RE: WJG Manuscript No 38355, entitled "Regulatory Polymorphism of CXCL10 rs1439490 with seronegative occult hepatitis C virus infection"

March 30th, 2018

Dear Editor,

Many thanks for your decision letter regarding our manuscript, WJG Manuscript No 38355. We also thank the reviewers for the constructive and pertinent comments, which have allowed us to improve the quality of our paper. We have revised the manuscript accordingly. All amendments are highlighted in yellow in the revised manuscript. In addition, point-by-point responses to the comments are listed at the end of this letter.

We hope that the revised paper is now suitable for publication in *WJG*. We are looking forward to hearing from you. Should you have any questions, please contact me.

Sincerely yours,

Jiangbin Wang
zrlwangjb@163.com

Point-by-point Response Editor & Reviewers' comments

Reviewer #1: I have read with deep interest the manuscript by Wang X et al, a very interesting study showing CXCL10 G-201A polymorphism is associated with occult hepatitis C. This is a well-done study, although some minor concerns should to be taken into account in order to improve the manuscript:

1. A list of abbreviations used might be included.

Response: A list of abbreviations used in the manuscript has been added (Page 4).

2. Clearly state the aim of the study and the primary outcome in the abstract. "OCI is a pattern of chronic HCV infection with unclear etiology" is the background, not the aim of the study.

Response: The Abstract has been revised as follows: "Aim: To examine the relationship between the single nucleotide polymorphism (SNP) CXCL10 rs1439490 and seronegative occult HCV infection (OCI)."

3. Methods for IL28B and CXCL10 should be described in the methods section rather than only a reference

Response: We have now included the methods for IL-28B and CXCL10 SNP examination (Page 10), as follows: "Peripheral blood samples from the patients were collected in anticoagulant EDTA-treated tubes, and genomic DNA was extracted using a Puregene SK8224 DNA isolation kit from Sangon Biotech (Shanghai, China), according to the manufacturer's instructions. The primers targeting specific fragments were designed and synthesized by Sango Biotech: CXCL10 promoter region G-201A rs1439490[20]: forward: 5'-TTCAGTAACATAAACCCCAACAA-3'; reverse: 5'-CACAAAGGAAGACAATAAGGGAG- 3'. CXCL10 promoter region C-1513T rs1440802: forward[20]: 5'-CTCACTTTGTCTCACCAATCTCA-3'; reverse: 5'-CAGAGAAATGAGAGACCTAAGTGTG- 3'. IL-28B rs12979860[22]: forward: 5'-CCTCTGCACAGTCTGGGATTC-3'; reverse: 5'-GCTCAGGGTCAATCACAGAAG-3'.

4. Please indicate if all patients included belonged to the same ethnic group. Patients from 5 hospitals have been included, and although all of them are located in the northeastern China, authors do not clarify if all patients had the same genetic background.

Response. All patients enrolled in our study were Han Chinese. We have added this description in the **Materials and Methods** section (Page 9,line 8).

5. GG genotype of CXCL10 G-201A is associated with OCI. The p value shown in the first paragraph of page 14 and in table 2 is the corresponding p value of the 2x3 contingency table comparing the three genotypes in OCI and CHC? Please indicate. Also, include the p value, OR and 95% CI when comparing the GG and non-GG genotype.

Response: Thanks! We have now divided the original Table 2 into two tables (**Table 2 and Table 3** in the revised manuscript). P values and OR (95% CI) have also been added in the individual tables.

Reviewer #2: *I congratulate the authors for this well written manuscript with an interesting topic.*

Response: Thank you so much for your recognition of the scientific merits of our study!

Reviewer #3: General opinion: *I think this article has a strong connection with previous researches within this population of patients, but as well it has its own contribution and scientific significance, which makes it a candidate to be published. Still, there is lack in methods describing, especially genotyping, which I think it is important. Finally, the clinical implication of the work should be more emphasized.*

Minor revision:

1. Aim should include interleukin-28B polymorphisms. It is important determinant that is used to select patients into groups. Probably authors wanted distinction between their work and work of other authors (ex. Bartolomé J et al), but it is not necessary, it is almost completely different. Also, the aim should be more précised and include measuring serum and liver CXCL10 levels, and HCV RNA levels.

Response: Due to the word limit for the abstract, we can't add these sentences to the Abstract.

However, we have included related descriptions in the **Introduction** (Paragraph 3 and paragraph 5 of the **Introduction**).

2. Aim should be stated more accurate in the abstract

Response: The aim has been revised as follows: "To examine the relationship between the single nucleotide polymorphism (SNP) CXCL10 rs1439490 and seronegative occult HCV infection (OCI)."

