Author's response to reviews

Title: Mouse model of carbon tetrachloride induced liver fibrosis: Histopathological changes and expression of CD133 and epidermal growth factor

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Author's response to reviews: see over
April 11, 2010

Melissa Norton, M.D.
Editor-in-Chief, *BMC Gastroenterology*

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Dear Dr. Norton,

Thank you very much for your letter on January 13, 2010 regarding our manuscript entitled “Mouse model of carbon tetrachloride induced liver fibrosis: Histopathological changes and expression of CD133 and epidermal growth factor” by Tsutomu Fujii and Bryan C. Fuchs, et al. We have made every effort to improve our paper following the reviewers’ suggestions. The changes made in the revised version are summarized in the accompanying Response to Reviewers.

We continue to believe that this manuscript is suitable for publication in *BMC Gastroenterology* and would be of interest to your readership. Thank you in advance for your further consideration of our revised submission.

Sincerely yours,

Kenneth K. Tanabe, M.D.

**Reviewer's report**
Title: Mouse model of carbon tetrachloride induced liver fibrosis:
Histopathological changes and expression of CD133 and epidermal growth factor

Version: 1 Date: 11 December 2009

Reviewer: Biserka Radosevic-Stasic

Reviewer's report:
The article presents interesting and novel data about expression of CD133 and epidermal growth factor (EGF) in chronically injured and neoplastic liver tissues in mice subjected to ingestion of carbon tetrachloride (CCl4). Results, based on histopathological analysis, quantitative real time-PCR determination, ELISA and Western blotting showed that intoxication with CCl4 after 15 weeks induces in the liver a fibrosis (without other signs of cirrhosis) and hepatocellular carcinomas, with high upregulation CD133 and significant downregulation of EGF protein and EGFR mRNA. The findings were followed by increased expression of representative markers of hepatic stellate cells (desmin and GFAP), suggesting these CD133+ cells contribute to liver regeneration and tumor progression in A/J mice. Since similar changes were not observed in humans and rats, it was concluded that species-specific differences exist in chronic liver diseases. The hypothesis needs to be confirmed by immunohistochemistry and FACS analysis, but the experiments were designed appropriately, the methodology was precise, and the discussion supports the results.

We thank the reviewer for the comments. In the new draft, we have added immunohistochemistry of serial sections of liver tissue stained with CD133 and two markers for hepatic stellate cells, desmin and a-SMA. The results show that CD133, desmin and a-SMA localize to the same regions in CCL4-treated mouse livers.

Discretionary Revisions
In the Material and Methods: (Animals and Experimental design) a link to Figure 1 should be added (probably after “Research Animal Care” …. top, second line…. “As shown on Fig. 1”

We have made this addition according to the reviewer’s suggestion.

In Results: (in section EGF and EGFR expression line 8: (Fig. 3C). “And” should be deleted.

We have made this deletion according to the reviewer’s suggestion.

In Discussion: (line 7) did not developed although HCC….. instead of did not developed. Although HCC…….
We have made this correction according to the reviewer’s suggestion.

**Level of interest:** An article of outstanding merit and interest in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**
'I declare that I have no competing interests'

**Reviewer's report**

**Title:** Mouse model of carbon tetrachloride induced liver fibrosis: Histopathological changes and expression of CD133 and epidermal growth factor

**Version:** 1  **Date:** 16 December 2009

**Reviewer:** Frank Tacke

**Reviewer's report:**
Fujii et al describe experiments in which mice were treated with CCl4 per gavage, resulting in liver fibrosis and tumor formation. They also found upregulated gene expression of EGF, TGF, HSC markers and CD133 and conclude that CD133 might be involved in fibrosis progression or tumor formation.

Major Compulsory Revisions
1. It is very hard to believe that treatment with CCl4 per gavage leads to liver fibrosis and HCC development. CCl4 has been extensively used in the literature (typically i.p. injections for 6-8 weeks), but usually does not result in HCC. If this should be true, the authors need to provide compelling experimental evidence. To prove fibrosis formation: hydroxyproline assay, collagen or a-SMA Western Blot, collagen immunohistochemistry. For tumor formation: clonal analysis, macroscopic picture, gene profile from tumorous vs non-tumorous tissue.

