Dear editor,

Thank you for your letter and for the reviewer's comments concerning our manuscript entitled "Alcohol promotes EMT-mediated premetastatic niche formation of colorectal cancer by activating the early interaction between LAMC2 and integrin β1". These comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. The revised manuscript has been uploaded, and the changes highlighted red in the revised manuscript.

The following pages are our point-by-point responses to each of the question. If possible, we sincerely hope that you can take some more time to read our reply and the revised manuscript. We really hope that our reply and the revised manuscript would meet the requirements of your prestigious journal and can be accepted for publication. Meantime, if you have any questions concerning the revised manuscript, please contact us without hesitate.

Yours sincerely,

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Reviewer #1:

Thank you very much for your valuable suggestions. We have revised the manuscript according to your suggestions. We sincerely hope that you can take some more time to read our reply and the revised manuscript.

Question 1: Thank you for this interesting manuscript. My only concern is related to the presumed direct link between alcohol consumption and the colorectal carcinogenesis. Specifically, how can you differentiate the effect of alcohol from the effect of other carcinogens? It is known that people with other pathology, i.e. obesity, smoking history are also more prone to developing cancer. For this reason, the table comparing the alcohol and non-alcohol group should include these factors, if available, to insure homogeneity of groups.

Answer: Thank you very much for your questions. As mentioned in "Introduction" section, it is reported that long-term alcohol consumption is associated with an increased risk of CRC metastasis [1]. In the presence of concomitant metastasis, the maximum survival rates after surgical intervention do not exceed 20% [2,3]. Therefore, this study focused on the relationship between alcohol consumption and CRC metastasis. In our study, patients with smoking, family history, blood lipids, obesity, and chronic diseases were excluded, which has been added into the section of "2.2 Patients and clinical data".

[1] Murphy N, Moreno V, Hughes DJ, Vodicka L, Vodicka P, Aglago EK, Gunter MJ and Jenab M. Lifestyle and dietary environmental factors in colorectal cancer susceptibility. MOLECULAR ASPECTS OF MEDICINE 2019: 2-9.

[2] Zheng K, Yu J, Chen Z, Zhou R, Lin C, Zhang Y, Huang Z, Yu L, Zhao L and Wang Q. Ethanol promotes alcohol-related colorectal cancer metastasis via the TGF-beta/RUNX3/Snail axis by inducing TGF-beta1 upregulation and RUNX3 cytoplasmic mislocalization. EBioMedicine 2019: 224-237.

[3] Mohr AM, Gould JJ, Kubik JL, Talmon GA, Casey CA, Thomas P, Tuma DJ and McVicker BL. Enhanced colorectal cancer metastases in the alcohol-injured liver. Clin Exp Metastasis 2017; 2: 171-184.

Question 2: Additionally, alcohol consumption exists over a spectrum. It would be interesting to differentiate the effects of heavy drinking from moderate drinking, in order to assess a potential alcohol quztity-effect relationship. The animal studies are described in an excellent way and clearly took a lot of effort to conduct.

Answer: Thank you very much for your suggestions. Does alcohol consumption exist over a spectrum, to differentiate the effects of heavy drinking from moderate drinking will be a very meaningful research. However, due to the limited clinical samples, it is currently difficult to further differentiate the effects of heavy drinking from moderate drinking. And we will continue to collect samples for future study.

Reviewer #2:

Thank you very much for your valuable suggestions. We have revised the manuscript according to your suggestions. We sincerely hope that you can take some more time to read our reply and the revised manuscript.

Question 1: The introduction should be focused on the aspects to be discussed in the work and reduced.

Answer: Thank you very much for your suggestion. We have rewritten the "Introduction" section.

Question 2: In the methodology it should be specified when the monoclonal antibodies are used in rats or in humans.

Answer: Thank you very much for your question. We have added the related description into "2.1 Antibodies and reagents": "All above the mentioned antibodies can be used in both in rats and in humans."

Question 3: The patient cohort study is left behind in this article, perhaps its presence is not decisive. In any case, neither the amount nor the time of alcohol consumption in the patients is specified.

Answer: Thank you very much for your question. In this study, animal study was used to explain the influence and potential mechanism of alcohol intake on CRC metastasis and exclude the influence of non-alcoholic factors on CRC metastasis as much as possible. Clinical study was taken to verify the influence and potential mechanism of long-term alcohol intake on CRC metastasis from the perspective of patients.

In addition, alcohol consumption in our study was ascertained from three items on the baseline and follow-up questionnaires: (1) 'On how many days each week do you usually drink alcohol?'. Participants reported their response as an integer. (2) 'About which alcoholic drink do you like often? a. wine, b. beer; c. 30% or over 30% alcohol liquor; d. any other'. (3) 'About how long have you kept the habit of drinking? '. CRC patients who never drank alcohol were added into the non-alcohol group, and CRC patients who drank alcohol over 3 times a week for more than 5 years were added into the alcohol group. Which has been added into "3.7 Baseline characteristics of CRC patients. A total of 63 patients with a preliminary diagnosis of colorectal cancer participated in the trial" : "According to the baseline characteristics and data on alcohol consumption of 63 CRC patients, 29 CRC patients who had been drinking alcohol over 3 times each week for more than 5 years were classified in alcohol group, while the remaining 34 CRC patients who have never drunk alcohol before were classified in non-alcohol group."

Question 4: The meaning of injecting the rat with DMH should be explained.

