University of Massachusetts
UMASS Medical School

Gyongyi Szabo, M.D., Ph.D.
Associate Dean for Clinical and Translational Sciences
Director, MD/PhD Program
Vice Chair for Research
Department of Medicine
364 Plantation Street, LRB 208
Worcester, MA 01605-4321
Tel: 508-856-5275
Fax: 508 856-4770
Gyongyi.Szabo@umassmed.edu

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Ze-Mao Gong
Scientific Editor
World Journal of Gastroenterology

Re: Resubmission of manuscript # 23771 entitled "*An adult mouse model of early hepatocellular carcinoma promoted by alcoholic liver disease".*

Dear Dr. Gong,

Our manuscript entitled "An adult mouse model of early hepatocellular carcinoma promoted by alcoholic liver disease" received an encouraging review from *World Journal of Gastroenterology*. The authors greatly appreciate the insightful review, valuable comments of the Reviewers and an opportunity to resubmit the manuscript. Our revised manuscript addresses all comments raised by both Reviewers. Below we have provided point-by-point responses to the questions raised by the Reviewers. All changes in the revised version of the manuscript have been underlined.

The answers to specific Reviewer's Comments are as follows:

Reviewer 1: 01560575

Major comments:

The fact is that this manuscript tries to demonstrate the accelerating effect of ethanol in chemo-carcinogenesis by DEN and that this mouse model does not essentially represent the model of hepato-carcinogenesis induced by chronic ethanol consumption, namely the hepatocellular carcinoma seen in alcoholic

cirrhosis, even if the histopathological features would resemble those of alcoholic hepatitis, fibrosis or cirrhosis. The authors should always be aware of this fact whenever they write sentences since there are many confusions some of which are critical and should be omitted some of which are listed below. Otherwise, this manuscript is well written.

Answer: We appreciate these comments and as per your suggestion, we have deleted sentences from the revised version of the manuscript.

Minor comments:

1. Page 18, lines 15. "By utilizing adult mice and the Lieber-DeCarli alcohol diet, our model displays the natural course and progression of alcoholic liver disease and shows acceleration of early hepatobiliary tumors after a chemical carcinogen exposure" should be changed to, "By utilizing adult mice and the Lieber-DeCarli alcohol diet, our model shows acceleration of early hepatobiliary tumors after a chemical carcinogen exposure with some histopathological resemblance to human alcoholic liver diseases". The natural course and progression of alcoholic liver disease takes about 6 years even in baboons fed Lieber-DeCarli diet that was proven by Lieber et al.

   Answer: We appreciate the reviewer's suggestion and have changed the sentence, as suggested.
2. Page 22, lines 22. The sentence, "Our model involves a sequencial step-wise progression of alcoholic liver disease to HCC" should be omitted.

   Answer: We thank the reviewer for this suggestion. This sentence has been removed.
3. Page 23, line 1. "This combination of carcinogen pre-exposure and chronic alcohol consumption presents one of the most unique phenomenons of chronic alcohol leading to progression of HCC that occurs in humans", should be omitted.

   Answer: We appreciate the reviewer's suggestion and have removed this sentence.

Reviewer 2: 00004603

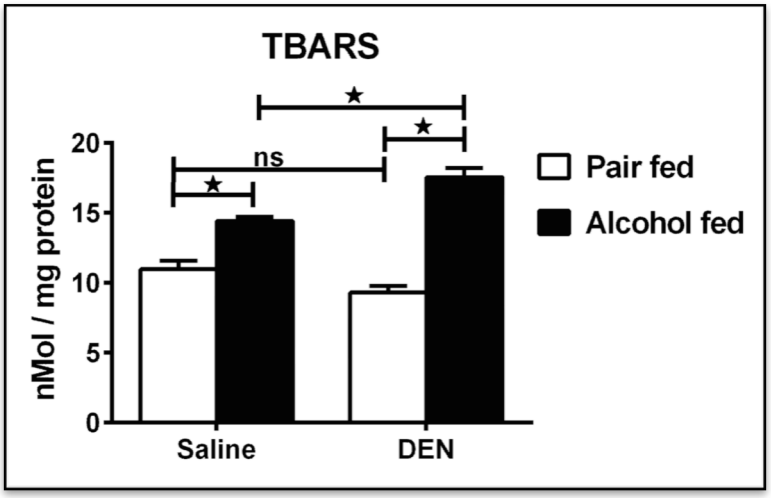
This is an interesting manuscript devoted to increased DEN-induced HCC development by alcohol. An important aspect of this study is that mice were fed not ethanol in water, but ethanol in Lieber De Carli diet (pair-feeding), which by itself induces liver injury.

