August 25, 2014

Dear Editor,

On behalf of the authors, I am pleased that World Journal of Gastroenterology has found our manuscript acceptable for publication, pending revisions. Please find appended our revised version of WJG-12640, "Overexpression of High-mobility Group Box 1 (HMGB1) Correlates with Lymph Node Metastasis and Poor Prognosis in Intrahepatic Cholangiocarcinoma". We appreciate the comments of our reviewers and editorial staff, and have revised the manuscript to address their concerns. With these revisions, we hope that this manuscript is now acceptable for publication. Please do not hesitate to contact me if you require any additional information.

Sincerely,
Bo Han, M.D., Ph.D.

Title: Overexpression of High-mobility Group Box 1 (HMGB1) Correlates with Lymph Node Metastasis and Poor Prognosis in Intrahepatic Cholangiocarcinoma

Author: Yunfei Xu, Fujian Ge, Jing Zhang, Xiaoqing Yang, Hong Su, Ancheng Zhao, Minghong Zhao, Yubao Yang, Bo Han

ESPS Manuscript NO: 12640

Point to point response

Reivewer 1.

1 Whether the "results define an important role of HMGB1 in the progression of cholangiocarcinoma", as proposed by the authors, remains uncertain. In vivo tumor models would be needed to strengthen the conclusions. The authors might either wish to expand functional studies to in vivo models or put drawn conclusions into perspective throughout the paper.

Reply: Thank you very much for your suggestion. We agree that lack of in vivo data is a major limitation of our study and we have discussed this point appropriately in the revised version. Based on your suggestion, we have carefully changed the drawn conclusions into perspective points and we hope that this publication can trigger the further investigation on the role of HMGB1 in IHCC in vivo. Thank you again for your suggestion.

2 Using immunohistochemistry (IHC) for the expression of HMGB1 and others, did the authors differentiate between the center of the tumor and the invasion front?

Reply: Thank you for your comment. In pre-trial IHC experiment, we used tissue sections and found no significant difference of HMGB1 expression between the center of the tumor and the invasion front. In the current study, we used the tissue microarray (TMA) for IHC analysis. We select the representing tumor area of the case, which is usually from the center of the tumor with absence of necrosis.

3. Please indicate whether the used antibodies were monoclonal or polyclonal! If

polyclonal antibodies were used, where there non-specific bands in western blotting or unspecific staining in IHC?

Reply: Thank you for your comment. The anti-HMGB1 antibody used in this study was from GenTex Inc (GTX101277, polyclonal rabbit antibody). We have updated the antibody's information in the revised manuscript. This antibody has satisfied specificity to the antigen. In the datasheet of this antibody, the IHC and western blotting images are also very clear (antibody website: http://www.genetex.com/HMGB1-antibody-GTX101277.html).

4. As indicated under "Materials & Methods", the semiquantitative IHC scoring system was based the intensity and distribution of cells. The authors might wish to refer to published papers using this score system.

Reply: Thank you for your suggestion. We have added new reference in new manuscript.

5. IHC slides with scores of 8 or higher were classified as "overexpression" – why 8 was the cutoff? Does a score of 8 or higher really define samples with an "overexpression"? What is the reference tissue? The authors might wish to better distinguish between "low expression" and "high expression"? Further, tumors above 12.7 were classified as "high" LMVD group – why 12.7 was the cutoff? For the sake of consistency, the authors might wish to define groups according to "lower cutoff" and "cutoff or higher".

Reply: Thank you for your comment. IHC score "8" was selected as the cut-off according to the previous study[1]. We have added this reference into the revised manuscript. In pre-trial IHC experiment with tissue sections, we carefully compared HMGB1 expression in different IHCC cases. The reference tissues were adjacent residual bile dutal epthelia. Slides without incubation of primary were selected as the negative control (score "0"). Based on our preliminary IHC data and the previous reference, we selected "8" as the cutoff for "overexpression".

The quantitation of vessel counts were performed according to the method described by Weidner et al[2]. The average score of LMVD of all samples was selected as the cut-off. The cut-off of LMVD was 12.7 and separated LMVD into high and low group[3].

6. The authors evaluated a possible link between HMGB1 and VEGF-C. It remains unclear, why they did not check for VEGF-D!

Reply: Thank you for your comments. VEGF-C and VEGF-D have similar functions and both are correlated with lymphatic vessel density and lymph node metastasis in malignancy. As far as we know, VEGF-C, but not VEGF-D, has been suggested to serve as an important prognostic factor in IHCC patients [4-6]. VEGF-C is thus more generally regarded as a biomarker of IHCC.

Additionally, there are several reports predicting the correlation between HMGB1 and VEGF-C[7, 8]. Characterization of the role of VEGF-D in IHCC merits further investigation. However, we focused on the link between HMGB1 and VEGF-C in our study. Thank you again for you advice.

