Author's response to reviews

Title: Truncated LEF-1 is one of the key regulators in the growth of colon carcinoma

Authors:

Shu-Hong Wang (wsh2003@126.com)
Ke-Jun Nan (nankj@163.com)
Yao-Chun Wang (Tanking@sina.com)
Wen-Juan Wang (Wangwenjuan1983@tom.com)
Tao Tian (Tiantao0607@163.com)

Version: 3 Date: 18 October 2011

Author's response to reviews: see over
Dear Dr Timothy Shipley,

Re: Manuscript reference MS: 6024328605352270

Please find attached a revised version of our manuscript “The balance between two isoforms of LEF-1 is a key regulator in the growth of colon carcinoma”, which we would like to resubmit for publication as an original article in BMC Gastroenterology.

Your comments and those of the reviewer were highly insightful and enabled us to greatly improve the quality of our manuscript. In accordance with the reviewer’s suggestion, we changed the title of our manuscript and modified Figures 1, 4, 5 and 7. In the following pages, please find our point-by-point responses to each of the comments of the reviewer as well as responses to your comments.

Revisions in the text are shown using red highlighting for additions, and strikethrough font highlights for deletions. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in BMC Gastroenterology.

We shall look forward to hearing from you at your earliest convenience.

Yours sincerely,
Dr Shu-Hong Wang PhD
Department of Medical Oncology
The First Affiliated Hospital of the School of Medicine of Xi’an Jiaotong University
Xi’an 710061, China
Tel: +86-29-85324086
Email: wsh2003@126.com
Responses to the Reviewer's comments

Major comment:

1 Question: In the methods section it is not specifically stated how many patient samples were obtained and analyzed. Was the total number evaluated 22 as stated in the results section? Additionally, the authors mention obtaining tumor samples and adjacent normal tissue. Yet in the results section (see Figure 1) three types of tissue samples were evaluated, colon carcinomas, adjacent tissue AND normal tissue. It is not clear from the information available what exactly the samples analyzed represent. This needs to be clarified.

Response: The total number of patient samples obtained and analyzed was 22. Every patient sample had been divided into three parts, namely tumor tissues, adjacent tissues and normal tissues. Adjacent tissues: Their distance from tumors was greater than 2 cm, but less than 5 cm. Normal tissues: Their distance from the tumors was greater than 5 cm. Every part of each sample was analyzed for the expression of LEF-1. The number of patients has been added in Methods.

2 Question: Moreover, in Figure 1, are the Western blots of the three tissue samples from two or more patients? Presumably these are representative results. The authors should indicate in each case, how many they are representative of. The authors should indicate in all these panels where appropriate molecular weight markers migrate.

Response: The expression of different LEF-1 isoforms was detected by western blot in every part of the 22 samples. In Figure 1, two representative sample results are shown. In Figure 1, molecular weights of LEF-1 isoforms and His tags have been added.

3 Question: In Figure 2, cells are compared after analysis of the cell cycle by flow cytometry. Rather surprisingly, no increments in the sub G0/G1 population are detected for both the SW480-dL and HT29-dL cells. This would be expected based on the result shown in Figure 3 where an increase in Annexin-V positive cells is observed for both these cell lines. The authors need to provide an explanation for this discrepancy and preferably should include the percentage cells in sub G0/G1 in the analysis shown in Figure 2.

Response: In fact, our results showed that the sub G0/G1 population increased significantly for both the SW480-dL and HT29-dL cells, as indicated in Figure 2E and 2F. These results have statistically significant differences, shown in Figure 2G and 2H.

4 Question: In Figure 4, the authors talk about a colony forming assay in panels A-D. In is not clear from the description provided what exactly the authors are measuring here. Is this anchorage-independent growth in a colony
forming assay? This needs to be explained better in all relevant sections. In panels B and D, the y-axis should probably be labeled “Colony numbers” rather than “Clone numbers”.