We thank the reviewer for their comment but respectfully disagree. Although it is true that CCl4 has been recently used in short durations to induce liver fibrosis, historically, CCl4 was identified as an agent that causes liver fibrosis and HCC after oral ingestion (Edwards and Dalton, *J Natl Cancer Inst* 1942;3:19-41). In fact, they showed that the strain A/J mice used in our study developed HCC at a 98.4% frequency when given oral gavage of CCL4 for 4 months. Many studies today still use this model both to induce fibrosis and also HCC. In our study, to prove fibrosis, we originally used the standard clinical Masson’s trichrome stain to detect collagen and
the results depicted bridging fibrosis (Ishak score 3 to 4 as determined by a skilled pathologist) but no cirrhosis as determined by the lack of regenerative nodules. To further address the issue of fibrosis in this revision, we have performed Sirius red staining for collagen. As shown in Figure 3, the results are consistent with the original trichrome stains – bridging fibrosis (Ishak score 3 or 4) is observed at Time point II with no evidence of cirrhosis, i.e. lack of regenerative nodules. The presence of HCC, as compared to other pathologies like regenerative nodules or adenomas, was again determined by a skilled pathologist through standard H&E staining. These results are depicted in Figure 2.

2. The paper is solely descriptive, and the up-regulation of HSC-related genes in a fibrosis model is not surprising. If the authors believe that they have HCC development, they should differentiate gene expression profiles at time-point III for tumorous vs non-tumorous tissue.

We thank the reviewer for their comment, but we believe that the presence of HCC was adequately determined pathologically.

3. It is completely unclear if the upregulation of CD133 has anything to do with HSC activation or proliferation. Therefore, clear immunohistochemical stainings are needed to assess CD133 in liver tissue and assess co-staining with activated HSC (e.g., by a-SMA staining). Many hematopoietic cells can express CD133 as well, this should be tested. Furthermore, it would be helpful to isolate primary cells from the treated livers, i.e. hepatocytes, HSC, endothelial cells and Kupffer cells, to assess which cell type expresses CD133. The use of cell lines is not helpful in this respect.

We thank the reviewer for the comments. Unfortunately, we were unable to perform co-stainings with CD133 (It is our understanding from others as well that co-staining with CD133 antibodies is very difficult). We therefore stained 5 um serial sections of liver tissue with antibodies to CD133, a-SMA and desmin (another HSC marker). The results show that CD133, a-SMA and desmin localize to the same regions in CCL4-treated mouse livers suggesting that HSC are the source of CD133. As the reviewer mentioned, hematopoietic cells can also express CD133 but we believe that they are not responsible for the increase in CD133 expression as VEGFR1 mRNA expression (a marker for hematopoietic cells) does not change in CCL4-treated mouse livers.

**Level of interest:** An article of limited interest

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests
Reviewer's report

Title: Mouse model of carbon tetrachloride induced liver fibrosis: Histopathological changes and expression of CD133 and epidermal growth factor

Version: 1 Date: 21 December 2009

Reviewer: helene gilgenkrantz

Reviewer's report:

Major points
1. Histological data have to be improved:
   - Why do the authors claim that there is no cirrhosis? It seems at least, although it should have been better seen on Sirius red Staining that there is bridging fibrosis. More data have to be presented to confirm this point. Moreover, PCNA or Ki67 should be included to demonstrate the presence or absence of regenerative nodules. These results will allow to correlate EGF/EGFR expression to hepatocyte proliferation.
   - Hepatocellular carcinoma is not well characterized. Higher power field as well as a macroscopic view should also be included.

We thank the reviewer for the comments. We claim the absence of cirrhosis based on the lack of regenerative nodules observed by trichrome staining. For this revision, we did perform Sirius red staining as shown in Figure 3. The results are consistent with the trichrome stains – bridging fibrosis is observed (Ishak score 3 or 4 as determined by a skilled pathologist) with no evidence of cirrhosis, i.e. lack of regenerative nodules. We performed a PCNA western blot (Figure 4) and the results demonstrate that PCNA increases with CCL4 administration (Time points I and II) and decreases with CCL4 withdrawal (Time point III) which suggests that CCL4 causes hepatocytes to proliferate just not within regenerative nodules. A macroscopic picture of HCC has been added to Figure 1 and a higher power field can be seen in Figure 2.

