List of Responses

Dear Editors and Reviewers:

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "Biomarkers and potential pathogenesis of colorectal cancer-related ischemic stroke" (Manuscript No. 42024). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval. All of the revisions are marked in red in revised manuscript and cited in the response letter. The main corrections in the paper and the responds to the reviewer's comments are as following:

Responds to the reviewers' comments:

(1) **Reviewer #1 (code:** 03730829**)**

The authors aimed to investigate the specific biomarkers and potential pathogenesis of colorectal cancer-related ischemic stroke (CRCIS). A good job they did; However; there are major issues:

Comment 1: The study was retrospective which introduces bias into the results of the research.

Response: Thanks for your valuable comment. As you mentioned, the present study was a retrospective one. In our study, we had recruited the patients according to the CRCIS inclusion and exclusion criteria strictly. The bias in the present study had been pointed out in revised manuscript. Please refer to the red section in the seventh paragraph of the DISCUSSIONS.

"The strengths of our study were multicenter enrollment and comparison with CRC patients without IS. The main limitations of this study included the

relative small sample size and some uncontrollable setting. Still, our results could promote further prospective larger population studies, which would be better to illuminate the biomarkers and pathogenesis of IS in CRC patients."

Comment 2: Sample size calculation and the power of the study are so important and should be added in the study design and in the methods section as you are investigating the specific biomarkers and potential pathogenesis of colorectal cancer-related ischemic stroke; so is this sample sufficient or not? It is important question to answer to get a valid conclusion.

Response: Thank you for your constructive comment. The design of the present study referred to previous studies as follow: (1) *Schwarzbach CJ et al. Stroke and Cancer The Importance of Cancer-Associated Hypercoagulation as a Possible Stroke Etiology. Stroke, 2012 Nov; 43(11): 3029-34, (2) Grazioli S et al. Cancer-associated ischemic stroke: A retrospective multicentre cohort study. Thromb Res, 2018 May; 165: 33-37, (3) Kassubek R et al. Identifying ischemic stroke associated with cancer: a multiple model derived from a case-control analysis. J Neurol, 2017 Apr; 264(4): 781-791.* Additionally, as you recommended, we had added sample size calculation in our revised manuscript. Please refer to the red section in the second paragraph in **MATERIALS AND METHODS**. As we had included 114 CRCIS patients and 114 CRC patients, the sample size in our study should be sufficient.

"To improve the power of the present study, the sample size need to estimate. Previous studies found that cancer-related IS was associated with elevated plasma D-dimer. However, whether it was associated with CRCIS is not clearly. Therefore, we hypothesize that D-dimer may also be associated with the occurrence of CRCIS. It was reported that the positive rate of D-dimer in colorectal cancer was 15% to 31%. In our 1:1 matched case-control study, for sample size estimation, we assumed the positive rate of D-dimer in CRC

patients was 20%, and risk of CRCIS was 3.5. Results showed that it needed 97 pairs when α=0.05 and β=0.2 (two-tailed).”

Comment 3: You arrived to conclusion that hypercoagulability induced by elevated carcinoembryonic antigen and increased neutrophil count are the main pathogenic factors in CRCIS. You should further explain in discussion to the readers the exact pathogenesis of hypercoagulability caused by increased neutrophil count and by increased carcinoembryonic antigen.

Response: Thanks for your constructive comment. As you recommended, we have further explained the exact pathogenesis of hypercoagulability caused by increased neutrophil count and by increased carcinoembryonic antigen in our revised manuscript. Please refer to the red section in the fourth and fifth paragraphs of the DISCUSSIONS.

“Although hypercoagulability may be associated with cancer-related IS, its underlying mechanism remains unclear. Interestingly, recent studies have suggested that mucins generated from mucinous cancer are associated with hypercoagulability and increase the risk of IS[28-30]. A study by Jovin *et al*[28] reported four patients with metastatic cancer, brain infarcts, and markedly elevated mucinous serum marker CA125 levels, and suggested a possible association between this protein and stroke. Moreover, the relationship between mucins and hypercoagulability were further confirmed by necropsy evidences from mucinous cancer patients with widespread intracranial arteriovenous thrombosis and multiple cerebral infarctions, in which the mucin within vessels and in microthrombus in the regions of infarction were found by microscopic examination[29]. Furthermore, animal experiments demonstrated that mucins secreted by cancer cells could trigger the reciprocal activation of platelets and neutrophils and lead to the formation of thrombus in the blood[30]. These findings indicated that mucins associated

hypercoagulability played an important role in cancer-related IS."

