Author’s response to reviews

Title: Weipiling ameliorates gastric precancerous lesions in Atp4a-/- mice

Authors:

Wei Liu (gzzydxliuwei@126.com)
ming Zhao (88435811@qq.com)
liang Liu (448688667@qq.com)
feng Pan (gzphf@126.com)
zhu Lin (lizhulin26@yahoo.com)

Version: 2 Date: 01 Oct 2019

Author’s response to reviews:

October 01, 2019, Guangzhou

Dear Editor Arun K. Yadav,

Thank you very much for your kind letters dated on September 15, 2019, and sending us the information concerning our manuscript ‘Weipiling ameliorates gastric precancerous lesions in Atp4a-/- mice’. Enclosed please find out our revised version with some changes according to the consultant’s suggestions.

We greatly appreciate the consultant’s suggestions, and have revised the manuscript (marked with red color), and would like to take the opportunity to point out as follows.

Ewa Nowakowska-Zajdel (Reviewer 2):

1. The research is very interesting, the method is clear. Authors write that Weipiling consist of eight herbs, which of them has anticancer effect?
2. As the Author write "Clinical and basic research shows that a WPL decoction can effectively relieve clinical symptoms, improve quality of life, and reverse precancerous lesions, but the specific mechanism governing this effect is unknown". Whether Weipiling was tested on cell lines? If we know that Weipiling effect on cell cancer is unknown, I am sure, that the research should be performed on cell lines first.

We greatly appreciate the consultant’s comments. We elaborate the effect of gastric mucosa protection of Weipiling may through regulating glucose metabolism in GPL mice, but the GPL vitro cell model is not established until now, this is also the limitation of this paper, so we do not explore the further evidence of mechanism in vitro study. Further, we have rewrite " The specific mechanism governing this effect is unknown, thus, the aim of this study was to investigate the effect of the WPL decoction on the GPL animal model and explore the molecular mechanism underlying its anti-malignant transformation of gastric mucosa and corresponding inhibition of gastric cancer.".

Se-Hee Kim (Reviewer 3): This investigation posited that Weipiling ameliorates gastric precancerous lesions in Atp4a-/- mice by inhibiting the expression of transporters and mTOR/HIF-1α pathways. The topic is interesting and the mouse model are employed to explain the effect of Weipiling. I recommend that this paper be accepted after several minor revisions. The details are listed below,

Major comments:

1. Background. Zeng et al (2018) simulated N-methyl-N’-nitro-N-nitrosoguanidine (MNNG)-induced GPL in male Sprague-Dawley rats. The authors may introduce the advantages of Atp4a-/- mice model compared to other mouse models in GPL study.
We greatly appreciate the consultant’s comments. In the article, we describe the advantages of Atp4a-/- mice model in the page 3 line 16, “Replication of disease models is an effective tool for studying disease pathogenesis and treatment. For the study of GPL, researchers used chemical drug induction and HP infection methods, but there are limitations in model replication time, large individual differences, and unstable models, thus affecting the study of GPL. There are indications that the acid secretory activity of the H, K-ATPase might be necessary for the viability and normal development of parietal cells and possibly for the differentiation of chief cells. As we reported before1, with the development of genetic engineering technology, knockout mice are gradually being used to study precancerous lesions. The phenotypic progression to IM observed in the Atp4a-/- mouse model is similar to the evolution of gastric cancer in humans2-3, as initially described by Correa. So, in this research, we choose the Atp4a-/- mice to simulate GPL. In future studies, the Atp4a-/- mouse should be a useful in vivo model for studying secretory membrane biogenesis and the role of the parietal cell in differentiation and maturation of gastric epithelial cells. (1. Liu W, Pan H, Wang Q, et al. The application of transgenic and gene knockout mice in the study of gastric precancerous lesions[J]. Pathology-Research and Practice, 2018; 2. Judd L M, Andringa A, Rubio C A, et al. Gastric achlorhydria in H/K-ATPase-deficient (Atp4a (−/−)) mice causes severe hyperplasia, mucocystic metaplasia and upregulation of growth factors[J]. Journal of gastroenterology and hepatology, 2005, 20(8): 1266-1278; 3. Spicer Z, Miller M L, Andringa A, et al. Stomachs of mice lacking the gastric H, K-ATPase α-subunit have achlorhydria, abnormal parietal cells, and ciliated metaplasia[J]. Journal of Biological Chemistry, 2000, 275(28): 21555-21565)

2. Results. The manuscript have many separate figures which can be grouped by topics. For example, figure 2 and 3 can be grouped into a single figure.