Answer: Thank you very much for your suggestion. We have added description into the "2.3 Animals, induction of CRC and treatments" section: "The rats were fed water (10 mL/kg b.wt.) every day and from the 6th week on, the rats were administered a subcutaneous injection of 1,2-dimethylhydrazine hydrochloride (DMH) at a dose of 30, 35, 25 or 20 mg/kg b.wt. once a week for 12 weeks in order to induce CRC. Alcohol group (DMH + ethanol): The rats were fed 30% ethanol (10 mL/kg b.wt.) every day, and from the 6th week on, they were administered a subcutaneous injection of DMH at a dose of 30, 35, 25 or 20 mg/kg b.wt. once a week for 12 weeks in order to induce CRC."

Question 5: It is necessary to explain how the histological sections are made.

Answer: Thank you very much for your question. Related descriptions about how the histological sections are made have been added into the section of "2.8 Immunohistochemical staining (IHC)": "The IHC was performed by the method described in previous studies [1,2], all colonic tissue were fixed with 4% paraformaldehyde and paraffin embedded, then thin 4μm sections were obtained and deparaffinized in xylene and rehydrated through decreasing grades of alcohol."

[1] Mansour DF, Abdallah H, Ibrahim B, Hegazy RR, Esmail R and Abdel-Salam LO. The Carcinogenic Agent Diethylnitrosamine Induces Early Oxidative Stress, Inflammation and Proliferation in Rat Liver, Stomach and Colon: Protective Effect of Ginger Extract. Asian Pac J Cancer Prev 2019; 8: 2551-2561.

[2] Huang G, Bao J, Shao X, Zhou W, Wu B, Ni Z and Wang L. Inhibiting pannexin-1 alleviates sepsis-induced acute kidney injury via decreasing NLRP3 inflammasome activation and cell apoptosis. LIFE SCIENCES 2020: 117791.

Question 6: It is recommended to indicate the purpose of the PLA technique.

Answer: Thank you for pointing out this issue. PLA technique was used in this study to detect the interactions between LAMC2 and integrin β1 in tissue samples, and the added description has been added into the section of "Duolink proximity ligation assay (PLA)" : "The Duolink PLA in situ was used in this study to detect the interaction between LAMC2 and integrin β1 in tissue samples, and it was performed as previously described by Ning Bai et al. Briefly, after washing, permeabilizing, and blocking as histological analysis, colon sections were incubated with primary antibodies against LAMC2 (1:200) and integrin β1 (1:400) overnight at 4°C.Then the slides were incubated with Duolink PLA Rabbit MINUS and PLA Mouse PLUS proximity probes for 1 h at 37 ℃. Ligation and amplification were conducted using the Duolink in situ detection reagent kit according to the protocol. Images were captured under the light microscope (Olympus, BX51)."

Question 7: Avoid the use of abbreviations in the different sections of the manuscript.

Answer: Thank you very much for your suggestion. We have checked and revised the manuscript.

Question 8: In section 3.7 make a correct wording of the section. Part of what is indicated should be explained in the methodology

Answer: Thank you very much for your suggestion. We have checked and revised it in section of "3.7": "According to the baseline characteristics and data on alcohol consumption of 63 CRC patients, 29 CRC patients who had been drinking alcohol over 3 times each week for more than 5 years were classified in alcohol group, while the remaining 34 CRC patients who have never drunk alcohol before were classified in non-alcohol group. As shown in Table 2, there are significant differences in clinical stage and tumor metastasis ($P<0.05$) between alcohol group and non-alcohol group. There were no significant differences in sex, age, degree of differentiation or T stage ($P>0.05$). The experimental results were consistent with the results of animal experiments. The degree of tumor deterioration in CRC patients in the alcohol group was significantly higher than that in non-alcohol group."

Question 9: 3.10, should not be discussed in results.

Answer: Thank you for pointing out this issue. We have checked and revised it.

Question 10: The discussion should be focused more.

Answer: Thank you very much for your suggestion. We have revised it.

Question 11: The figures should be more explanatory, indicating the meaning of the abbreviations.

Answer: Thank you very much for your suggestion. We have added the meaning of the abbreviations into figure legends.

Question 12: The sample sizes should be indicated in the figures.

Answer: Thank you very much for your suggestion. We have added the sample sizes into figure legends.

Question 13: The different stains and markings should be indicated in the figure. The figures should be explained.

Answer: Thank you very much for your suggestion. We have added the stains and markings into figure legends.

Question 14: The units of measurement should be included in the figures.

Answer: Thank you very much for your suggestion. We have added the units of measurement into the figures.

Question 15: The significant comparisons in the graphs are tedious to understand. The authors could mark with a bar the compared groups.

Answer: Thank you very much for your suggestion. We have added the description into the figure legends.

Question 16: Fig 7a is not well understood.

Answer: Thank you very much for your suggestion. Fig 7a represent the survival analysis of integrin β1 and LAMC2 in CRC, we have made it bigger to make it easier to understand.

Question 17: Fig 7b, the group "normal cancer" is not understood.

Answer: Thank you very much for your suggestion. In Fig 7b, the group " cancer normal " means “cancer adjacent tissue” group, which has been added into figure legends.

Question 18: A general scheme of the purpose and techniques used in rats and in humans would help to understand the purpose of mixing both studies.

Answer: Thank you very much for your suggestion. In this study, animal study was used to explain the influence and potential mechanism of alcohol intake on CRC metastasis and exclude the influence of non-alcoholic factors on CRC metastasis as much as possible. Clinical study was taken to verify the influence and potential mechanism of long-term alcohol intake on CRC metastasis from the perspective of patients.