3. Genotyping methods should be given more detailed not only the one sentence and reference. ''SNP analysis primers were based on published reports (CXCL10 rs1439490 and rs1440802 [18], IL-28B rs12979860 [20]) and synthesized by Sango Biotech (Shanghai, China)''

Response: We have added a detailed description of the methods used for genotyping (Page 10), as follows: "Peripheral blood samples from the patients were collected in anticoagulant EDTA-treated tubes, and genomic DNA was extracted using a Puregene SK8224 DNA isolation kit from Sangon Biotech (Shanghai, China), according to the manufacturer's instructions. The primers targeting specific fragments were designed and synthesized by Sango Biotech: CXCL10 promoter region G-201A rs1439490[20]: forward: 5'-TTCAGTAACATAAACCCCAACAA-3'; reverse: 5'-CACAAAGGAAGACAATAAGGGAG- 3'. CXCL10 promoter region C-1513T rs1440802: forward[20]: 5'-CTCACTTTGTCTCACCAATCTCA-3'; reverse: 5'-CAGAGAAATGAGAGACCTAAGTGTG- 3'. IL-28B rs12979860[22]: forward: 5'-CCTCTGCACAGTCTGGGATTC-3'; reverse: 5'-GCTCAGGGTCAATCACAGAAG-3'.

4. Abbreviations should be checked again for the explanations.

Response: All abbreviations have been carefully checked.

5. Authors (Introduction part) need to explain the actual basis of CXCL10 gene polymorphisms and why they decide to investigate. '' In addition, lower serum levels of IFN gamma inducible protein-10 (CXCL10) were found in IL-28B C/C OCI patients than in CHC patients [17]. The importance of CXCL10 expression during chronic HBV infection has been recently emphasized. Two single nucleotide polymorphisms (SNPs) of CXCL10 (G-201A and C-1513T) were reported to have high allele frequency in chronic HBV infection in Chinese populations'' It is stated in Discussion, I think

it should be also stated in Introduction part. ''G-201A locates within the CXCL10 promoter region and is proximal to the NF-κB1/2 binding sites. G-201A SNP was associated with the expression of CXCL10 in PBMC and chronic HBV disease progression''

Response: We agree with the reviewer and have added the suggested descriptions to the **Introduction** (Paragraph 4 in the **Introduction** part). "G-201A is located within the CXCL10 promoter region and is proximal to the NF-κB1/2 binding sites. The G-201A SNP is associated with the expression of CXCL10 in PBMC, underpinning the mechanism of chronic HBV disease progression. Based on this large cohort study, and the observation that both HCV and HBV promote development of hepatic lesions and fibrosis by inducing inflammatory infiltration rather than by damaging hepatocytes directly, we hypothesized that CXCL10 G-201A may also affect the disease manifestation of CHC. However, to date, there is no such report."

6. This clinical application of this genotyping should be detailed and emphasized.

Response: We have added more discussion of the potential clinical application of the genotyping of CXCL10 (G-201A) (last paragraph in the **Discussion**), as follows: "Our results suggest the potential clinical significance of CXCL10 G-201A genotyping in identifying OCI during chronic HCV infection. In addition, clarifying the correlation between CXCL0 rs1439490 and liver necroinflammation or fibrosis stage may also guide *IFN-based* antiviral treatment of CHC patients."

7. English is correct.

Response: We have checked throughout the manuscript for grammatical errors or typos.

Reviewer #4:

- In the introduction section, the authors should highlight the role of the selected SNPs "CXCL10 rs1439490, rs1440802, and IL-28B rs12979860" in others viral infection or similar diseases.

Response: Thanks for the suggestion! It has been well-established that IL-28B is closely associated with HCV infection (*Hepatology* 2012;55:20-29; *Journal of Hepatology* 2011; 55: 692-701; *Hepatology* 2010; 52:1216-24; *Gastroenterology* 2010; 139: 1865–1876). However, the correlation of IL-28B with prognosis of HBV-related disease and treatment efficacy remains debatable

(reviewed in *World J Gastroenterol* 2014; 20: 12026–12030). The main viewpoint is that IL-28B is not positively correlated with HBV. We thus didn't emphasize the relationship between IL-28B polymorphisms and HBV infection. As for the CXCL10 SNPs rs1439490 and rs1440802, there are only three reports of the role of these SNPs with HBV infection, and two of them are of a small cohort. We thus only cited one large cohort study by Deng et al (*Gastroenterology* 2008; 134:716-726) in the introduction section.

- *The references should be updated and more recent references should be added.*

Response: We have added some new references in the revised manuscript (Refs 5, 6)

- *The authors should more develop the discussion part.*

Response: We have added expanded the discussion of this part (Paragraphs 1 and 4 of the **Discussion** part).

- *Table 1 should be reorganized and detailed:*

• *Clinical, Virological, Biochemical and Histologic Characteristics of Patients must be developed and reorganised.*

Response: We have added the biochemical parameter GGT in Table 1. The virological and histologic characteristics of patients have been described in the **Results** part.

