Author's response to reviews

Title: Metabolic Markers and Microecological Characteristics of Tongue Coating in Patients with Chronic Gastritis

Authors:

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Author's response to reviews: see over
Response letter

Dear editor,

First of all, we would like to thank you and the reviewers for interesting, thorough, and helpful comments and suggestions. We have included most of them into the new version of our paper. The figure and table in paper were modified according to the reviewers. Below, we first summarize the major changes, and then the detailed responses to all of the reviewers are presented.

Response to the reviewers:

Reviewer #1(Shao Li)

Comment 1:
The investigation was performed on the chronic gastritis group (70 cases) and the normal controls (20 cases). It is well known that TCM doctors always use tongue coating as a very important index to determine the TCM Syndromes of patients. However, it is not clear whether the chronic gastritis patients in this study suffered from different Syndromes with different tongue coating. I suggest that the TCM Syndromes should be taken into consideration in this work.

Our response:
TCM believes that the tongue coating is a very sensitive index that reflects physiological and pathological changes in the organs, especially in the spleen and the stomach; tongue coating is closely
related to TCM syndrome. The manuscript based on the relationship between tongue coating and the organs. Next, we plan to study the correlation between TCM syndromes with tongue coating.

Comment 2:
Authors used both metabonomics and microbiological techniques to determine the biological basis of tongue coating in chronic gastritis patients. They should clarify the scientific logic of the connection of both metabonomics and microbiological techniques as well as both human body and micro ecological conditions.

Our response:
Tongue-coating appearance is one of the most important features in Chinese traditional medicine diagnosis. Tongue coating is very sensitive index that reflects physiological and pathological changes in the organs, especially the spleen and the stomach. The microbiome on the tongue coating is one of the main microbiomes of the human body. Densely populated by a variety of microorganism communities, the human gut microbiome is considered essential to maintaining natural host-environment interactions involved in nutrient absorption, epithelium regeneration, energy metabolism and immune response. We speculated that the microbiome composition is a key factor that affects the appearance of the tongue coating, and then we reasoned that the microbiome composition on the tongue coating could be associated with
metabolism and characteristics of stomach diseases. Many scientists referred to the physiological and pathological characteristics of human body resulted from the relation of human metabolism and microbial. The relevant literatures had been mentioned, e.g. Metabonomic analysis of saliva revealed generalized chronic periodontitis signature (Mario Aimetei, 2012), the metabolic profile of saliva of the samples were analysis with LC-MS and GC-MS (He hong-bing, 2012); microbiological sample (saliva) taken from the gingival crevice was analyzed checkerboards DNA-DNA hybridization technique (Zeigle, 2012)

Comment 3:
In page 7, authors claimed that they performed bacterial genomic DNA extracted for 20 normal controls and 20 patients with the thickest and greasiest tongue coating. They should clarify why and how to select the cases from 70 patients and also show some details about the tongue coating of the other 50 cases.

Our response:
The manuscript consists of two parts about metabonomic and microbiological techniques. In part of metabonomic, 70 chronic gastritis patients and 20 normal controls were selected; in part of microbiological techniques, 20 chronic gastritis patients and 20 normal controls. Because lack research funding, we selected 20 patients suffered from atrophic gastritis with the thickest and greasiest tongue coating from 70
patients. 50 cases of 70 chronic gastritis patients had superficial gastritis without the thickest and greasiest tongue coating, their mean ages were 43.95 ± 13.36 years. Thickest and greasiest tongue coating images were provided in the Fig7.

**Comment 4**

There are some related studies on the biological basis of the TCM Syndrome in chronic gastritis patients as well as patients’ tongue coating. For example,” Integrating next-generation sequencing and traditional tongue diagnosis to determine tongue coating micro biome. Scientific Reports 2012;2:936”, “Imbalanced network biomarkers for traditional Chinese medicine Syndrome in gastritis patients. Scientific Reports 2013;3:1543”. The manuscript would benefit from citing these closely related studies and discussing the results with them.

**Our response:**

Thanks very much for your kindly suggestion. We have read the papers carefully you suggested. The manuscripts will benefit from the two articles. We cited some views of two papers.

**Comment 5**

English and clarity throughout the text should be improved significantly.

**Our response:** The manuscript has been polished.

**Comment 6**

The Chinese characters in Fig5 should be presented in English.
Our response: Fig5 had been modified.

Reviewer #2(Yoshihisa Yamashita)

Comment 1: .

