

Dear editor:

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 Editor/Reviewers' warm work earnestly, and hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in the eminent journal.

We shall look forward to hearing from you at your earliest convenience.

Best regards,

Sincerely,

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Response for reviewer 1, **code:** 02440657

COMMENTS TO AUTHORS:

The authors aim to explore the exact interaction between Notch and Transforming growth factor β (TGF- β) signaling in liver fibrosis. They established liver fibrotic rats model by concanavalin A (ConA) and isolated peripheral blood mononuclear cells (PBMCs) from model rats. It is meaningful, the design is reasonable, and the methods are appropriate. Several points need to be clarified. **Major: 1.** Since the topic is liver fibrosis, it is better that if data of the collagen fiber stain such as masson or sirius red is provided to confirm the formation of fibrosis, as well as serum ALT and AST levels which can reflect the liver function. **2.** The number of each experiment of PCR, Western Blot

should be displayed in the figure legends. 3. Recent research about TGF- β signaling and fibrosis, as well as the relationship between Notch1, Hes1, Hes 5, TGF- β 1, Smad3, need to be more illustrated in the "Discussion" part. **Minor: 1.** In conclusion of "abstract", "up-regulation" should be changed into "up-regulate". **2.** In the first paragraph of INTRODUCTION, the final sentence is incomplete. "However, whether similar regulator occurs in liver fibrosis, and what happened between the two signaling during the restore stage of liver fibrosis" can be changed into "However, whether similar regulator occurs in liver fibrosis, and what happened between the two signaling during the restore stage of liver fibrosis, still need to be further clarified."

Thank you for your reviews very much. We made responses as follows:

Major: 1. We supplemented the serum levels of ALT, AST, and Albumin. We performed masson stain in our previous works and added the reports as citation in our manuscript.

Major: 2 and 3. We revised manuscript according to the comments and highlighted all the changes in manuscript.

Minor: 1 and 2. We revised in manuscript as well.

Response for reviewer 2, code: 03536939

COMMENTS TO AUTHORS:

The authors aimed to investigate the relation between Notch and TGF-beta pathways. These two pathways have crucial roles in hepatic fibrogenesis, in the activation of hepatic stellate cells (HSC) and a fraction of macrophages activated classically. Therefore, the interaction between the two pathways is in the interest of recent hepatic fibrogenesis-related research. In the presented article, the authors investigated this issue in relation to peripheral blood mononuclear cells (PBMC) isolated from Concanavalin A-treated fibrotic rats. The respective inhibitors were investigated in a maintained cell culture of these PBMC cells. Therefore, this article provides a particular answer only that is

associated with the white blood cells being present in the circulation of the hepatic fibrosis-induced rats, presuming that the PBMC fraction is characteristic and indicative of the fibrotic state, and that the PBMC fraction may have a role in hepatic fibrogenesis. A crucial issue here is the association of increased Notch and TGF-beta in PBMC with the development of hepatic fibrosis in the respective rat. Did the author monitor this association on a weekly basis and correlate this in the course of fibrogenesis? The second issue is the important role of HSC fraction in liver fibrosis. Since it is possible to isolate HSC fraction from liver or there are HSC cell lines available, did the author perform (does the author plan to perform) similar inhibition experiment on HSCs? In addition, did the author check whether rat fibroblast or macrophage cell lines are available? The third issue regards the found inhibitory effects and the drawn conclusions. The authors observed downregulation of the same members of Notch and TGF-beta pathways irrespective of the inhibition of either Notch or TGF-beta. This does not necessarily indicate the direct interplay between the two pathways. Therefore, it would be necessary to study this process in liver and monitor other targets of the two pathways, such as α -SMA, COL1 α 1. In addition, overexpression studies are needed with Notch and TGF-beta pathway members and targets. Furthermore, the sentence "Our study demonstrated that Notch signaling mediated by TGF- β /Smad signaling pathway, resulting in liver fibrosis" is in my view an exaggeration. The fourth issue is associated with the presentation of the topic and the rationale of the study regarding the investigation of PBMC in relation to hepatic fibrosis and the presentation of Notch and TGF-beta interplay, which are not thorough in my view. The fifth issue is the English language polishing throughout of the text. Further comments: - Role of Notch signaling in Introduction: what is pattern formation??? - Authors used only rats; however, mice also appears in the Materials and methods - DMSO was the control group but the authors did not mention that the inhibitors were dissolved in which percent of DMSO - Jagged1

