ANSWERING REVIEWERS

May 13, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 10285-Research Report.doc).

Title: Inhibition of KL-6/MUC1 glycosylation limits aggressive progression of pancreatic cancer

Author: Xu HL, Zhao X, Zhang KM, Tang W, Kokudo N

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 report 1: This is an interesting study on the role of MUC1 glucosylation in pancreatic malignancies. Nevertheless in general the investigation lacks convincing depth that could be improved by for example a second method for confirmation of claimed effects in each case. Some specific points/ questions: - The finding that all pancreatic ca cases were positive for the glucosylation ab (albeit in different degrees) is quite interesting. 1)More details should be provided regarding the intensity of staining and if any quantitative measurement was applied. Also whether the pathologist grading was blinded on the patients' diagnosis. Were there any controls with benign pancreatic tissue diagnoses studied? If not it would be advisable to study and present such controls. Was any attempt of clinical prognostic correlation undertaken? 2)The part on EMT is particularly interesting but authors have presented only very initial data. Immunoflorescence data on vimentin should also be presented. In fig 6b, in contrast to what the authors claim, e-cadherin seems to be down-regulated and vimentin up-regulated with tunicamycin in Panc-1 cells. Also presenting quantification of the western data would be of help. - No attempt has been made for a mechanistic clarification of the seen effects, i.e. what are the intra-cellular pathways affected. - Addition of in vivo experiments would be a major asset for the paper. - Some language polishing is needed. This is particularly required for the abstract.

Answers:

1) A) Regarding the intensity of staining: The percentage of stained cells was determined in 10 random microscopic fields of each tissue sample or in the entire region if the tissue sample consisted of fewer than 10 fields (magnification 200×). In the evaluation of KL-6/MUC1 staining, cases presenting positivity in more than 10% of cancer cells observed were considered positive according to the previous report (Zhang W, et al. Oncol Rep 2008; 20:1013–9.]. We have added these points in the "*Immunohistochemical staining of KL-6/MUC1*" in the "MATERIALS AND METHODS".

   B) The pathologist staining grading was blinded on all the patients' diagnosis.

C) The staining of benign pancreatic tissues has been evaluated in our previous study, you can found the related data in our previous article (Xu, HL, et al. Life Sci 2011; 88 1063–9). We are now trying to evaluate the clinical prognostic value of KL-6/MUC1 in benign pancreatic tissues and pancreatic cancer.

2) A) We have also found that the part on EMT is particularly interesting, but we have presented only very initial data, further studies about mechanistic clarification of the seen effects will be performed in our lab. Thanks for the good suggestion. We will try to illustrate the intra-cellular pathways affected, both in vitro and in vivo.

B) In fig 6b, changes of E-cadherin and vimentin expression in panc-1 cells after N-inhibitor treatment might not so obvious (P>0.05). We have revised this point in the manuscript, please find it in the "Possible proteins involved in decreased invasive abilities of pancreatic cancer cells" in the RESULT section. We have presented the quantification of the western data, please found it in figure 6C.

C) We have polished our language of this manuscript, and please find the language certificate in our revised manuscript.

Reviewer report 2: In this paper, the authors have studied the presence of KL-6/MUC1 epitope on two types of pancreatic cancer, PDAC and IPMN. They found that KL-6 is expressed in PDAC but not in IPMN. Then they inhibited O-glycosylation and N-glycosylation to try to correlate a type of glycosylation to biological properties of pancreatic cancer cells. They found differences that suggest that targeting glycosylation may be useful to control aggressive behavior of the tumor. Despite very interesting data, the paper can not be published as it is and would be greatly enhanced with a few additional experiments listed in the major points. In general, authors' conclusions are too fetched forward and often have to be modulated as they do not have the real proof for what they conclude. For example when they compare data in the two pancreatic cancer cell lines, they often conclude that the effects are the same in both cell lines when we can see clear differences. Major points: 1- The paper is on KL-6/MUC1 glycosylation (it is stated as early as in the title) but nowhere there is data on MUC1 to correlate with KL-6 stainings. It is important as KL-6 motifs may be found on other membrane glycoproteins. MUC1 immunohistochemical staining must be added in figure 1 to correlate with KL-6 and eventually show co-localization. In figure 1B, KL-6 positive staining is shown at the apical pole of normal epithelial cells. It is important to show MUC1 staining is there as well to be able to conclude that KL-6 staining corresponds of MUC1 peptide staining. 2- Figure 2: What are the controls to compare the effects of tunicamycin and BAG? Are cells incubated with solvent used to dissolve tunicamycin or BAG? This is not stated. Without the control we can not conclude to any effect since it is a comparative study. Add this information both in the material and methods section (proliferation assays) and in figure 1. 3- Figure 3E: conclusion that KL6/MUC1 staining decreased in both cell lines must be modulated. It is not clear that there is a decrease in figure 3E. Same remark in figure 4. Inhibition following BAG treatment is quite clear whereas that following tunicamycin is more subtil (B and E), all cells are not spherical and individualized as shown for BAG (C and F). These differences between