**Response:** A plate colony-forming assay has been widely used to examine the proliferation of tumor cells. The process of the plate colony-forming assay mainly refers to the article “Clonogenic assay of cells in vitro” by Franken NA et al. in Nat Protoc. 2006; 1(5):2315-9. Briefly, tumor cells were plated in 35-mm plates at a density of 500 cells per well in the complete medium. Cells were cultured at 37°C in 5% CO₂ for 14 days. After fixation using methanol for 10 min, the cells were stained with Giemsa stain for 15 min and colonies (with more than 50 cells) were photographed and counted by Image Pro Plus 6.0 software. Each experiment was repeated at least three times, and data were analyzed with one-way ANOVA analysis and LSD-t test. In panels B and D, the y-axis “Clone numbers” has been replaced by “Colony numbers”.

5 **Question:** In Figure 5, the authors analyze expression of their his-tagged constructs in tumor-derived cells. From the methods section, it is not clear how these cells were obtained. Moreover, all tagged proteins shown are of the same size. According to the results in Figure 1, LEF-1-dL is notably smaller. The authors need to explain better what is being demonstrated. Also molecular weight markers should be included.

**Response:** In Figure 5, tumor tissues were first cut into small pieces and then digested by 1% collagenase IV at 37°C for 1 hour. Single cells were produced using 200 mesh and were lysed by RIPA buffer for western blots. As mentioned in Methods, two isoforms of LEF-1 were cloned into the plasmid of pCDNA3.1/V5-His. In this expression system, His tag and LEF-1 variants were expressed independently for their genes did not fuse together, which could avoid the influence of His tag protein on the function of LEF-1. Therefore, all proteins were of the same size when His tag was detected. In Figure 5, the molecular weights of His tag have been added.

6 **Question:** In Figure 6, tumor growth is documented for the different HT29 and SW480 variants. In Figure 7, vascularization of tumors is characterized using different markers in one set of tumors. However, the information provided is confusing. In the figure panels, HT29 is mentioned while the figure legend states that tumors derived from both HT29 and SW480 cells were analyzed. This needs clarification. Also, the authors should evaluate whether reduced tumor vascularization observed for cells expressing the LEF-1-dL variant is also significantly reduced in tumors of the same size. The current analysis detects differences in tumors of different sizes.

**Response:** In Figure 7, vascularization of tumors only formed by HT29 was detected, and we did not examine that of SW480. This error in the figure legend has been corrected. Before tumor samples were sectioned into a thickness of 10 µm, we cut them into small pieces with approximately equal
size (about 100 mm$^3$) at random. In fact, vascularization of different tumors was compared in the same tumor sizes.

7 Question: The conclusion at the end of the discussion is that “the balance between the two forms of LEF-1 might have important consequences for normal growth of colon cancer cells and cancer”. This statement doesn’t really coincide with the message provided by the title “Truncated LEF-1 is one key regulator in the growth of colon carcinoma”. The title needs to be altered to reflect better the findings of the paper.

Response: The title “Truncated LEF-1 is one key regulator in the growth of colon carcinoma” has been changed to “The balance between two forms of LEF-1 is a key regulator in the growth of colon carcinoma”.

8 Question: The English of the manuscript needs major revision preferably by a native speaker. There are a number of confusing statements throughout the text.

Response: According to editor’s suggestion, our manuscript has been edited by an Edanz editor.

Minor comments:

1 Question: Figure 1. should be HeLa rather that Hella.

Response: This error has been corrected in Figure 1.

2 Question: Throughout the text several abbreviations are not explained (CSFE, SDF-1, CXCR4, MMPs, CDK etc). Also is the abbreviation Hif-1a or HIF-1a?

Response: CSFE, SDF-1, CXCR4, MMPs, and CDK have been explained in Abbreviations. Hypoxia-inducible factor 1a should be abbreviated as HIF-1a and we have changed all incidences of “Hif-1a” to “HIF-1a” in the manuscript and Figure 7.