• *Abbreviation in table 1 of "ALT (IU/L), HOMA-IR "must be added and indicated*

Response: Abbreviations have been added in the table notes of **Table 1**.

• *What did you mean in table 1 by: "Genotype 1 and non Genotype 1" or "Non-C/C genotype"? Please clarify and indicate the genotype with exact genetic notation (name of the appropriate nucleotide bases).*

Response: We have now explained the genotypes in the table notes. HCV has six genotypes and HCV genotype 1 is the primary genotype in China. In addition, the disease prognosis and treatment of HCV genotype 1 and other genotypes differ significantly. We thus classified the genotypes as genotype 1 and non-genotype 1 in **Table 1**.

-This part in table 1: IL-28B SNP rs12979860 C/C genotype Non-C/C genotype 97 (94.2%) 6 (5.8%) 128 (82.6%) 27 (17.4%) 0.007 Should be added in table 2

Response: We have merged this part of Table 1 into Table 2. In addition, IL-28B rs12979860 SNP have C/C, C/T and T/T genotypes and C/C is the primary SNP in China. We thus classified the IL-28B rs12979860 as C/C and non-C/C genotype. This explanation has now been added to the table notes of **Table 2**.

- Table 2 should be written and divided in different part, according to genetic co- dominant and dominant model related to the distribution of different genotype of CXCL10 rs1439490, rs1440802, and IL-28B rs12979860. P value and IC (OR95%) should be added and the reference group should indicate.

Response: Thanks for the suggestion! Table 2 has now been divided into two tables (**Table 2 and Table 3**). P values, OR and 95% CI have all been included in the new tables.

- Multivariate analysis and adjusted model for different confounding factors should be added to the results in Table 2.

Response: The purpose of **Table 2** is to compare the distribution of the three SNPs in OCI and CHC groups. We have revised the table and included the p values, OR and 95% CI in the revised table.

- What is the statistical criteria to include Factors in the multivariate model? - The choice of patients' number and the statistical power of this study must be indicated and added in the material and methods section.

Response: We have added this description in the **Statistical analysis** part.

- Then, the combination and interaction between CXCL10 rs1439490, rs1440802, and IL-28B rs12979860 should be indicated in a separate table.

Response: We here revealed that serum CXCL10 levels in OCI patients were lower than in CHC patients, and serum CXCL10 levels did not differ significantly between IL-28B rs12979860 polymorphism. IL-28B polymorphisms may affect endogenous IFN-λ levels, thus, IFN was less likely to play a determining role in OCI occurrence. In addition, the distribution of CXCL10

rs1440802 did not differ significantly between the two groups. We thus focused on the association of CXCL10 rs1439490 with OCI but did not investigate the interactions between the 3 SNPs.

- *The interpretation of results should be modified and scientifically introduced based on modified and added results.*
- *The title and the legend of each figure should be detailed.*
- *The legend of each figure should be detailed.*

Response: The related detailed descriptions have been added.

- *In case of liver lesions study, Lesions were graded (necroinflammation) and staged (fibrosis score) according to the Metavir scoring system. The authors should detail the METAVIR scoring in the manuscript.*

Response: The METAVIA scoring has been added in the **Materials and Methods** part.

- *Does the liver biopsies are read by an experienced pathologist? Authors should specify.*
Sections were scored by two independent observers.

Response: Yes, the liver biopsies were read by experienced pathologists. The stained sections were scored by two independent observers. We have added theis detail to the revised manuscript **Methods** section (Page 12).

- *What are the significances to make a Logistic regression analysis of factors associated with seronegative occult occurrence of HCV as indicated in table 5. Table 5 should be revised.*

Response: IL-28B polymorphisms may affect endogenous IFN-λ levels and be associated with low viral replication in some patients. However, IFN has also been reported not to play a determining role in OCI occurrence: IL-28B C/C OCI patients were found to have lower serum levels of CXCL10 than IL-28B C/C CHC patients. Therefore, regulation of OCI and the associated disease progression likely involves additional host immune factors. CXCL10 G-201A may influence the secretion of CXCL10, and subsequently the binding of CXCL10 to CXCR3 on the surface of Th1 cells. As such, the CXCL10-CXCR3 axis mediated adaptive immune response is compromised. This concession would affect the spontaneous clearance of the virus, but on the other hand, may cause less liver

damage, manifested as mild HCV infection. We therefore performed multivariate logistic regression analysis and revealed that CXCL10 G-201A G/G genotype and IL-28B rs12979860 C/C genotype significantly influenced the occult occurrence in patients with HCV infection.

- *In case of the measurement of Serum CXCL10 Levels, what is the sensitivity of the assay?*
Response: The sensitivity of the assay was 38-1340 pg/ml. We have added this detail to the revised **Methods** section (Page 11).