"Additionally, neutrophil extracellular traps (NETs) that generated from neutrophils were also found to associate with hypercoagulability and thrombosis diseases (including IS) in patients with cancer[32, 33]. In solid tumor models, it was found that cancer can induce an increase of peripheral blood neutrophils. And increased neutrophils may release NETs in the vascular, which may promote hypercoagulability by stimulating platelet activation[34]. Therefore, NETs generated from neutrophils was considered to be a novel coagulation-promoting mechanism in cancer patients[35, 36]."

Special thanks to you for your good comments and constructive suggestions.

(2) **Reviewer #2 (code:** 02982391**)**

This is a well designed manuscript and it addresses an important topic in gastroenterology, neurology and haematology. It is a potential good research paper for this journal. I have a few comments for the authors.

Comment 1: The authors may need to explain further why did they decided to choose these biomarkers in their study and did not test other potential biomarkers such as soluble ST2, Serum IL6, miR-146b (Chen et al 2018).

Response: Thanks for your constructive comment. In our retrospective study, the patients' clinical data were all collected base on the examination items that they had done during the time of hospitalization, which were only satisfied with the needs of clinical diagnosis and treatment. Few CRCIS patients have done examination items such as soluble ST2, serum IL6 and miR-146b. Moreover, the biomarkers that were chose in our study were all variables with statistically significant. However, the potential biomarkers that your mentioned will be tested and studied in our future prospective studies.

Comment 2: Other researchers have found D-dimer as a potential risk factor in this group of patients and I think this paper adds to our knowledge in this area. The authors may need to read and cite these two studies: Wang JY, Zhang GJ, Zhuo SX, Wang K, Hu XP, Zhang H, Qu LD. D-dimer >2.785 μg/ml and multiple infarcts ≥3 vascular territories are two characteristics of identifying cancer-associated ischemic stroke patients. Neurol Res. 2018 Nov;40(11):948-954. Li J, Gu C, Li D, Chen L, Lu Z, Zhu L, Huang H. Effects of serum N-terminal pro B-type natriuretic peptide and D-dimer levels on patients with acute ischemic stroke. Pak J Med Sci. 2018 Jul-Aug;34(4):994-998. doi: 10.12669/pjms.344.15432.

Response: Thank you for your comment. We have read the two articles you suggested carefully. As you recommended, we have added the relevant content and cited the article wrote by Wang JY, *et al* in our revised manuscript. The study of Li *et al* was not involved in IS patients with cancer. Therefore, it was not cited in our revised manuscript. Please refer to the red section in third paragraph of the DISCUSSIONS.

"Recently, Wang and his colleagues found that the plasma D-dimer value of 2.785 μg/ml was the cutoff in identifying cancer-related IS patients [26]."

Comment 3: The authors did not study several other biomarkers, therefore I would recommend that they make changes to their conclusions about the index or only limiting the scope of their conclusions by just reporting their findings. May be in the future there will be a study on a bigger number of patients covering 10 or so markers and in such situations you may discuss CRCIS Index.

Response: Thanks for your comment. It is a very good and professional question. As you recommended, we have limited the scope of the conclusions. Please refer to the red section in sixth and eighth paragraphs of the

DISCUSSIONS and in *CONCLUSION* of Abstract.

"As the area under the ROC curve of the CRCIS Index was highly accurate, and the sensitivity and specificity of the CRCIS Index were high, we suggest that CRCIS Index could serve as a potential biomarker. However, due to the retrospective design of the present study, the other biomarkers mentioned in previous studies that may be related to cancer-related IS were not studied[12,13]. The role of CRCIS Index in CRCIS patients may need to be confirmed in future studies with larger samples and more biomarkers. Nevertheless, our study provided a meaningful method to the further investigation about the index of CRCIS in future studies."

"In summary, our findings suggest that hypercoagulability induced by elevated CEA and neutrophils may be an important cause of CRCIS. CRCIS index which serves as a biomarker of CRCIS needs to be confirmed in future study."

"***CONCLUSION***

Hypercoagulability induced by elevated CEA and neutrophils may be an important cause of CRCIS, CRCIS index which serves as a biomarker of CRCIS need further study. "

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper.

We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

Once again, thank you very much for your comments and suggestions.