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Prof. Ruo-Yu Ma, MD, PhD

Co-Editor-in-Chief

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Dear Prof. Ruo-Yu Ma:

NMR-based metabolomics and metabolic pathway networks from patient-matched esophageal carcinoma, adjacent noncancerous tissues and urine

(ID: 46020)

We are pleased with the strong support of Referees for our work, and with their constructive comments on our manuscript. Those comments are all valuable and very helpful for revising and improving our paper. We have studied comments carefully, and have made correction which we hope meet with approval. Revised portion are marked in red in the paper. The detailed responds to the reviewer's comments have addressed as indicated below.

Point-to-point responses to reviewers' comments

Reviewer #1 (Comments to the Author):

1. ***The authors identify certain metabolites to be changed in esophageal cancer tissue compared with healthy tissue and this is validated only in 16 patients (8 EC and 8 HC). I think this group is too small for validation and this strongly limits the conclusions from this exploratory research.***

Response: Many thanks for the kind commend. In this initial study, orthogonal partial least squares discriminant analysis (OPLS-DA) was applied for disease classification (through the use of score plots) and biomarker detection (through the use of loading plots). To further assess the predictive ability of the model for unknown samples, we randomly selected 80% of urine samples ("training set", 33 EC patients and 32 HCs) to construct an OPLS-DA model, which was then

used to predict the remaining 20% of samples ("testing set", 8 EC patients and 8 HCs). As can be seen in Figure 2A-c, the testing sets of EC patients and HCs were correctly located in their corresponding region of the training sets. As a follow up to this study, a larger quantity of samples of esophageal cancer are needed to validate our initial findings.

2. Stage of the tumour may be an important factor in the changes observed, especially in stage IV disease. In the 17/41 patients, the tumour stage was not unknown. This should be further specified or at least an attempt should be made to have limit these missing data.

Response: We concur with this comment and Table 1 has been updated to specify the tumor stage. Accordingly, the session of "patient recruitment and sample collection" has been updated to include the following sentence " The EC patients were diagnosed by microscopy, biopsy, or surgical resection, and the disease stage was determined according to the American Joint Committee on Cancer (AJCC) staging system for esophageal tumors: stage I/ II, 15 patients; stage III, 11 patients; stage IV, 15 patients", which has been highlighted in red.

Table 1. Summary of clinical and demographic features for study subjects and tumor characteristics

	EC	HC	χ^2	*P**
No. of subjects	41	40		
Age (median, range), yr	60, 39-77	59, 28-78		
Sex			6.77	0.12
Male	31	19		
Female	10	21		
Cancer stage				
Stage I/II	15	-		
Stage III	11	-		
Stage IV	15	-		
CEA (ng/mL)				
Positive	2	-		
Negative	31	-		
Not check	8	-		
CA 19-9 (U/mL)				
Positive	2	-		
Negative	18	-		
Not check	21	-		
Location				
Cervical	1	-		

Upper thoracic	5	-
Middle thoracic	24	-
Lower thoracic	11	-
Symptoms		
Dysphagia	40	-
Gastroesophageal reflux	27	-

CA 19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen.

3. Figure 1 is not clear to me. This may be further clarified by indicating the important parts of this figure, or can be omitted and explained in the text to improve readability.

Response: Many thanks for this comment. Figure 1 were representative 1D ^{1}H-NMR spectra of urine specimens obtained from EC patients, healthy controls, and patient-matched esophageal tissue extracts obtained from ECT and ANT. The major metabolites in the spectra were identified according to previous studies and the Human Metabolome Database (http://www.hmdb.ca/). In all urine and esophageal tissue spectra, the aliphatic region at 0.8–4.2 ppm included numerous signals from the following water-soluble metabolites: glutamate, glutamine, acetoacetate, citrate, cis-aconitate, choline, creatine, creatinine and glycine, which are known to be involved in many biochemical processes, especially in energy metabolism. We hope this addresses the reviewer's concerns.

