Response to Reviewers' comments

We are thankful to all two reviewers the editors for reviewing the manuscript and providing us with their comments and suggestions. Overall, our manuscript was well received by the reviewers and our manuscript is described as “great interest”. We have carefully revised the manuscript by incorporating all suggestions and answering the comments. The point-by-point responses to the reviewers’ comments are provided below.

Reviewer 1

We are thankful to reviewer for all suggestions and comments that help us improve the quality of the manuscript. Responses were provided as follows.

Add the unique of this study compared to other studies discuss the same issue.

To address this comment, we have added comparisons with other studies in the discussion (Page 11, Line 31).
“Interestingly, compared with previous studies12,14, we found that IL-1β but not IL-6, NLRP3, TNF-α is a central player in fibrogenesis and progression of fibrosis in chronic HBV patients. Moreover, our results showed that the expression of PDGF, a known HSCs activation factor15,16, did not increase with the progress of fibrosis. When we treat the human stellate cells LX-2 with the cytokines in vitro, we found that either IL-1β or TGF-β1 can induce the expression of PDGF. These results indicated that IL-1βmay play a critical role in the progression of fibrosis in chronic HBV patients.”

Add more on the basic of this disease in the introduction -Discus role of imaging using these ref -Razek AA, Massoud SM, Azziz MR, El-Bendary MM, Zalata K, Motawea EM. Prediction of esophageal varices in cirrhotic patients with apparent diffusion coefficient of the spleen. Abdom Imaging 2015; 40: 1465-9 -Besheer T, Elbendary M, Elalfy H, Abdelmaksoud, M, Mohamed S, Abdel Razek A. Prediction of Fibrosis Progression Rate in Patients with Chronic Hepatitis C Genotype 4: Role of Cirrhosis Risk Score and Host Factors. J Interferon Cytokine Research 2017; 37: 97-102. English language correction through the manuscript -Update of references as most of references are old using these ref -Besheer T, Elalfy H, Abd El-Maksoud M, et al. Diffusion-weighted magnetic resonance imaging and micro-RNA in the diagnosis of hepatic fibrosis in chronic hepatitis C virus. World J Gastroenterol 2019; 25: 1366-1377. =Besheer T, Arafa M, El-Maksoud MA, et al. Diagnosis of cirrhosis in patients with chronic hepatitis C genotype 4: Role of ABCB11 genotype polymorphism and plasma bile acid levels. Turk J Gastroenterol 2018; 29: 299-307.

Following the reviewer’s suggestions, we have added more introduction on basic research related of liver fibrosis and update more recent references, including all references that reviewer mentioned.

“Staging the hepatic fibrosis of chronic liver disease is a key indicator to judge the condition, decide the treatment strategy and evaluate the treatment effect. With the development of non-invasive approaches, especially the imaging technology, the accuracy of the assessment of liver fibrosis has been improved.” (PMID: 25732406, 28068153, 30918429, 29755014, 27621571, 28251119) (Page 5, line 8).

"Recent studies have showed that various factors such as metabolic factors, growth factors, inflammatory factors, and microRNA all have an impact on the progress of fibrosis." (PMID: 23516981, 26957957, 26559292, 28983598, 23042547, 30918429, 30582979) (Page 5, line 22).

Reviewer 2

great interest I read the paper " Hepatic microenvironment underlies fibrosis in chronic hepatitis B patients" by Feng et. al. The manuscript's introduction highlights the current lack of knowledge regarding hepatic microenvironment physiology and, in particular, its involvement in the progression of liver disease. This study results show potential in bringing more knowledge regarding hepatic microenvironment factors in chronic HBV infection and impairment of associated pathways. However, there are several concerns, which need to be addressed.

We are thankful to the reviewer for all the great feedbacks that help sharpen the focus and improve the quality of the manuscript. Responses were provided as follows.

Major comments

1. The aim of the study should be formulated more specifically. It was stated that authors aimed to profile the microenvironment factors, however, authors evaluated a series of pre-selected markers related to fibrosis and inflammation. Authors should either reformulate the aim to demonstrate their particular interest in these markers and the involved pathways or to apply high-throughput methods to fit the original aim.

We now have changed the "profile" in the manuscript to "examine a subset of pre-selected" (Page 3 Line 13, Page 6 Line 3), or "examine" (Page 8, Line 1). Indeed, the primary goal of this study is to develop an in vitro model to better recapitulate the microenvironment of the HBV patients, and in order to do that, we screened or examined a series of preselected factors known to have critical functions in fibrogenesis, or inflammation etc to identify the ones that are significantly upregulated in HBV patients with advanced fibrosis.