According to the consultant’s suggestions, figure 2 and 3 have been grouped into a single figure on the page 19 figure 2.

3. In figures 2 and 3, the authors showed the representative images for H&E and AB-PAS staining, respectively. However, the authors may provide the quantitative data from 5 or 6 independent images to clarify the effect of Weipiling in figure 2 and 3.

We greatly appreciate the consultant’s comments, a detailed explanation of figure 2 and 3 has been added on page 9 of the article as:” Our results indicated that WPL treatment may produce regulatory effects on the IM of gastric mucosa, which further verifies the AB-PAS staining result in figure 2-B.” We have further illustrated the results of figure 3 on page 10 of the article “Thus, WPL could effectively inhibit dysplasia of gastric mucosa in GPL mice as shown in figure 2-A.”

4. In figure legends for figures 4~11, the authors do not include the sample sizes.

According to the consultant’s suggestions, the sample sizes have been supplied in the article on the figure legends.
5. In figures 4~8, including western blot analysis would support the IHC data further.

We greatly appreciate the consultant’s comments, on the page 11, We added further explanations. “The cancerization of gastric mucosa epithelial cell is a gradual process. The results of HE and AB-PAS staining suggest that the gastric mucosa of Atp4a-/− mice display the intestinal metaplasia and the adenomatus dysplasia with a back-to-back gland pattern. To study the biological features, we used CDX2, MUC2 for cell differentiation, p53 for cell cycle control, Ki-67 for proliferation. Immunohistochemical staining for CDX2, MUC2, Ki-67, P53 and PTEN was performed, and their expression levels were evaluated in the mucosa. The results, including western blot analysis, showed the characteristics of intestinal metaplasia, dysplasia and abnormal energy metabolism of gastric mucosa in mice with GPL.”

6. Discussion. There was no mention of the limitations of this study. Also, mention how your results compare to other studies that were published recently.

We greatly appreciate the consultant’s comments, we have supplied the text on the page13 and 14. The limitations of this study are that the vitro cell model of GPL is not established until now, so we elaborate the effect of gastric mucosa protection of Weipiling may through regulating glucose metabolism in GPL mice. In recent years, More and more researchers study the GPL, however, they used the rats treated with MNNG to demonstrate GPL 1-3. this method has the limitation that the pathologic degree of the time-consuming and non-uniform digress of pathological degree of the model. In this article the Atp4a-/− mice was used to established GPL, the gastric mucosa of 10-week old mice showed the intestinal metaplasia, dysplasia, which is more closely rated to the clinical patients of the premalignant lesions of the clinical gastric cancer. (1. Xu J, Shen W, Pei B, et al. Xiao Tan He Wei Decoction reverses MNNG-induced precancerous lesions of gastric carcinoma in vivo and vitro: Regulation of apoptosis through NF-κB pathway[J]. Biomedicine & Pharmacotherapy, 2018, 108: 95-102; 2. Zhang C, Cai T, Zeng X, et al. Astragaloside IV reverses MNNG-induced precancerous lesions of gastric carcinoma in rats: R egulation on glycolysis through mi RNA-34a/LDHA pathway[J]. Phytotherapy research, 2018, 32(7): 1364-1372; 3. Cai T, Zhang C, Zhao Z, et al. The gastric mucosal protective effects of astragaloside IV in mnnng-induced GPL rats[J]. Biomedicine & Pharmacotherapy, 2018, 104: 291-299.)

Minor comments:

1. In the figures 2 and 3, images are missing the appropriate labels.

We greatly appreciate the consultant’s comments, we have modified the label of Figure 2 on the page 19.

