Author’s response to reviews

Title: Attenuated expression of histamine receptor H4 in colorectal cancers: a potential correlation with histamine-mediated tumor growth

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Author’s response to reviews: see over
Reviewer 1

Major Compulsory Revisions:
1. The authors ascribe the down-regulation of H4R expression to increased histamine production associated with cancerogenesis, but instead of proving this statement in their model they refer only to the literature. Histamine production or HDC activity should be measured in these cells. Would an inhibitor of HDC like #-FMH increase H4R expression in CRC samples?

Answer: We did not mean that the down-regulation of H4R is a consequence of histamine accumulation in CRC tissue. We have modified some statements in the manuscript to avoid misunderstanding. In our preliminary experiments using CRC cell lines, we found that elevated histamine could not cause significant alteration of H4R expression in CRC cells. So it was not mentioned in the manuscript.

On the other hand, we did make some effort to investigate the mechanisms of regulation of H4R expression. Since no CpG island was observed on the proximal region (2000 bp) of H4R promoter. DNA methylation might not play an important role in the regulation of HRH4 expression. DNA deletion at chromosome position 18q22 in gastrointestinal adenocarcinomas has been reported by different group. We wondered whether there were copy number variations (CNVs) of HRH4 in CRCs, which might have a correlation with gene expression.

It was found that the copy number loss of HRH4 was present in collected CRC samples (17.8%, 19 out of 107), while there was not a statistical difference of HRH4 CNV among cancer groups at different Duke's stages. And real-time analysis showed that the copy number loss of HRH4 at least plays a partial role in the down-regulation of HRH4 expression in CRCs while there are other mechanisms involved. Now we are examining the CNVs of HRH4 in gastric cancer (GC) samples. The results obtained also indicated the presence of HRH4 copy number abnormalities in GCs. These will be discussed in our next manuscript about CNVs of HRH4 in gastrointestinal malignancies. Actually, we have focused on the CNVs of certain genes in CRC or GC recently (Fang Z et al. Detection of APC Gene Deletions in Colorectal Malignancies Using Quantitative PCR in a Chinese Population. Pathol Oncol Res. 2011 Feb 26. [Epub ahead of print]; Zhang C, Fang Z et al. Acta Biochim Biophys Sin (Shanghai). 2010 Nov;42(11):834-8).

2. If histamine is actually produced and secreted by these cells, how do the authors explain the effect of exogenous histamine? Is histamine production diminished after transfection of the H4R gene?
Answer: As we described above, we did not mean there is a correlation between H4R expression and histamine production in CRC cells. In our in vitro studies, we used the commercial product of histamine purchased from Sigma, which should be regarded as exogenous histamine here.

Actually we previously did examine the concentration of histamine that produced by Lovo cells in culture medium (2ml medium in 6cm dish, about $2 \times 10^6$ cells) using Histamine ELISA Kit. The concentration is relatively low and there is not a statistical difference between Mock-Lovo (0.629±0.212µM) and H4R-Lovo (0.598±0.193µM ) cells. So we didn’t mention it in the manuscript.

A previous study reported that the median histamine concentration in colorectal cancer tissue was 8.4 micrograms/g [7.6 x 10⁻⁵M], ranging from 0.3 microgram/g to 20.6 micrograms/g (Reynolds JL, Eur J Surg Oncol., 1997 Jun;23(3):224-7). It is hard to determine the concentration of exogenous histamine used in this work. So we referred to a previous work from your group. (Petit-Bertron AF et al. PLoS One 2009, 4:e6504.)

Minor Essential Revisions:
1. 10 mg/ml of 5-FU seems a very high dose for so little effect on apoptosis, especially for a drug that is currently used for this type of cancer. Is this a spelling error?
   Answer: We have checked the experiment record and found that we did make a mistake in writing the manuscript. We have tried 0.1mM~50mM 5-Fu on the Lovo cells and found that 10mM 5-Fu could induce appropriate cell death rate in Lovo cells after 36h.