4. In the abstract it is stated that colonic tissue was used, I think the authors mean esophageal tissue 2. The Variable Importance (VIP) needs more extensive explanation in the discussion 3. The results section of the abstract must be more specific 4. The discussion can be compacted substantially.

Response: Many thanks for the good suggestion. As requested, the abstract has been revised, which has been highlighted in red, and the "Discussion" section has now been shortened by about 23%. The detailed explanation of VIP can be found in the session of "Pattern recognition (PR) analysis and cross validation"in methods, which has been addressed in red.

Reviewer #2 (Comments to the Author):

1. Please tell me the reason why EC patient urine metabolites, compared with HCs, including elevated acetoacetate, glutamate, cis-aconitate, citrate and reduced creatinine, glycine, hippurate , taurine, glucose.

Response: Acetoacetate, glutamate, cis-aconitate, and citrate are important TCA intermediates, and their elevated amounts could reflect high TCA cycle activity to maintain tumor promotion, Tumor-microenvironment co-operation may occur in cancer cells, which exhibit a high rate of anabolic metabolism, by which they take up large amounts of glucose, glycine and other nutrients to fuel the TCA cycle and oxidative phosphorylation. Hence, decreased urinary glucose, glycine observed in this study were in agreement with the ingested nutrients that fuel the TCA cycle to support cell proliferation. Creatinine is the downstream product of creatine phosphate metabolism (one of energy metabolism), and its downregulation may indicate disruption of energy homeostasis due to tumor promotion. The observed depletion of hippurate in EC urine suggests a disruption in the intestinal epithelium and diffusion of gut microbes associated with esophageal tumourigenesis. The observed depletion of taurine suggest a disruption in taurine metabolism and diffusion of gut microbes associated with EC tumor.

Accordingly, the discussion session has been rewritten, which has been addressed in red.

2. Which is the most sensitive predictor of esophageal cancer in urine metabolite?

Response: In our initial study, creatinine was found to be the most sensitive predictor of esophageal cancer in urine metabolite, with AUC of 0.790.

3. Please tell me the different metabolomic profile between stage1,2 EC and stage 3,4

Response: Many thanks for this suggestion. As requested, we re-analyzed the urine data by OPLS-DA model, which revealed that early and later stage of EC could be clearly distinguished based on their metabolomic profles. Successive analyses identified distinct disturbances to urinary metabolites at later stage EC patients (stage 3/4), compared with those in stage 1/2 EC patients, including reduced levels of creatinine, glucose and hippurate and elevated quantities of acetoacetate, cis-aconitate, citrate and glutamate. However, due to the small amount of samples,

the sensitivity and specificity of these metabolites is low, with AUC value of <70%. Therefore, we decide not to put these data in the revised manuscript. Further study would consider the changes of urinary metabolites characteristically occur in the earlier stage of EC based on larger samples.

4. According to author's data, amino acid in tissue level is specific parameter, high level of valine, leucine,glutamate, acetate, alam\nine,choline, succinate , citrate and low level of glucose,creatinine,glycine, threonine, creatine, glutamine, taurine. How about serum level of amino acid in EC patient? Is the tissue level of amino acid in EC patients same pattern of serum level of amino acid in EC patient?

Response: Many thanks for this suggestion. We have no direct evidence from this current manuscript to support the serum metabolite changes in EC patient. But in our same group, another one are now investigating the serum samples of EC patients to see whether same pattern of serum level of amino acid can be found in EC patients.

In the final analysis, we would like to thank the editor and the reviewers for their insightful comments and constructive feedback which have enabled us to significantly enhance our manuscript. We hope that we were able to address all their concerns and answer all their questions to their full satisfaction. We look forward to a receiving a favorable response regarding the publication of our manuscript in your esteemed journal.

Yours sincerely

Jiahao Liang (for the authors)