2. To explain why they found a downregulation of EGFR and EGF during the progression of fibrosis in mice, while some previous papers had shown an upregulation of EGFR in cirrhotic rats and in human cirrhosis, the authors suggest species differences. This conclusion is not convincing:
   Indeed, in a DEN-induced fibrosis/cirrhosis/CHC sequence in rat, Schiffer et al have found exactly the same results, namely:
   - a down regulation of EGFR protein and mRNA
   - a down regulation of EGF
   - an upregulation of TGFa
   The authors have to reinterpret their results with this view since equivalent results in a rat model have been obtained (HEPATOLOGY 2005;41:307).
   Moreover, to confort these data, a western blot showing the active
phosphorylated form of EGFR should be given.

We thank the reviewer for the comment. Indeed, our results are consistent with the Schiffer report as now discussed in the paper. We did perform a western blot for p-EGFR and the results demonstrate that EGFR signaling increases with CCL4 administration and decreases upon withdrawal. Overall, our results suggest that EGFR downregulation is the result of increased signaling – which is consistent with ligand-mediated receptor endocytosis – as a result of proliferating hepatocytes during fibrosis as indicated by increased PCNA staining. In fact, total EGFR goes down while p-EGFR and PCNA go up with CCL4 administration whereas total EGFR goes up and p-EGFR and PCNA go down after CCL4 withdrawal.

3. Discussion: “EGFR may possibly participate in the development of liver cirrhosis through a previously unrecognized mechanism” is not true: amphiregulin has been shown to participate in this process (Perugonia et al Hepatol 2008; 48: 1251). It should be interesting to show the expression of other EGFR ligands such as amphiregulin or HB-EGF in their model.

We thank the reviewer for pointing out this paper that we missed. In fact, we did real-times for both HB-EGF and AREG. Although levels of HB-EGF do not change, we observed a dramatic increase in AREG during CCL4-induced liver fibrosis suggesting that AREG is involved in the development of liver fibrosis.

4. The authors claim that CD133+ HSC are recruited during chronic liver injury, while only a correlation between up-regulation of different transcripts is shown. To confort this point and the fact that CD133 is downregulated in non-cancerous tissue after Ccl4 withdrawal and not in HCC, an immunohistochemistry for CD133 should be included. Finally, there is no experimental evidence that CD133 HSC have contributed neither to liver regeneration nor to tumor progression (last sentence of the abstract) as it is claimed.

We thank the reviewer for the comments. Unfortunately, we were unable to perform co-stainings with CD133 (it is our understanding from others as well that co-staining with CD133 antibodies is very difficult). We therefore stained 5 um serial sections of liver tissue with antibodies to CD133, a-SMA and desmin (two HSC markers). The results show that CD133, a-SMA and desmin localize to the same regions in CCL4-treated mouse livers suggesting that HSC are the source of CD133. The last sentence of the abstract has been removed.

Minor essential revisions

5- Material and Methods: the authors should indicate when mice have been killed regarding the last oral CCl4 administration.

We have made this addition according to the reviewer’s suggestion.
6- Discussion: the authors reviewed nine reports of cirrhosis induced by Ccl4 in mice. Their conclusion about this study is unclear. Moreover, in these papers, either HCC develop on a cirrhotic liver, or cirrhosis is incomplete and the animals do not develop HCC. How do the authors explain that in their hands, mice develop HCC without cirrhosis?

In our review of nine reports in the literature, we want to make the point that the authors concluded that CCL4 induces cirrhosis but we believe that the livers were not cirrhotic based on our assessment of the depicted photomicrographs. We have clarified this point further in the Discussion.

7- Last paragraph in the Results Section: Since all cell lines tested are hepatocytes or hepatoma cells, the authors wanted to determine whether Ccl4 promotes CD133 expression in existing PARENCHYMAL cells. Moreover, the authors should indicate whether there is a differential expression between non-tumorigenic and cancer cell lines of the same species.

We have made this addition according to the reviewer’s suggestion. In this small sampling, we did not observe differential expression between non-tumorigenic and cancer cell lines and have added this statement in the revision.

8- The authors should indicate that CD133 up-regulation has also been demonstrated in human fibrosis, in the ductular reaction of chronically damaged human livers (Tsuchiya et al, Hepatol Res 2009; 39: 1080). They also have to discuss the different observations of CD133-positive and CD133-negative hepatocellular carcinoma in humans (BMC cancer 2009; 9: 324/ Ma et al, gastroenterol 2007/ Yin et al Int J Cancer 2007…)

We have further discussed the reports of CD133 in human HCC as suggested by the reviewer.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
'I declare that I have no competing interests'