2. In the introduction, the authors brought focus on the absence of the appropriate in vitro model to study microenvironmental mechanisms. However, the study does not take steps to solve this problem. It is recommended to rephrase the introduction, focusing on problems in which this study has contributed.

We thank the reviewer for the suggestion and have now rephrased the introduction as follows (Page 5, line 24):
Therefore, expose the roles that the hepatic environment played in the patient will greatly facilitate the establishment of suitable in vitro environment to recapitulate more hepatitis patients-specific pathogenic factors. Such microenvironment factors are important for developing the in vitro fibrosis assays, which can be used for identifying novel drug targets and drug screening.

3. For the present cohort (Table 1), the Pearson Chi-square test was used. However, since the overall size of the cohort is small, and subgroups <5 exist, it is recommended to use Fisher's exact test instead.

Following the reviewer's suggestion, we recalculated the P value using the Fisher's exact test and

updated it in Table 1 (Page 8, line 17).

4.The study characterizes several microenvironmental inflammation and fibrosis related factors in a cohort of chronic HBV infection patients and compares the expression between no or mild fibrosis and advanced fibrosis. The overexpression of IL-1β, IL6, NLRP3, TNF-α, and TGF-β1, correlated with fibrosis degree, which was previously reported (PMID: 23516981, 26559292). On the other hand, lack of PDGF overexpression, a known HSCs activation marker is contradicting previous studies (PMID: 28983598). For this reason, the study may be improved by: 1) extending this study by including also non-HBV patients to demonstrate the possible contribution of HBV infection into pathway dysregulation; 2) including chronic HBV patients receiving antiviral treatments with signs of fibrosis regression to demonstrate the contribution of HBV on pathway dysregulation.

As the reviewers pointed out, previous studies (PMID: 23516981, 26559292) have reported that growth factors and inflammatory factors play a key regulatory role in fibrogenesis. Here we tested the expression of related growth factors and inflammatory factors in HBV-infected patients with less fibrosis and advanced fibrosis. Compared with previous studies, we found that IL-1β but not IL-6, NLRP3, TNF-α is a central player in fibrogenesis and progression of fibrosis in chronic HBV patients. Previous study (PMID: 28983598, 23042547) has demonstrated that PDGF is a key regulator of HSC activation using LX2 cell line infected HBV, however, we found that PDGF did not show significantly difference between the two groups of patients. Our *in vitro* results demonstrated that IL-1β and TGF-β1 can activate the expression of PDGF in LX2 cells, which indicates that IL-1β and TGF-β1 may play a more critical role than PDGF. In order to address the concern of reviewer, we have cited relevant references for more discussion on these contents (Page 5, line 22; Page 11, line 31; Page 12, line 19).

We are very grateful for the two improvement suggestions suggested by reviewer, so we will collect more samples from non-HBV infection patients and chronic HBV patients receiving antiviral treatments in future studies to demonstrate the contribution of HBV on pathway dysregulation. We added this part in the discussion (). Due to the following reasons, we would like to first analyze the samples from chronic HBV patients without antiviral treatment in this study.
1) It will take 1-2 years to obtain the new ethical documents and recruit sufficient volunteer donors for collecting samples receiving antiviral treatments; 2) Since more than 80% of liver fibrosis patients in China are HBV infected patients, collect non- HBV infection patients samples will take longer; 3) Due to the impact of COVID 19 epidemic, new sample collection is currently not possible.

5. Figure 1 (“Reticular fiber and HE staining of HBV patient liver samples”) demonstrates an increase in collagen fiber content and in infiltrate size following the stage of fibrosis. However, it does not contain novelty since it’s an established fact (PMID: 28251119, 26957957) it does not represent the main results of the study. Therefore, it is recommended to place this figure into the supplementary materials and revise the other figures to better demonstrate the main results of this study.

As suggested by the reviewer, we have now moved Figure 1 into supplementary materials as Figure S1 (Page 22, line2) and split Figure 2 into Figure 1 and Figure 2 to highlight the detection results of IL-1b and TGFb (Page17, line3; Page 18, line2).

6. The table of primers (page 7) should be moved to the Supplementary materials. Instead, the choice of markers for PCR analysis could be discussed in more detail.

