whole Ndc80 complex is up-regulated in hepatocellular carcinoma by qPCR.

Thanks for your suggestion and we have detected the expression levels of Nuf2, Spc24 and Spc25 in 12 pairs of HCC and adjacent tissue samples by real time quantitative PCR assays. As illustrated in figure A and B, the expression of Nuf2 and Spc24 was significantly enhanced in human HCC tissues in comparison to adjacent tissues. In figure C, there was no significant difference in the level of Spc25 expression between HCC and adjacent tissue samples.



As important components of the NDC80 kinetochore complex, Nuf2 and Spc24 are essential for kinetochore-microtubule attachment and chromosome segregation. Nuf2 was reported to be overexpressed in various kinds of carcinomas including hepatocellular carcinoma, lung cancer, gastric cancer,

ovarian cancer and colorectal cancer. Liu et al reported silencing of Nuf2 can inhibit tumor growth and induce apoptosis in human hepatocellular carcinomas [1]. Zhu et al reported high Spc24 expression as a new adverse independent prognostic factor in HCC [2].

1 Liu Q, Dai SJ, Li H, Dong L, Peng YP. Silencing of NUF2 inhibits tumor growth and induces apoptosis in human hepatocellular carcinomas. Asian Pacific journal of cancer prevention : APJCP 2014; 15(20): 8623-8629

2 Zhu P, Jin J, Liao Y, Li J, Yu XZ, Liao W, He S. A novel prognostic biomarker SPC24 up-regulated in hepatocellular carcinoma. Oncotarget 2015; 6(38): 41383-41397

3) The authors selected to use SMMC-7721 cells as a representative of hepatocellular cancer. However, it is unclear NDC80 gene and protein are upregulated in SMMC-7721 compared to control hepatocellular cells. The authors need to show NDC80 genes and protein are overexpressed in SMMC-7721 cells like the authors found in clinical samples in Fig. 1A.

4) Related to 3), it seems like NDC80 gene is significantly up-regulated in Hep3B and Huh-7 cells compared to SMMC-7721 cells. The authors should use one of these cells and repeat the experiments the authors performed using SMMC-7721 cells.

Thanks for your suggestion. The cell line employed by HCS must be lentivirus-friendly (multiplicity of infection, MOI<10) and enjoy a vigorous proliferation. Hence, SMMC-7721 was the most suitable cell model.

Minor concern

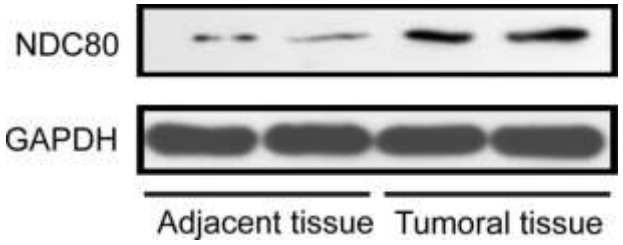
1) In the introduction, the authors should not use “our country” since the readers are all over the world. You can say “Especially in China, …” or other instead.

Thanks for your suggestion and we have rewritten the sentence as “Especially in China with a high incidence of hepatitis B, the magnitude of the problem

should never be underestimated."

2) In the results, first subtitle should be "NDC80 gene is overexpressed…" since the authors have tested only gene expression.

Thanks for your suggestion and we have added the content about the level of NDC80 protein expression in HCC and adjacent tissues in Results (Figure 1B).



Response to Reviewers 2

Major comments:

1. The NDC80 expression varied greatly in four hepatoma cell lines, can the authors give the possible reasons for this difference?

Thanks for your suggestion. Chromosomal aberration including gene amplification, gene deletion, and heteroploidy is a common event in carcinogenesis, which introduces gene dosage differences between normal cells and transformed cell lines. In addition, expression profiles of transcription factors could be difference in hepatoma cell lines. Therefore, the NDC80 expression varied greatly in four hepatoma cell lines.

2. In Fig 1 A & B, the authors give 'relative expression' for each quantitative RT-PCR NDC80 mRNA levels but we noticed that NDC80 expression were significantly lower in any hepatoma cell lines as compared to HCC tumoral tissue, please clarify it. (i.e. The authors need to label GAPDH level as its control for references.)

Thanks for your suggestion and we have explained as shown below.

Relative expression analysis: GAPDH was used as the reference gene. The relative levels of gene expression were represented as $\Delta C_T = C_T(NDC80) - C_T(GAPDH)$ and the fold change of gene expression were calculated by the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T = (C_T NDC80 - C_T GAPDH)experiment - (C_T NDC80 - C_T GAPDH)control$. C_T referred to the mean cycle threshold. Using the $2^{-\Delta\Delta C_T}$ method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene. In figure1 B, for SMMC-7721 cell sample, $\Delta\Delta C_T$ equals zero and 2^0 equals one, so that the fold change in NDC80 gene expression relative to other three cell samples equals one. It can better compare the expression level of NDC80 mRNA in four hepatoma cell lines.

HCC tumors and cell lines share remarkably similar gene expression profiles.

However, since the environment of cells growing in vitro is different from that in a heterogeneous tissue, the prolonged process of cell culture may induce substantial changes of the expression of some genes. Gene expression levels were in general low in comparison with those in human tissues.

3. Please explain why SMMC-7721 cell line but not any other cell lines was selected for functional studies.

Thanks for your suggestion. The cell line employed by HCS must be lentivirus-friendly (multiplicity of infection, MOI<10) and enjoy a vigorous proliferation. Hence, SMMC-7721 was the most suitable cell model.

4. The authors need to put (Figure 3B) in proper position in the text and label cell count unit in Figure 3B left figure.

Thanks for your suggestion and we have corrected as suggested. In figure 3B left figure, ordinate represents the specific number of the cells.

5. It would be interesting to see if different etiologies of HCC (i.e. HBV, HCV, etc.) shown different NDC80 gene expression in the tumor tissues (or cells), but in Table 1 the authors did not list etiologies of HCC as one of the clinical variable.

Thanks for your suggestion and we have added the content in Table 1.

Clinical Variable	NO. of patients (%)
Number of patients	47
Mean age ± SD (years)	56 ± 11
Gender (Female/Male)	
Female	18 (38.30%)
Male	29 (61.70%)
Serum AFP (ng/ml)	
≤400	36 (76.60%)
>400	11 (23.40%)
HBV infection	
Positive	38(80.9%)
Negative	9 (19.1%)
Largest tumor diameter, mean ± SD (cm)	5.7 ± 3.4
TNM stage (I/II/III)	
I	2 (4.25%)
II	30 (63.83%)
III	15 (31.92%)
Lymph node metastasis	
Positive	20 (42.55%)
Negative	27 (57.45%)

Liu B et al reported that the expression level of NDC80 was remarkably up-regulated in HBV-related HCC tissues. To some extent we confirmed their results.

Minor comments:

1. Recitation of appropriate references are strongly recommended for references 1~7.

Thanks for your suggestion.

2. For Major comments 5, the authors may cite the following article for one of the references and give their comments to it: Liu B et al. ShRNA-mediated silencing of the Ndc80 gene suppress cell proliferation and affected hepatitis B virus-related hepatocellular carcinoma. Clin Res Hepatol Gastroenterol. 2016 Jun;40(3):297-303. doi: 10.1016/j.clinre.2015.08.002. Epub 2015 Sep 14

Thanks for your suggestion and we have added the content in Table 1 and cited the related reference.