Our response: The manuscript page numbers have been marked

Comment 2:
Page 5, lines 29-30: What type of cells did the authors count? How did the authors count the cell number after centrifugation and how did they adjust. Is the supernatant finally collected tongue coating sample? The reviewer cannot image what are tongue coating samples.

Our response:
We counted the epithelial cells in order to adjust the same concentration (10^6/ml cells of each tongue coating) by saline in OLYMPUS microscope. The supernatant were finally collected tongue coating samples. Tongue coating samples were prepared by centrifugation at 3000 rpm for 10 min; the supernatants are mainly metabolites which were generated by the epithelial cell in the tongue coating. Tongue coating samples can be seen in Fig 7.

Fig 7 Sampling images of tongue coating from the centre of the tongue, an area
regarded as tongue coating in the traditional tongue diagnosis.

**Comment 3:**

Page 6, lines 2-7: What derived from tongue coating was analyzed by LC-MS? Washed solution of tongue coating cells was analyzed?

**Our response:**

The supernatants derived from tongue coating were analyzed by LC-MS. We tested the extracellular substances of tongue coating. The supernatants are mainly tongue coating metabolites. Tongue coating is an important physiological fluid that contains a highly complex of substance. Tongue coating locally produced proteins, as well as other molecules from the systemic circulation. Such highly resolute and complementary techniques have revealed the complexity of tongue.

**Comment 4:**

Page 8, lines 24-26 & Fig. 1: Figure 1 shows similar peak patterns between upper and lower panels. Although the authors insisted difference in the peak height, it was just 1.5 fold in this figure. Is there any significant difference between gastritis and control groups?

**Our response:**

These were significant differences between gastritis and control group. We found similar substances between normal controls and chronic gastritis group (Fig1). The differences between gastritis and control group were substance quantity by LC-MS. Table 1 showed
the substance changes between normal controls and chronic gastritis group.

Fig1. Typical UPLC/MS metabolic fingerprinting total ion chromatogram of human tongue coating samples from the chronic gastritis group and the normal controls

Comment 5:

Page 11, Table 1: tR/min of compound is very closed, eg, 4.06, 4.07, and 4.08 for Vitamin D2, 3-Ketolacotse, and Metarhodopsin, respectively and 8.14 and 8.18 for Prostaglandin A2 and Leukotriene A4, respectively. On the other hand, two peak patterns in Fig. 1 showed 0.04 of difference in tR/min. How did the authors exactly align the different peak patterns?
How much error did author accept when the peak patterns were aligned?

No description concerned in this matter was found in this paper. This definition is critical for evaluating the results in Figs 2 and 3.

**Our response:**

We analyzed the different peak patterns by Markerlynx software. It is very difficult to see the differenced peak patterns with the naked eyes. Small peaks can be detected and calculated. Though retention time was closes, the substances were different. Data analysis was done with 98% confidence interval. Parameters were set as follows:

**Instrument:** ZQ  
**Column:** T3

| A: 5mM AcNH4+0.1%FA | B: Acetonitrile | Column T: 45°C | Sample T: 4°C |

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**Polarity** ES+  
**Multiplier (V)** 650 -649  
**Calibration** Dynamic 1  
**Capillary (kV)** 3.60 3.60  
**Cone (V)** 30.00 -32.11  
**Extractor (V)** 1.00 -2.56  
**RF Lens (V)** 1.0 -1.0  
**Source Temperature (℃)** 120 119  
**Desolvation Temperature (℃)** 300 298  
**Cone Gas Flow (L/Hr)** 50 47  
**Desolvation Gas Flow (L/Hr)** 600 595  
**LM 1 Resolution** 14.0  
**HM 1 Resolution** 14.0  
**Ion Energy 1** 0.5  
**Polarity** ES-  
**Multiplier (V)** 650 -649  
**Calibration** Dynamic 1  
**Capillary (kV)** 3.60 3.60  
**Cone (V)** 30.00 -32.11  
**Extractor (V)** 1.00 -2.56  
**RF Lens (V)** 1.0 -1.0  
**Source Temperature (℃)** 120 119  
**Desolvation Temperature (℃)** 300 298  
**Cone Gas Flow (L/Hr)** 50 47  
**Desolvation Gas Flow (L/Hr)** 600 595  
**LM 1 Resolution** 14.0  
**HM 1 Resolution** 14.0
Function 1

- Scans in function: 818
- Cycle time (secs): 0.600
- Scan duration (secs): 0.200
- Inter Scan Delay (secs): 0.100
- Start and End Time (mins): 0.000 to 10.000
- Ionization mode: ES+
- Data type: Enhanced Mass
- Function type: Scan
- Mass range: 70 to 1000
Comment 6:

6. Page 12, Fig. 4: Label for left figure is not available. The reason why these samples were selected in this figure is not clear. What is M2? Anyhow the figures show no meaning.

