Author's response to reviews

Title: Decreased PCSK9 expression in human Hepatocellular Carcinoma

Authors:

Mamatha Bhat (mamatha.bhat@mcgill.ca)
Marc Deschenes (marc.deschenes@muhc.mcgill.ca)
Victoria Marcus (victoria.marcus@muhc.mcgill.ca)
Nicolas Skill (nskill@iupui.edu)
Xianming Tan (xianming.tan@clinepi.mcgill.ca)
Jeanne Bouteaud (jeanne.bouteaud@mail.mcgill.ca)
Sarita Negi (sarita.negi@gmail.com)
Zuhier Awan (zuhier_awan@yahoo.com)
Reid Aikin (reid.aikin@mail.mcgill.ca)
Janet Kwan (janet.kwan@mail.mcgill.ca)
Ramila Amre (ramila.amre@muhc.mcgill.ca)
Sebastien Tabaries (sebastien.tabaries@mcgill.ca)
Mazen Hassanain (mazen.hassanain@mcgill.ca)
Nabil G. Seidah (nabil.seidah@ircm.qc.ca)
Mary Maluccio (mmaluccio@iupui.edu)
Peter Siegel (peter.siegel@mcgill.ca)
Peter Metrakos (peter.metrakos@mcgill.ca)

Version: 3 Date: 7 February 2015

Author's response to reviews: see over
Letter of Response

Dear Ms. Manibo,

Thank you for your interest in our manuscript MS: 2284420631227453 entitled "Decreased PCSK9 expression in human Hepatocellular Carcinoma".

We have attempted to address the comments as thoroughly as possible by performing further experiments, as detailed below and in the revised manuscript.

Reviewer #2

Major Compulsory Revisions

1. The strongest data have been obtained from immunohistochemistry, it would be important to reinforce the quantification by adding representative pictures from HCC and control tissues stained for PCSK9.

We thank the reviewer for this helpful comment. Representative pictures of HCC and cirrhosis stained for PCSK9 have been added to the revised manuscript as Figure 1E. Additionally, having performed the LDL-receptor staining on the same tissues, we have added representative pictures within the new Figure 2.

2. A western-blot analysis of PCSK9 in HCC and HCC tissues should be added to validate the immunostaining experiments.

We used formalin-fixed, paraffin-embedded tissue blocks to create the tissue microarray, and hence could not use these tissues to perform western blot analysis. This is unfortunately the reason why only immunohistochemistry was possible.

3. Finally, as PCSK9 expression is reduced in HCC but the circulating PCSK9 levels is increased in HCC patients compared to non HCC control patients, it is necessary to measure in HCC the real effect on cell surface LDLr. Thus, it is required to quantify by immunohistochemistry and western blot the LDLr protein levels in HCC and adjacent tissues.

LDL-receptor levels have been assessed on the tissue microarray by immunohistochemistry, and the results have been added as tracked changes in the abstract as well as the results section. We did find an inverse correlation between PCSK9 and LDL-R expression in HCC, which confirmed our hypothesis. However, we could not assess LDL-receptor levels by Western blot, as explained above.
Minor Essential Revisions

1. The authors should precise whether patients included in the study received any pharmacological treatment.

The patients did not receive any pharmacological treatment, and this has been clarified on p.3, line 9.

Reviewer#3

1. Discussion pag. 9: basically the authors do not explain how can be that the higher level of PCSK9 in the serum of HCC bearing patients, is attributed to a “…mechanism by which tumors assist PCSK9 secretion in order to allow for…” since they do not observe statistical differences btw mRNA PCSK9 of HCC when compared to matched adjacent tissue or to control liver. There may by a particular mechanism leading HCC to secret PCSK9 once synthesized, or what? In my opinion, this point needs to be clarified extensively.

The reviewer has raised an excellent point. In fact, we have reflected on this point extensively as a group. Given that the serum PCSK9 levels are not correlated with tumor PCSK9 expression, there is no reason to say that tumors secrete PCSK9. We simply conclude that there is no correlation whatsoever between the tumor PCSK9 expression and serum levels. Hence, one can clearly say that serum PCSK9 levels are not reflective at all of the presence or absence of HCC. We have substituted a paragraph as follows to account for this:

"In our study, the systemic levels of PCSK9 did not correlate well with the presence or absence of tumor. In the literature, the reported mean values of human plasma PCSK9 concentrations are quite variable, ranging from a low of 80 ng/mL[30], 150 ng/mL[14] or 200 ng/mL[31] to a high of 4.1mg/mL[32] or 6.1 mg/mL[33]. This variability arises due to the different antibodies against PCSK9 used in the various assays. The assay used in our study has a mean of 77-80 ng/mL, meaning that the HCC patients had a ~12% higher value on average, which is not significantly different from normal values. The half-life of PCSK9 has been determined to be only 5 min in vivo[34], which implies that the liver is continuously producing high levels of PCSK9. However, at least based on our findings, the ongoing malignant process in the liver with modulation of PCSK9 levels locally has no impact systemically."

The Authors, refer a similar amount of LDLR in surrounding and HCC (reference n. 15), while the PCSK9 is lower in HCC, how can they explain this observation since PCSK9 favors LDLR degradation?

We thank the reviewer for the comment, please see the detailed response above to question #3 of Reviewer 2.
2. PCSK9 IHC, pag 7: How the Authors explain the absence of differences in PCSK9 expression btw. poorly and well differentiated tumors, while PCSK9 expression correlates with more advanced fibrosis stages?

These are two separate correlations, given that hepatic fibrosis (liver scarring) is a separate entity from tumors and their degree of differentiation. Certainly, advanced hepatic fibrosis is a precursor to development of cancer.