Shyamalima Gogoi (Reviewer 4): Table 1, page 4, authors are requested to rewrite the scientific names of the plants properly (species name)
We greatly appreciate the consultant’s comments, we have rewritten the scientific names of the plants properly as follow on the page 4.

Table 1

Components of the WPL formulation.

<table>
<thead>
<tr>
<th>Chinese herbal name in Pinyin name</th>
<th>Species name</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huang-qi</td>
<td>Hedysarum multijugum</td>
<td>20</td>
</tr>
<tr>
<td>Tai-zi-shen</td>
<td>Pseudostellaria heterophylla (Miq.) Pax</td>
<td>15</td>
</tr>
<tr>
<td>Bai-zhu</td>
<td>Atractylodes macrocephala Koidz</td>
<td>10</td>
</tr>
<tr>
<td>Fu-ling</td>
<td>Poria cocos (Schw) Wolf (Poria)</td>
<td>12</td>
</tr>
<tr>
<td>San-qi</td>
<td>Panax notoginseng (Burk.) F. H. Chen (P. notoginseng)</td>
<td>3</td>
</tr>
<tr>
<td>E-zhu</td>
<td>Curcuma zedoaria (Christm.), Roscoe</td>
<td>10</td>
</tr>
<tr>
<td>She- she- cao</td>
<td>Hedyotis diffusa Willd</td>
<td>15</td>
</tr>
<tr>
<td>Hou-gu-jun</td>
<td>Hericium erinaceus (Rull ex F.) Pers</td>
<td>15</td>
</tr>
</tbody>
</table>

Page 5, line 15, is there any specific reason to use only male mice if their kindly mention.

We greatly appreciate the consultant’s comments. Literature research is done before carrying out this experiment. In humans, environmental factors such as smoking, alcohol consumption, geographic location and infection as well as genetic background make gender differences in human gastric cancer difficult to detect. In contrast, in inbred genetically engineered mouse studies, gender has been found to influence the hyperplastic response. H/K-ATPases are required for parietal cell viability and normal development of the gastric mucosa, we have founded that loss of the α-subunit confers an age and gender-dependent (female-biased) gastric hyperplasia with incomplete intestinal metaplasia 1,2. Atp4a−/− female mice develop increased number of fundic gland polyps with increasing age3. In order to avoid the impact due to gender differences in results, we use the male Atp4a−/− to study the GPL. (1. Judd L, Andringa A, Rubio C, Spicer Z, Schull G, Miller M. Gastric hlorhydria in H/K-ATPase-deficient (Atp4a(/)) mice causes severe hyperplasia, mucocystic metaplasia and upregulation of growth actors. J. Gastroenterol. Hepatol. 2005; 20: 1266–78; 2. Spicer, Z.; Miller, M.L.; Andringa, A.; Riddle, T.M.; Duffy, J.J.; Doetschman, T.; Shull, G.E. Stomachs of mice lacking the gastric H,K-ATPase alpha -subunit have achlorhydria, abnormal parietal cells, and ciliated metaplasia. J. Biol. Chem. 2000, 275, 21555–21565; 3. Rubio C A, Miller M L. Fundic gland cysts in Atp4a−/− mice mimic fundic gland polyps in humans[J]. in vivo, 2009, 23(6): 979-981.)
Page 5, line 40, 41 it is not clear why only sacrifice of the model group mice was mentioned. what about the control, WPL and GPL groups?

We greatly appreciate the consultant’s comments, it was our mistake that was now modified on the page 5: At the end of the 10th week, all the experimental mice were humanely terminated with sodium pentobarbital.

13. Page 6, line 44, 45 authors are requested to mention the kit name that have been used.

We greatly appreciate the consultant’s comments, we have supplied the kit name on the page 6. The prepared specimens were stained with haematoxylin and eosin (H&E) (Beyotime, C0105, China) or alcian blue-periodic acid-Schiff (AB-PAS) (BASO.615041, China).

We sincerely hope that the revised manuscript meets your approval and the approval of the reviewers, and that it is now acceptable for publication in your esteemed journal.

Thank you very much for your consideration of this paper.

Yours sincerely,

Hua-feng Pan, PhD, MD

Guangzhou University of Chinese Medicine,

Guangzhou, 510405, China