Our response:

16S rRNA gene region V3 PCR–DGGE diagrams show that the strips are numerous. Some stripes are existed in two groups; some stripes are unique in normal group or chronic gastritis group. 20 normal controls and 20 atrophic gastritis patients, the following figure shows some of the samples (Fig4).

![PCR–DGGE atlas of 16S rRNA gene V3 region between the chronic gastritis group and the normal controls](image)

**Fig.4.** PCR–DGGE atlas of 16S rRNA gene V3 region between the chronic gastritis group and the normal controls

(A) Normal group, M1-marker, M2-control, 2-19: normal group sample

(B) Chronic gastritis group, M1-marker, M2-control, 2-19: chronic gastritis sample
Comment 7:

Page 12, Figure 5: The authors emphasized 10 strips characteristics for discriminating between gastritis and control groups. However these strips seem to be individually specific. The reviewer is unable to feel consistency of these strips in gastritis or control group. In addition, Chinese character is not appropriate for labeling the figure.

Our response:

16S rRNA gene region V3 PCR–DGGE gel were scanned by imaging system (Tanon-3500, Tanon), the data were showed in supplementary material 1. The gel and Image J were used to obtain the DGGE digitalized map for multivariate statistical analysis (PCA, PLS–DA ) to determine any significant difference between the strips in each group. 10 strips characteristics for discriminating between gastritis and control groups are got by Statistical analysis.
Fig5 Comparison of PCR–DGGE diagrams of 16S rRNA gene V3 region between the normal controls and the chronic gastritis group

(A) Normal group, M1-marker, M2-control, normal group samples -- 1, 5, 9, 12, 16, 20

(B) Chronic gastritis group, M1-marker, M2-control, Chronic gastritis samples -- 11, 12, 13, 14, 15, 16, 17, 18, 19, 20

Comment 8:

Page 13, Figure 6: There is no description for alignment of digitalized DGGE maps. This definition is critical for evaluating the results in Fig 6.
The information on DGGE strips selection in PLSDA analysis should be also clarified. The authors should identify bacterial origins of DGGE strips with high VIP values in PLS-DA analysis in the same way with metabolomics.

**Our response:**

Digitalized DGGE maps were described in Fig6. Each sample was composed by a set of DGGE strips data. Each pot was a sample in PLSDA analysis. (Fig 6)

![Fig6. PCA and PLS-DA of the 16S rRNA gene V3 region of the normal group and the chronic gastritis group](image)

(a) PCA results, red dots-normal samples, green dot- chronic gastritis patients

(b) PLS-DA results, red dots-normal samples, green dot- chronic gastritis patients
Reviewer #3 (santosh patil )

Comment 1:  Major Compulsory Revisions- Not Required

Comment2. Minor Essential Revisions

A Abstract-5thline grammatical error
b .Introduction paragraph 21stline-grammaticalerror
c.18thline-grammaticalerror
d. Data and methods-criteria for diagnosis-grammaticalerror
e. Methods-paragraph1-grammaticalerror
f. Figure 4-legend missing
g.Discussion1stparagraph,6thline-grammaticalerror
h.7th paragraph-quote reference
Last line- glucose mentioned twice
i. References- Author names and journal names not in format
j .Limitations of the study are not clearly stated

Our response:

Abstract-5thline grammatical error------ had been modified.
B.introduction paragraph 21stline-grammaticalerror-- had been modified.
C.18thline-grammaticalerror-- had been modified---had been modified.
d. Data and methods-criteria for diagnosis-grammatical error-- had been modified.

e. Methods-paragraph1-grammaticalerror-- had been modified.

f. Figure 4-legend missing--- had been modified.

g. Discussion1stparagraph, 6thline-grammaticalerror--- had been modified.

h. 7th paragraph-quote reference-- had been modified.

lastline- galactose mentioned twice-- had been modified.

i. References- Author names and journal names not in format-- had been modified.

Comment3: Discretionary Revisions- Not Required