tunicamycin and BAG effects (figures 3, 4) should be discussed in the discussion section. It is clear from these experiments that inhibition of N-glycosylation does not alter cell properties as does inhibition of O-glycosylation with BAG. 4- Figure 6: again effects of tunicamycin on KL6 expression (decrease?) are not as clear cut as those with BAG. Moreover, there is no precise calculation of the number of cells expressing or not KL6 after the treatments. This should be done to give some depth to the conclusion. Observation on one field shown to the reader is not sufficient. Figure 6B: showing Ecadherin and vimentin expression by western-blotting is quite preliminary data to talk about EMT process. More is needed. Again modulate the conclusions. Especially increase of Ecadherin and decrease of vimentin in Panc-1 treated with tunicamycin is difficult to see/believe. It is the inverse that we see. Please explain/discuss. 5- Discussion should emphasize the differences between the two inhibitors that target either O- or N-glycosylation, it is obvious that the consequences are not the same. Make a parallel with oligosaccharidic structures present on MUC1/mucins in general? Discuss. Minor points: 1- Figures 4 and 5: What are the control groups? Please define. We do not know what control corresponds to. 2- Manuscript must be proofread by a native speaker to correct grammatical errors here and there. 3- Be careful with the terms used: to conclude at the end of the discussion that KL6 plays an important role… is a bit too fetched forward as no mechanistic is shown is the paper. Keep the conclusions to "possible involvement/implication" but certainly not a role.

Answers:

Major points:

1) MUC1 mucin, one kind of mucin glycoprotein, is abundantly expressed at the surface of epithelial cells in many tissues. MUC1 molecule has many oligosaccharides in the extracellular domain, and these oligosaccharide moieties have a great deal of variety. Therefore, the qualitative change of oligosaccharides in MUC1 has great importance. Although the processing of the full length MUC1 core protein is similar in both normal and tumor cells, there is a remarkable diversity in oligosaccharide moieties between normal and cancer cells. Thus, it has been considered to be important to detect the specifically structured MUC1 in cancer cells and to clarify its role and clinical significance.

   The expression of MUC1 can be detected using different antibodies, such as CD227, DF3, and CA15-3 antibodies, and these antibodies recognized MUC1s were named CD227, DF3, and CA15-3. Since different antibodies can recognize different motifs in MUC1, their diagnosis values were different. Krebs yon den Lundgen-6/Mucin 1 (KL-6/MUC1), a type of MUC1 categorized as cluster 9, is recognized by KL-6 monoclonal antibody, and its epitope includes sialo-oligosaccharide moiety in MUC1 molecules. This mucin was first established in the serum of patients with intestinal pneumonia but has recently been detected in various cancer tissues. Using a novel compound library of synthetic MUC1 glycopeptides, the specific epitope structure of anti-KL-6 MAb was a heptapeptide sequence Pro-Asp-Thr-Arg-Pro-Ala-Pro (PDTRPAP), in which the Thr residue is modified by Neu5Ac alpha2,3Gal beta1,3GalNAc alpha (2,3-sialyl T antigen, core 1-type O-glycan) (Ohyabu N, et al. J Am Chem Soc. 2009;131(47):17102-9.).

2) In Figure 2: the cells without drugs treatment were use as the control groups. And the inhibitory rates were calculated compared with the control groups. The tunicamycin and BAG were both dissolved in DMSO, and then diluted in the culture medium before adding to the cells. After adding to the culture, the final concentrations of DMSO were <0.1%.

   In Figure 1, PBS was used instead of primary antibody as negative control.

   We have add these information in the material and methods section (Cells and cell culture conditions, proliferation assays, and Immunohistochemical staining of KL-6/MUC1) and in.

3) In Figure 3 and Figure 4, the effects of tunicamycin treatment were not so obvious as the effects of BAG. The reason why BAG was more effective to affect KL-6/MUC1 expression may be that KL-6/MUC1 was a more highly O-glycosylated cell surface glycoprotein. These differences

between tunicamycin and BAG effects (figures 3, 4) were discussed in the discussion section, please find it in the revised manuscript.

4) In Figure 6, again effects of tunicamycin on KL-6/MUC1 expression are not as clear as those with BAG. The reason may also be that KL-6/MUC1 was a more highly O-glycosylated cell surface glycoprotein. These differences were discussed in the discussion section, please find it in the revised manuscript.

   For precise calculation of the number of cells expressing KL-6/MUC1 after the treatments, the fluorescence intensity of KL-6/MUC1 and E- cadherin were shown in Figure 6A-g and h.

   In fig 6b, changes of E-cadherin and vimentin expression in panc-1 cells after N-inhibitor treatment might not so obvious (P>0.05). We have revised this point in the manuscript, please find it in the "Possible proteins involved in decreased invasive abilities of pancreatic cancer cells" in the RESULT section. We have presented the quantification of the western data, please found it in figure 6C.

   We have found that the part on EMT is particularly interesting, but we have presented only very initial data, further studies about mechanistic clarification of the seen effects will be performed in our lab.

5) We have emphasized the differences between the two inhibitors that target either O- or N-glycosylation. Please find it in the revised manuscript.

Minor points:

1) In Figures 4 and 5, the cells without drugs treatment were the control groups. We have added these points in the "Cell adhesion assay" and "Transwell chamber assay" of the "MATERIALS AND METHODS" section.
2) We have used the copyediting service provided by professional English language editing company, and please find the language certificate in our revised manuscript.
3) We have revised our conclusion in the discussion: "This study indicated an important involvement of KL-6/MUC1 glycosylation in pancreatic cancer metastasis and invasion, which may be related with EMT process."

3. References and typesetting were corrected.

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

Sincerely yours,

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