- *What is the nature of the association between fibrosis progression and necroinflammatory activity scores on liver biopsy?*
- *The necroinflammatory process is it implicated in the fibrogenesis process or not and Why?*
Response: Liver necroinflammation is primarily induced by causative factors (e.g. HCV infection) in the long-term, which can result in diffusive degeneration and necrosis of hepatocytes, destruction of the liver lobular structure, infiltration of inflammatory cells, the release multiple cytokines, and increased production of collagen. Hepatocytes have a unique potential to regenerate, however, due to the rupture or collapse of the hepatic lobule fiber scaffold, the regenerated hepatocytes cannot grow along the original scaffold but reform new hepatic lobules. In this circumstance, liver necroinflammation promotes the differentiation and proliferation of fibroblasts and the secretion of collagen fibers, leading to changes in the proportion and distribution of different types of collagens. The accumulated collagen fibers then not only wrap the regenerated hepatic nodules, but also re-divide the remnant hepatic lobules into pseudo-lobules. With the increase in pseudo-lobules, fibrogenesis processes from fibrosis to cirrhosis. Therefore, the higher liver necroinflammation score, the faster fibrogenesis process. It is not only the initiation factor of hepatic fibrosis and cirrhosis, but also runs through the whole pathological process of the related liver diseases.

Reviewer #5:
Overall the data are sound. The study is well presented and the paper is well written. The discussion is fine. However, this study does not represent a substantial contribution to the field but rather confirms previously published evidence from other groups. An interesting observation is that IL-28B

rs12979860 C/C patients with CXCL10 rs1439490 G/G genotype show lower serum and liver CXCL10 levels. Is it a cause or a consequence of low viral replication? Monitoring serum and liver levels of IL-28 would greatly improve the impact of this study.

Response: Many thanks for the reviewer's comments and insightful suggestion! The previous study by Deng et al (*Gastroenterology* 2008; 134:716-726) concerns the relationship between CXCL10 polymorphisms and HBV infection. We here report the correlation between CXCL10 SNPs and the occurrence of serum negative HCV infection (occult HCV infection, OCI). To our knowledge, our study is the first to examine CXCL10 expression in OCI. We revealed that OCI group exhibited significantly lower serum CXCL10 levels than CHC group, irrespective of IL-28B rs12979860 C/C or non-C/C. Because of the prevalence of IL-28B rs12979860 C/C genotype within both groups, we next compared the serum and liver CXCL10 levels in IL-28B rs12979860 C/C patients with different CXCL10 G-201A SNPs. Interestingly, we observed that IL-28B rs12979860 C/C patients with CXCL10 rs1439490 G/G genotype exhibited lower serum and liver CXCL10 levels. OCI may result from sporadic exposure to trace amounts of HCV, which can induce low levels of IL-28B, mildly activate the JAK/STAT pathway, and repress the HCV replication, but not eliminate the virus. We observed a higher prevalence of IL-28B rs12979860 C/C in OCI than CHC patients. Bartolomé et al. revealed that among OCI patients, intrahepatic HCV RNA load was significantly lower in those with the IL-28B C/C genotype than those with CT + T/T genotypes (*Journal of Medical Virology* 2016; 88: 268-274). IL-28B polymorphisms may affect the endogenous IFN-λ level, and thus are associated with low viral replication. However, low expression of CXCL10 in the context of IL-28B C/C genotype-associated high endogenous IFN expression remains to be understood.

In general, HCV infection can induce expression of chemokines, such as CXCL10 through the PRR signaling pathway and secondary paracrine signal pathways induced by IFNs, and thus promote establishment of an adaptive immune response. This response is able to suppress or clear the virus, however, it also triggers immunopathological reactions and liver injury by intrahepatic recruitment of lymphocytes. Host genetic factors may affect these processes. For example, the CXCL10 G-201A SNP may influence the secretion of CXCL10, and subsequently the binding of CXCL10 to CXCR3 on the surface of Th1 cells. As such, the CXCL10-CXCR3 axis mediated adaptive immune response is compromised. This concession would affect spontaneous clearance of the virus, but on the other hand, may reduce liver damage. In the long-term, extremely low levels of HCV replication still

promotes liver disease. **In summary,** host genetic factors are implicated in the low CXCL10 levels, which may allow low level replication of HCV in OCI infection. The low expression of CXCL10 suggests that endogenous IFN expression is less likely to play a determining role in OCI occurrence. In addition, the OCI group exhibited significantly lower serum CXCL10 levels than CHC group irrespective of IL-28B rs12979860 C/C or non-C/C. Therefore, we didn't examine the serum and liver levels of IL28B. Nonetheless, we will take this good suggestion to monitor the serum and liver levels of IL-28 in future related studies where appropriate.