is listed in Table 1, but it is missing from the text in Materials and methods - The PCR profile is not mentioned. Did the authors use the one recommended by the manufacturer? - A possible way to indicate the degree of Celsius sign by using a small o letter in superscript followed by a capital C letter - “gel electrophoresis (SDS-PAGE) gel” - the gel word after the parenthesis is not needed - Did the author use variable amount of proteins as indicated (30-100 ug)? How did the author normalize the western blot data? - I was very pleased to see densitometry charts in the article, but what program was used for this? - The ECL system provided by GE Healthcare is not for alkaline phosphatase-conjugated secondary antibodies as it is stated in the text but for HRP-conjugated antibodies. - Standard deviation is abbreviated as S.D. - The used statistical tests are missing from the Figure legends.

Thanks reviewer's comments very much. We made responses as follows:

A crucial issue: We tested the association in 4 weeks, and our research group previous monitored that every week, including the liver function. We found the two pathways were overactive.

The second issue: The reviewer put forward a very good comment on checking rat fibroblast and macrophage cell lines. This present study is our preliminary research, we plan to perform similar inhibition experiment on HSCs and liver. We just maybe paid attention on the adaptive immunity in liver fibrosis.

The third issue: Thanks again for the reviewer's comment. We revised the sentence “Our study demonstrated that Notch signaling mediated by TGF- β /Smad signaling pathway, resulting in liver fibrosis”, see it in manuscript. The Notch intracellular domain (NICD) combine with Smad3 could enhance the transcription of their target genes. The DAPT and TGF-beta inhibitor are specific to Notch and TGF-beta pathways respectively. Moreover, we observed other members and targets of the two pathways between model and control group. We performed the subsequent experiments according to the significant members and targets. Some other responses were revised in the part of

discussion, we highlighted them.

The fourth issue: It is necessary to explore the mechanism in liver tissue, but the Notch have important role in immune cell, which promote T-cell differentiation; TGF-beta are mainly secreted by T regulatory cells in in the periphery. We aim to research the microenvironment of periphery in liver fibrosis, so the PBMCs are selected as the experimental subject. There are similar researches involved in Notch signal in relation to PBMCs in Immunology.

References

Zheng G, Liu L, Zhu K, Wei J. [Expression and significance of Notch receptors in the mouse model of allergic rhinitis]. Lin chuang er bi yan hou tou jing wai ke za zhi = Journal of clinical otorhinolaryngology, head, and neck surgery 2014; 28: 1585-1589 [PMID: 25764759]

Pei J, Tang Z, Zang G, Yu Y. Blockage of Notch1 signaling modulates the T-helper (Th)1/Th2 cell balance in chronic hepatitis B patients. Hepatol Res 2010; 40T: 799-805 [PMID: 20626467 DOI: 10.1111/j.1872-034X.2010.00680.x]

The fifth issue: We had revised the language throughout of the text.

Further comments: Thanks the reviewer's guidance in details.

We revised "pattern formation" and "mice" into "organ formation" and "rats".

The inhibitors which we bought from MedChem Express had been dissolved in 0.1% DMSO.

"Jagged1" had been added in Materials and methods.

Revised the PCR profile, see the part of RT-PCR in Materials and methods.

The degree of Celsius sign had been revised.

"gel electrophoresis (SDS-PAGE) gel" revised into "gel electrophoresis (SDS-PAGE)".

We revised the part of *Western blot analysis* in Materials and methods seriously, and checked the machine type of Imager, see it in manuscript.

The protein concentrations in supernatant were determined by BCA

colorimetric protein assay kit, so there were different amount of proteins samples.

We normalized the western blot data by β -actin.

The densitometry of Western blot calculated by a multimedia color image analysis system (Image-Pro Plus 6.0).

The abbreviation of Standard deviation was revised as SD.

The used statistical tests were added in the Figure legends, see the highlight in figure legends.

Response for reviewer3, code: 02937521

COMMENTS TO AUTHORS

Comments to the authors Dear authors I read with great interest your manuscript entitled: Notch mediated by TGF- β /Smad pathway in Concanavalin A-induced liver fibrosis rats I find the topic interesting, the manuscript is well structured, and the results and conclusion are important and may have a good impact on the liver fibrosis patients and their outcome. I find the manuscript only needs some revision and editing of the English language, and changing the title into more informative and interesting one. Regards

Thanks reviewer's positive comments. We have revised the English language.