7. In table 1 "*" indicates values being analyzed by Fisher's exact test. This symbol could be misleading, as it is usually used for levels of significance. Please change!

Reply: Thank you for the advice. We have changed the symbol to "#"

8. Figure 2 shows box blots for groups "HMGB1 Positive" and "HMGB1 Negative". Indeed, the groups the authors refer to might be "HMGB1 high" and "HMGB1 low".

Reply: Thank you for your advice. We have changed "HMGB1 Positive" and "HMGB1 Negative" to "HMGB1 high" and "HMGB1 low" in the revised version.

9. Figure 3 shows Kaplan Meier curves. By accident, group HMGB1+VEGF-C+ is missing in the legend.

Reply: Thank you for pointing out this error. We have changed Figure 3 into right version.

Reviewer 2

1. The number of patients is quite small, making it difficult to draw the conclusions hinted at by the authors.

Reply: Thank you for your comments. Cholangiocarcinoma is a rare malignancy. We have mentioned this limitation in the discussion of the revised manuscript and revised some conclusions to be more objective and accurate.

2. The turning point of 12.7 for LMVD becoming a relevant prognostic Parameter seems rather deliberately constructed.

Reply: The quantitation of vessel counts were performed according to the method described by Weidner et al[2]. The average score of LMVD of all samples was selected as the cut-off. The cut-off of LMVD was 12.7 and separated LMVD into high and low group[3].

3 The DISCUSSION section lacks critical evaluation of the presented data, weak points of the study as well as potential bias factors should be discussed.

Reply: Based on the reviewer's suggestion, we have discussed the evaluation of the current data, weak points as well as potential bias in the revised manuscript by an entire paragraph.

4. Some minor language flaws persist.

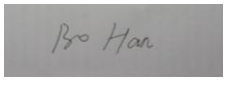
Reply: Thank you for your comments. We have invited a native speaker to review our article and revised the language flaws.

3) References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

Bo Han, M.D., Ph.D.



Reference:

1 Yang X, Wang W, Wang C, Wang L, Yang M, Qi M, Su H, Sun X, Liu Z, Zhang J, Qin X, Han B. Characterization of EGFR family gene aberrations in cholangiocarcinoma. *Oncology reports* 2014; **32**(2): 700-708

[PMID: 24927194 DOI: 10.3892/or.2014.3261]
2 Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *The New England journal of medicine* 1991; **324**(1): 1-8 [PMID: 1701519 DOI: 10.1056/NEJM199101033240101]
3 Mobius C, Demuth C, Aigner T, Wiedmann M, Wittekind C, Mossner J, Hauss J, Witzigmann H. Evaluation of VEGF A expression and microvascular density as prognostic factors in extrahepatic cholangiocarcinoma. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology* 2007; **33**(8): 1025-1029 [PMID: 17400419 DOI: 10.1016/j.ejso.2007.02.020]
4 Sirica AE, Dumur CI, Campbell DJW, Almenara JA, Ogunwobi OO, Dewitt JL. Intrahepatic Cholangiocarcinoma Progression: Prognostic Factors and Basic Mechanisms. *Clinical Gastroenterology and Hepatology* 2009; **7**(11): S68-S78 [DOI: 10.1016/j.cgh.2009.08.023]
5 Park BK, Paik Y-H, Park JY, Park KH, Bang S, Park SW, Chung JB, Park YN, Song SY. The Clinicopathologic Significance of the Expression of Vascular Endothelial Growth Factor-C in Intrahepatic Cholangiocarcinoma. *American Journal of Clinical Oncology* 2006; **29**(2): 138-142 [DOI: 10.1097/01.coc.0000204402.29830.08]
6 Sirica AE, Dumur CI, Campbell DJ, Almenara JA, Ogunwobi OO, Dewitt JL. Intrahepatic cholangiocarcinoma progression: prognostic factors and basic mechanisms. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* 2009; **7**(11 Suppl): S68-78 [PMID: 19896103 PMCID: 3795391 DOI: 10.1016/j.cgh.2009.08.023]
7 He W, Tang B, Yang D, Li Y, Song W, Cheang T, Chen X, Chen L, Zhan W, Li W, He Y. Double-positive expression of high-mobility group box 1 and vascular endothelial growth factor C indicates a poorer prognosis in gastric cancer patients. *World journal of surgical oncology* 2013; **11**: 161 [PMID: 23866030 PMCID: 3734148 DOI: 10.1186/1477-7819-11-161]
8 Qiu Y, Chen Y, Fu X, Zhang L, Tian J, Hao Q. HMGB1 promotes lymphangiogenesis of human lymphatic endothelial cells in vitro. *Med Oncol* 2012; **29**(1): 358-363 [PMID: 21181308 DOI: 10.1007/s12032-010-9778-7]