Author's response to reviews

Title: Co-expression of nuclear and cytoplasmic HMGB1 is inversely associated with infiltration of CD45RO+ T cells and prognosis in patients with stage IIIB colon cancer

Authors:

Rui-Qing Peng (gz13724083175@126.com)
Ya Ding (dingya@mail.sysu.edu.cn)
Xiao-Jun Wu (wuxiun@mail.sysu.edu.cn)
Chun-Yan Li (lichyanun@163.com)
Xing-Juan Yu (yuxj@sysucc.org.cn)
Xing Zhang (xingzhang@hotmail.com)
Zhi-Zhong Pan (panzhzh@mail.sysu.edu.cn)
De-Sen Wan (wds-fk@yahoo.com.cn)
Li-Ming Zheng (zhenglm@mail.sysu.edu.cn)
Yi-Xin Zeng (zengyix@mail.sysu.edu.cn)
Xiao-Shi Zhang (zxs617@hotmail.com)

Version: 3 Date: 17 November 2009

Author's response to reviews: see over
Dear Editor-in Chief:

Thank you for the review on our manuscript (MS: 1625007225303036). Now I answer the comments as follow:

For comment from Editor:

1. Please also document in the Methods section of the revised version of the manuscript the details of the institutional review board that granted ethical approval for the study, and the type of consent sought from the participants.

   Answer: This manuscript was a retrospective study, so there was no need to be granted ethical approval for the study, or sought consent from the participants.

For comment from Reviewer 1

1. The main criticism is related to the method used by the Authors to estimate the density of immunological cells inside the neoplastic tissue. The method is extremely subjective. In addition, several data have been omitted and should be added to replicate the described results.

   • In particular, how the Authors counted the immune cells? What is the extension/size of the tissue sections that were? I.e., the Authors indicate only 10 different fields but not report what is the extension of each field.
How the Authors distinguished and counted the cell clusters in the analyzed tissues? What criteria dictate a cut-off of 16 cells for CD3 density and of 24 cells for CD45RO density?

**Answer:** I had added some data in the Methods section to replicate the described results. We counted the immune cells referring to Hussein’s method (*J. Clin. Pathol.* 2006, 59:972-977). Two observers counted the immune cells infiltrated into the stroma of cancer center at the same time and the same field, using a multiple-lens microscope. Then they marked down the numbers of the immune cells of each field. The results were expressed as the mean. If inconsistency existed, a third pathologist served to achieve consensus. The size of each tissue section was about 1cm × 1.5cm. The extension of each high-power field (×400) was about 300µm×300µm. The CD antigens were stained brown on the membrane of the small and round immune cells located in the stroma of cancer center, which were easy to be distinguished. The criteria dictated a cut-off for CD3+ and CD45RO+cells density was according to the minimum-\(P\) value introduced by Galon (*Science*. 2006, 313: 1960-1964), in which the difference in survival between the “high” and “low” groups was the largest.

2. How the Authors addressed the problem of the heterogeneous
distribution of immunological cells inside the tumour? It would be interesting to know whether the Authors had assessed the issue of intra-sample and inter-sample variability.

Answer: In the immunohistochemical assay the staining was usually heterogeneous. So we chose 10 areas of the highest density in the cancer, which would be a representative for the whole section. The intra-sample and inter-sample variability did exist, so we did the immunohistochemistry in the same condition and counted the cells by two pathologists in order to decrease the error.

3. Additionally, the Authors recognized different subset of immunological cells (including CD4, CD8 and CD56) but omit to discuss why these cells have not been compared with HMGB1 expression and its immunolocalization.

Answer: CD3+ cells contains different subset of immunological cells (including CD4, CD8 and CD56). We did the immunostaining of these CD antigens in ten of the tissue sections to see which subset would play important role in this group of patients. The results showed CD8+ cells outnumbered CD4+ cells while CD56+ cells were rare, which meant T lymphocytes would be more important than NK cells in controlling the progression of this group of patients. So we did not discuss the relationship between these cells and HMGB1 expression as
well as its immunolocalization.

4. Figure 5 is not sufficiently clear to demonstrate a double immunohistochemistry for HMGB1 and CD45RO. The Authors should improve their staining procedure.

Answer: We did the double immunohistochemistry for HMGB1 and CD45RO again and submitted another figure (Fig 5). The sections were incubated with a rabbit anti-human HMGB1 polyclonal antibody (1:1000; Abcam, Cambridge, MA, USA) and a mouse anti-human CD45RO monoclonal antibody (1:100; Zymed, San Diego, CA, USA) at the same time, while the other procedure kept the same.

5. The Authors used the terms nuclear and nucleolar throughout the manuscript. However, because these terms indicate two different cellular localization, they should be more consistent and define with a single term the immunolocalization of HMGB1.

Answer: HMGB1 was mainly located in the nucleus of colon cancer cells. We had looked over the manuscript and changed “nucleolar” to “nuclear” as well as “nucleolus” to “nucleus”.

6. The tumoural tissue cannot be easily recognizable in Figure 1D.

Answer: We added an arrow in Figure 1D to point out the tumoural
tissue.

7. In the Legend of Figure 1 and 2, the Authors stated that “all of the antigens were stained on the membrane”. However the term stained should be changed with the most appropriate “immunolocalized”.  
Answer: We had changed the term “stained” with “immunolocalized” in the Legend of Figure 1 and 2.

For comment from Reviewer 2

1. Please take out the last sentence of the abstract since other articles show that in various circumstances, HMGB1 is necessary for the outcome (depending on posttranslational modifications and clinical setting)  
Answer: We had taken out the last sentence of the abstract as you suggested.

2. Ref 64 should be replaced by the original article from the same authors published in Nature Med 2007.  
Answer: We had replaced Ref 64 by the original article from the same authors published in Nature Med 2007 as you suggested.