There are couple of questions/suggestions to this study:

1. Although many chemical/histological parameters were determined, the design of the study does not allow distinguishing between the factors that drive HCC progression and those which accompany HCC development. However, more detailed pathogenic aspects are probably planned for future studies.
   Answer: Yes, indeed, we are planning to dissect the role of various factors such as Sonic Hedgehog, EMT, miR-122 in future studies that are beyond the scope of this paper.
2. The progression to HCC is confirmed by MRI. Considerable attention is attracted to liver macrophages and neutrophils, which claimed to play a role in HCC pathogenesis, The negative side of these studies is the characterization of macrophages as M1 and M2, which both are activated and thus, it is not clear what it can add to understanding of the mechanisms of alcohol-shaped HCC development and why in Discussion alcohol-induced switch to only M2 phenotype is emphasized.
   Answer: We appreciate the reviewer's helpful critique. Tumor-associated macrophages (TAMs) that help promote tumor progression have been shown to possess a distinct M2 phenotype (Sica A. et. al., EUROPEAN JOURNAL OF CANCER 42 (2006) 717–727). Given the role M2 macrophages play in supporting tumor progression, we emphasized on their potential contribution to HCC in our model in the discussion section. However, as per the comment by the reviewer, we have revised the Discussion to include the role of M1 macrophages. Investigation of M1/M2 MΦ markers is a novel finding in the alcohol-related HCC in our model.

3. Since it is known that induction of oxidative stress and ER stress in hepatocytes leads to HCC development, it will be reasonable to measure some parameters of oxidative stress is alcohol-fed mice exposed to DEN (like 4HNE-expression in hepatocytes, TBARS, glutathione levels).

   Answer: We appreciate the reviewer's comment and as shown below, we have measured the TBARS in our samples and have included the data below. The alcohol + DEN mice had significantly higher amount of ROS as compared to alcohol alone suggesting that the hepatocytes in alcohol + DEN mice are exposed to significantly higher stress / ROS which may be responsible for their accelerated transformation and HCC progression. This aspect is now included in the Discussion.



4. It has been shown in the literature that liver steatosis, but not alcohol per se affects HCC development. Do you think that in your case the increased HCC induction by DEN in alcohol-fed mice is attributed to alcohol metabolism or it requires liver fat accumulation as a consequence of ethanol metabolism?

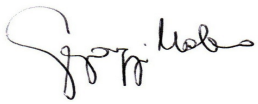
   Answer: The authors thank the reviewer for bringing up a very good point. We think that both alcohol itself and steatosis (due to alcohol or other etiology) are likely to promote HCC induction. Previous studies published by Dr. Mckillop's group had administered 20% v/v alcohol in drinking water for 24 or 48 weeks post DEN injection. Their data reported tumor lesions at 48 week only and a steady-state steatosis (Brandon-Warner et.al., Alcohol Clin Exp Res, Vol 36, No 4,

2012: pp 641–653). However, alcohol in drinking water does not cause significant changes in serum ALT, or significant steatosis (Cook, R. T. *et al*. *Alcohol. Clin. Exp. Res.* **31**, 1746-1758 (2007)). Hence it is possible that alcohol or its metabolites themselves, are responsible for enhanced inflammation and proliferation of cells.

Another study published by Dr. Karin's group showed in a dietary obesity model that the steatosis is an essential component towards HCC acceleration (Park EJ et. al. *Cell* 140, 197–208, 2010). Our unpublished data based on the NASH-HCC animal model that is similar to the one described in Dr. Karin's paper, also points to the role of liver steatosis as an important step towards HCC progression. Thus, it is possible that liver fat accumulation that is a consequence of ethanol metabolism, is also contributing towards the accelerated HCC development. We have included a new figure (Figure 8) that outlines the schematics of multi-factorial effects of ethanol on HCC development.

Overall, we have responded to all of the comments raised by the Reviewers, which we believe have significantly improved the clarity of the manuscript and added to our understanding the role of alcohol in accelerating hepatocellular cancer. With these revisions we sincerely believe that the manuscript now reaches the high scientific standards for publication in *World Journal of Gastroenterology.*

Your consideration of our manuscript for publication in *World Journal of Gastroenterology* is greatly appreciated.



Yours truly,
Gyongyi Szabo, MD, PhD