2. Figure legends are not very clear, especially fig. 4 panel C (Fig. 4B instead of 5A?) and Fig. 5 B and C (3 instead of C). 2. The designation of the proliferation assay is not homogenous (colonogenic or colony-forming assay)?
   Answer: We made a mistake here. There used to be 6 figures in this article. We have deleted one part and left some description uncorrected. They have been revised now. Thanks for your careful work.

   The “colonogenic assay” has been replaced by “colony-forming assay”. We did use the latter in the present study. Since colony-forming ability is closely related with proliferation ability in tumor cells, some experiments directly use it to assess proliferation(Chen et al. BMC Urology 2005 5:8; Kuo, P.L., Y.L. Hsu, and C.Y. Cho, Mol Cancer Ther, 2006. 5(12): p. 3209-21.). This might be improper. Here we selected a modified method as described in the “Pierlorenzo et al. Cancer Res. 2008 Aug 15;68(16):6770-8.”
3. There are a number of spelling errors like "whose characteristics of which" on page 5 and "more lower" on page 12.
   Answer: They have been revised.

4. The discussion is not really to the point.
   Answer: We have tried to make some improvements. Thanks for your advice.
Reviewer 2:
Major Compulsory Revisions:
The title and abstract must be revised to reflect the fact that most of the data being presented is derived from over-expression systems and less from the colorectal cancer dataset. As it stands, I feel the title and abstract are somewhat misleading.
Answer: We have tried to make revision to the title.

The previous work by Boer et al., showing decreased expression of HRH4 in colorectal tissues, also reported a reduction in HRH1. Is this true of this biopsy dataset?
Answer: Actually we did examine the mRNA levels of HRH1 in these samples using real-time PCR, there was a decrease in HRH1 expression. Since it is not closely correlated with this subject, we didn’t mention it in the manuscript.
The results were shown below, which might be added to another manuscript.

![Graph showing mRNA expression levels of HRH1 in ANT and CRC tissues](image)

While the protein is reduced by Western Blot on whole tissue extracts, it would be important to see immunohistochemistry to determine specific localization in the tissues. Since most colorectal tissue contains inflammatory and structural cells in or around the tumor tissue that will be extracted also during the biopsy, this could be an alternative source of the differences than the tumor cells themselves.
Answer: As shown in figure1E, H4R is positively expressed on the surface of enterocytes of colon and rectum tissues. There is no appreciable staining of H4R in invasive CRC tissues we selected.
We tried to find out some structural cells around the tumor tissues. Here is figure of H4R staining representing the diminishing expression of H4R in transforming colon tissues, which might provide additional proof of negative regulation of H4R in CRCs.

Over-expression of HRH4 could alter the expression of the other histamine receptors (including HRH3). This needs to be determined in order to determine the specificity of the effects of histamine and clozapine.

Answer: We examined the protein expression levels of HRH3 in Mock-Lovo and H4R-Lovo cells, and the results were shown in new figure2A. There was not a significant alteration of HRH3 in H4R-Lovo. This might because we chose a stable line with moderate H4R expression but not the ones with very high H4R expression. Thus clozapine could be used as specific activator of H4R in the present study.

That the cAMP effects are not via PKA is somewhat surprising since this kinase has been previously described as important for mediating the effects of signals via HRH4. The only known alternative pathway would be via EPAC. Are changes in EPAC observed?

Answer: Now we have re-analyzed the influence of EPAC-RAP activity on p21/p27 regulation in H4R-Lovo cells. The results obtained showed that activation of EPAC-RAP pathway had an influence on p38/MAPK pathway but had little effect on the regulation of p21/p27 expression (data not shown here).

On the other hand, we are now trying to make further experiments to understand the signaling pathway involved in H4R-mediated cell growth control and cell apoptosis. Interestingly, we found PI3-kinase activity was influenced by alteration of cAMP/PKA pathway in H4R-Lovo cells. For the treatment with cAMP inhibitor or
activator could alter the phosphorylation level of Akt at both the Ser473 and Thr308 sites in Lovo cells. PI3K/Akt pathway as well as cAMP/Akt pathway was involved in H4R-mediated regulation of cell apoptosis induced by 5-Fu in Lovo cells. These will be presented in the next manuscript.