Following the reviewer's suggestion, we have now moved the table of primers to Supplementary Materials as Table S1 (Page7, line 17; Page 21). And discussed the choice of maker genes (Page7, line 13).

Minor comments

"Chronic hepatitis B virus (HBV) is a leading cause of liver morbidity and mortality worldwide." Missed word: "Chronic hepatitis B virus (HBV) **infection** ...".

The word "infection" has been added in the manuscript (Page 3, line 3).

"We set out to profile the microenvironment factors of chronic hepatitis patients that may underlie fibrosis, with a focus on fibroblast activation." It should be specified that the profiling was performed specifically in chronic viral hepatitis B patients following the title and the focus of the study.

We have now rephrased the sentence as "We set out to profile the microenvironment factors of chronic HBV patients that may underlie fibrosis, with a focus on fibroblast activation." (Page 3, line 14).

"Chronic hepatitis B virus (HBV) is a leading cause of liver morbidity and mortality worldwide." Missed word: "Chronic hepatitis B virus (HBV) **infection** ...".

The word "infection" has been added in the manuscript (Page 5, line 2).

"Continuous viral infection associated inflammation and direct liver damages caused by viral components are accompanied by the transformation of **fibroblasts** into activated myofibroblasts, fibroblast propagation, and deposition of extracellular matrix (ECM)" An exact cell type should be specified (Hepatic stellate cells? Portal fibroblasts?).

Following the reviewer's suggestion, we have now rephrased the sentence as "Continuous viral infection associated inflammation and direct liver damages caused by viral components are accompanied by the transformation of Hepatic stellate cells (HSCs) into activated myofibroblasts, fibroblast propagation, and deposition of extracellular matrix (ECM)" (Page 5, line 6).

"Due to the lack of HBV **virus** receptors ..." The usage of the word "virus" in this case is a tautology and should be omitted.

The word "virus" has been omitted from the manuscript (Page 5, line 19).

"Hepatic microenvironment consisting of resident Kupfer cells, Hepatic stellate cells (HSCs), as well as the infiltrated immune cells and a variety of secreting factors including growth factors, cytokines and chemokines, and extracellular matrix ..." The list of the cell populations of the hepatic microenvironment is not complete without mentioning hepatocytes and liver sinusoidal endothelial cells, which are the part of hepatic microenvironment as well.

We have now rephrased the sentence as “Hepatic microenvironment consisting of hepatocytes, liver sinusoidal endothelial cells, resident Kupfer cells, Hepatic stellate cells (HSCs), as well as the infiltrated immune cells and a variety of secreting factors” (Page 5, line 29).

“... in diseases such as chronic **alcoholism** ...” The "alcoholism" term is considered as reductionistic and stigmatizing. Alternatives should be used instead ("chronic alcohol intake", "alcohol-use disorders", etc).

We have now rephrased the sentence as “... in diseases such as chronic alcohol intake...” (Page 6, line 2).

“We set out to profile the microenvironment factors in **chronic patient** with different degrees of fibrosis ...” It should be clarified that the cohort consisted not of patients with chronic liver disease as a whole, but specifically of patients with chronic hepatitis B infection, as it noted in the "Materials and methods" section.

We have now rephrased the sentence as “We set out to screen a subset of preselected microenvironment factors in chronic HBV patient with different degrees of fibrosis ...” (Page 6, line 4).

“The tissues were fixed in formalin and embedded in paraffin for heamatoxylineosin (H&E) and **reticular fiber staining as previously described** [6].” 1) Since it was never discussed before, the exact staining procedure should be mentioned; 2) The terms "fibrotic tissue", or "collagen type III" (if meant by authors), are more common than "reticular fiber". Therefore, authors should consider rephrasing it throughout the manuscript.

We have now replaced “reticular fiber staining” as “silver impregnation staining” (Page 6, line 14; Page 8, ling 5; Page22, line 2, line 3) and replaced "reticular fiber" as "fibrotic tissue" (Page 8, line 5).

“Inflammatory factors and TGF-β up-regulated **in the patients with advanced fibrosis**” Should be specified in according to the cohort parameters: “... in the patients **with chronic HBV infection and** advanced cirrhosis”

Following the reviewer’s suggestion, we have now rephrased the sentence as “Inflammatory factors and TGF-β up-regulated in the patients with chronic HBV infection and advanced cirrhosis” (Page 9, line 4).