Manuscript NO: 59620

Title: Alteration of fecal tryptophan metabolism correlates with shifted microbiota and may be involved in the pathogenesis of colorectal cancer

Answering Reviewer 05095461

Thank you very much for your comments and suggestions, which are very valuable for us to improve and revise our paper. We have made revisions or explanations point by point. We highlighted the changes in the manuscript by using the track changes mode in MS Word.

Specific comments:

1. The role of tryptophan metabolism in CRC and its correlation with gut microbiota has attracted wide attention (Transl Res, 2016, 167(1):67-79. doi: 10.1016/j.trsl.2015.07.003.). The hypothesis mentioned in this manuscript has existed (We hypothesized that there was an alteration of gut Trp metabolism mediated by microbiota and that it might be involved in the pathogenesis of cancer in patients with CRC).

Answer: We carefully read the literature you mentioned above(Transl Res, 2016, 167(1):67-79. doi: 10.1016/j.trsl.2015.07.003.). Although the role of tryptophan metabolism in CRC has attracted wide attention, most of the studies focused on tryptophan metabolism in colon tissues[1] or serum[2]. However, we focused on the fecal tryptophan metabolism. The noninvasiveness makes the results easily verifiable in a larger sample in the future.

2. The fecal Trp metabolism was correlated with shifted microbiota in CRC patients. However, there is no evidence that they have participated in the pathogenesis of CRC. May be CRC induced the changes of microbiota and fecal Trp metabolism.

Answer: Thank you very much for reminding us of this crucial issue. Our results only showed fecal Trp metabolism correlated with shifted microbiota, intestinal barrier function and tissue Trp kynurenine pathway (KP), there is no evidence that they have

participated in the pathogenesis of CRC. Since previous animal[3][4] and vitro[5] studies showed that fecal tryptophan metabolites had an inhibitory effect on colon tumorigenesis. We hypothesized that fecal tryptophan metabolism might be involved in the human CRC. There is an interaction between fecal metabolism and human body. It is possible that CRC induced the changes of microbiota and fecal Trp metabolism. Our conclusion that alterations of gut tryptophan metabolism and microbiota have participated in the pathogenesis of CRC is not exact. First, we corrected the conclusion and relevant details in the manuscript (title, abstract, and discussion sections). Second, the discussion on this question has been added in the 1st paragraph on P 19. Third, the conclusions based on this observational study prevent us from determining the causal relationships. We will later conduct intervention studies and animal experiments to verify their relationship.

3. The indexes of intestinal barrier function include ZO-1, Claudin, Muc, and so on. Just detection of ZO-1 only in mRNA level is not very convincing.

Answer: ZO-1, the main scaffolding protein of tight junction, has been used to assess intestinal barrier function in a variety of studies[6][7]. Just detection of ZO-1 only in mRNA level is not very convincing, so we added the protein level of ZO-1 in the 1st paragraph on P 11, 4th paragraph on P 13, and figure 2 on P 30. Since the study was preliminary explore the relationship between fecal tryptophan metabolites and intestinal barrier function, other intestinal barrier indexes were not detected. Other indexes of intestinal barrier function will be thoroughly assessed in our subsequent validation experiments.

4. The manuscript has some typos and format errors.

Answer: We have carefully checked the entire manuscript for consistent formatting, typos and corrected the errors.

Answering Reviewer 00004011

These comments are all valuable and very helpful for revising and improving our paper. All the authors have studied the comments carefully and revised the manuscript based on them. Thank you very much. Our manuscript is not clear regarding methodology issues. We have made revisions and explanations point by point. We highlighted the changes in the manuscript by using the track changes mode in MS Word.

1. The authors should describe in more details the specimens collected from each patient.

Answer: In the revised manuscript, we described the method of specimen collection from each patient in more detail in 4th paragraph on P 7 and 1st paragraph on P 8.

2. It is not clear if tissue materials used for RT-PCR are from the same patients that fecal samples were collected.

Answer: The tissue materials used for RT-PCR and IHC are from the same patients that fecal samples were collected. It has been corrected in the subjects and sample collection sections.

3. Did the patients received chemotherapy? or other therapy? since therapy influence the microbiota and microbiome function.

Answer: Thank you very much for reminding us of this very important issue. I'm sorry we didn't describe this in the methods section. All the patients included in our study were newly diagnosed and did not receive any treatment. It has been added on line 2nd paragraph on P 7.

References

[1] **Crotti S,** Bedin C, Bertazzo A, Digito M, Zuin M, Urso E D, Agostini M. Tryptophan Metabolism as Source of New Prognostic Biomarkers for FAP Patients. *Int J Tryptophan Res* 2019; **12:** 1178646919890293 [PMID: 31798304 DOI: 10.1177/1178646919890293]

[2] **Chen I C**, Lee K H, Hsu Y H, Wang W R, Chen C M, Cheng Y W. Expression Pattern and Clinicopathological Relevance of the Indoleamine 2,3-Dioxygenase 1/Tryptophan 2,3-Dioxygenase Protein in Colorectal Cancer. *Dis Markers* 2016; **2016:** 8169724 [PMID: 27578919 DOI: 10.1155/2016/8169724]

[3] **Diaz-Diaz C J**, Ronnekleiv-Kelly S M, Nukaya M, Geiger P G, Balbo S, Dator R, Megna B W, Carney P R, Bradfield C A, Kennedy G D. The Aryl Hydrocarbon Receptor is a Repressor of Inflammation-associated Colorectal Tumorigenesis in Mouse. *Ann Surg* 2016; **264:** 429-36 [PMID: 27433903 DOI: 10.1097/SLA.0000000000001874]

[4] **Kawajiri K**, Kobayashi Y, Ohtake F, Ikuta T, Matsushima Y, Mimura J, Pettersson S, Pollenz R S, Sakaki T, Hirokawa T, Akiyama T, Kurosumi M, Poellinger L, Kato S, Fujii-Kuriyama Y. Aryl hydrocarbon receptor suppresses intestinal carcinogenesis in ApcMin/+ mice with natural ligands. *Proc Natl Acad Sci U S A* 2009; **106:** 13481-6 [PMID: 19651607 DOI: 10.1073/pnas.0902132106]

[5] **Megna B W**, Carney P R, Nukaya M, Geiger P, Kennedy G D. Indole-3-carbinol induces tumor cell death: function follows form. *J Surg Res* 2016; **204:** 47-54 [PMID: 27451867 DOI: 10.1016/j.jss.2016.04.021]

[6] **Assimakopoulos S F**, Akinosoglou K, de Lastic A L, Skintzi A, Mouzaki A, Gogos C A. The Prognostic Value of Endotoxemia and Intestinal Barrier Biomarker ZO-1 in Bacteremic Sepsis. *Am J Med Sci* 2020; **359:** 100-107 [PMID: 31812215 DOI: 10.1016/j.amjms.2019.10.006]

[7] **Ruan Y C**, Wang Y, Da S N, Kim B, Diao R Y, Hill E, Brown D, Chan H C, Breton S. CFTR interacts with ZO-1 to regulate tight junction assembly and epithelial differentiation through the ZONAB pathway. *J Cell Sci* 2014; **127:** 4396-408 [PMID: 25107366 DOI: 10.1242/jcs.148098]