3. Discussion pag. 10: The hypothesis on the presence of mutation of PCSK9 needs to be verified.

This was a hypothesis on possible mutations in SREBP genes leading to dysregulated expression of PCSK9. However, we respectfully feel that analyzing for mutations in SREBP is beyond the scope of our study.

4. Methods, pag. 4: usually more than one pathologist take care to analyze the sections.

We did in fact have two liver pathologists involved in this study, Dr. Victoria Marcus and Dr. Ramila Amre, who are both listed as co-authors. Dr. Marcus selected the tissue cores for incorporation into the tissue microarray, and Dr. Amre verified their accuracy as described in the following added sentence on p. 4, 2nd paragraph: "Once the tissue microarray was constructed, a second pathologist (RA) confirmed the accuracy of the HCC and cirrhosis tissue specimens."

5. 6 fresh frozen HCC are real few samples

We agree that this is a small number of samples. However, these 6 fresh frozen HCC samples were used to simply verify whether the mRNA and protein levels of PCSK9 were correlated in any way, which they were.

6. Pag8: How do the Authors explain the last sentence where they found patients with cirrhosis and higher PCSK9 and patients with chronic liver disease without cirrhosis and lower PCSK9?

As mentioned earlier, we realized that the serum PCSK9 levels did not correlate at all with the tumor PCSK9 levels. Hence, we would not attribute any significance to the differences in PCSK9 expression between patients with cirrhosis and those without cirrhosis.

7. In addition, several English expression need to be check in order to render the text more clear to rad:

- Methods: line 1: this has been modified to read as follows: "Using the McGill University Health Centre Liver transplant database, we established a list of patients having undergone liver transplant or partial hepatectomy for HCC."

Page 5 first line: modified to read as follows: "We additionally permeabilized the cells with Triton 0.1% in PBS buffer and unmasked antigens by heat treatment with 10 mM sodium citrate buffer (pH 6.0)."

- Results: line 1-2: modified to read as follows: "Forty patients with HCC consented to inclusion in the tissue microarray portion of this study."
- Discussion: line 11: modified to read as follows: "Moreover, there has been limited study of tumor metabolism as a potential therapeutic target for HCC."

Reviewer #4

Major Revisions:

1. Concerning the immunohistochemistry data, some controls are lacking. Indeed, the authors only compared PCSK9 expression in HCC and in adjacent cirrhotic liver. What is the expression of PCSK9 in normal liver? At this time, it remains unclear whether PCSK9 is downregulated in HCC or overexpressed in fibrotic liver?

The main purpose of this paper was to determine PCSK9 expression patterns across tumors and background liver. If one wished to compare expression of PCSK9 in normal liver, comparable tissue would need to have been obtained from each patient. However, the nature of HCC is such that it tends to arise in a background of cirrhotic hepatic parenchyma, and this is what we obtained as patient specimens. In our PCR experiment though, we did find that PCSK9 expression was highest in control tissue, with tissue adjacent to tumor and HCC having lower and the lowest expression levels respectively (Figure 4).

2. The patients are heterogeneous with a mix of hepatitis B & C, NASH, alcoholic cirrhosis, etc...It should be remind here that it has been suggested that PCSK9 can play a role in the pathophysiology of hepatitis C by controlling the expression of some specific receptors involved in the uptake of the viruses (LDL-R, CD81). The authors should perform a regression analysis to determine whether the etiology of liver disease alters the results of PCSK9 expression in HCC.

This was performed, and we discovered that etiology of liver disease did not affect PCSK9 expression in HCC.

3. It's a pity that plasma PCSK9 levels were not measured in the same population than those for PCSK9 expression in HCC. The number of controls is very small...What are the clinical characteristics (etiology of liver disease) of the patients?

The demographic and clinical characteristics, including etiology of liver disease, have been added to p.8, first paragraph. The etiology of liver disease for the patients with HCC was as follows: 15 with Hepatitis C, 6 with Hepatitis B, 9 with alcoholic liver disease, and 10 with Non-alcoholic steatohepatitis.

The etiology of liver disease for the patients without HCC was as follows: 13 with Hepatitis C, 5 with Hepatitis B, 2 with alcoholic liver disease, 9 with Non-alcoholic steatohepatitis, 5 with cryptogenic cirrhosis, 1 with Primary sclerosing cholangitis and 2 with Primary biliary cirrhosis.
4. Since it has been demonstrated that the effect of PCSK9 in liver regeneration is dependent of cholesterol intake in mice, the plasma lipid parameters of the studied patients for PCSK9 expression analyses must be described.

As clarified earlier, the level of PCSK9 in tumors was completely independent of the levels in serum. Hence, we would not expect plasma lipid parameters to be correlated in any way with with PCSK9 expression in tumors.

Minor points:
1. The quality of the figure 2 is poor

We have reinserted a figure with higher dots per inch resolution.

2. For Q-PCR analyses, more than only 1 house-keeping (18S) gene must be used.
We did also use GAPDH as a housekeeping gene, and this has been clarified on p.6, line 4 as a tracked change.

3. The discussion is very speculative (notably concerning the link between PCSK9 and LDL-R expression, in the absence of LDL-R expression data....)

As answered above, we have now assessed LDL-receptor levels on the tissue microarray by immunohistochemistry, and the results have been added as tracked changes in the abstract as well as the results section. We did find an inverse correlation between PCSK9 and LDL-R expression in HCC, which confirmed our hypothesis.

We thank you for your interest in our manuscript, and hope you will consider it for publication in BMC Gastroenterology.

Yours sincerely,

Mamatha Bhat, MD

Peter Metrakos, MD

Assistant Professor of Medicine, Division of Gastroenterology
McGill University Health Centre
687 Pine Avenue West,
Montréal, Québec
Canada H3A 1A1