May 9, 2014

Dear Dr. Ma,

Thank you very much for giving us this opportunity to revise our manuscript entitled "A novel approach to identify the hepatitis B virus pre-S deletions associated with hepatocellular carcinoma". We also appreciate the reviewers for their expert opinions. We have followed their suggestions and revised the manuscript carefully. The changes are highlighted in the revised version. We hope that the current version of the manuscript addresses all the concerns from the reviewers and is now acceptable for publication in World Journal of Gastroenterology.

Please find enclosed the edited manuscript in Word format (file name: Esps Manuscript NO.9420-review.docx).

Title: A novel approach to identify the hepatitis B virus pre-S deletions associated with hepatocellular carcinoma

Author: Zhi-mei Zhao, Yan Jin, Yu Gan, Yu Zhu, Tao-yang Chen, Jin-bing Wang, Yan Sun, Zhi-gang Cao, Geng-sun Qian, Hong Tu

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 9420

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

(1) In the revised manuscript, the title was corrected for "A novel approach to identify the hepatitis

B virus pre-S deletions associated with hepatocellular carcinoma".

(2)The number of the word in abstract is adjusted according to the editor's suggestion.

(3) Core tip and comments are added in the ms.

(4) The manuscript is typed in 1.5 line spacing and in Book Antiqua font of size 10 with ample margins.

2 Revision has been made according to the suggestions of the reviewer

(1) Answer to Reviewer 02528327

We appreciate the thoughtful insights of the reviewer, whose suggestions helped us improve the manuscript.

1. ***The authors adopted the method of capillary gel electrophoresis (CGE) for the identification of deletions in the preS region of the hepatitis B virus (HBV) envelope gene. This method is established for the detection of deletions, insertions and duplications of DNA sequences, and has been utilized for various applications in different areas in life sciences. This is acknowledged by the authors in the introduction section, and also referenced. The sequence encoding for preS1 and preS2 domains of the viral envelope protein was selected because of the reported association of mutations in the pre-S region with the development of the hepatocellular carcinoma (HCC) in patients chronically infected with HBV. The manuscript clearly aims to establish a CGE methodology for the preS1+2 sequence but still, should also mention in the introduction section that deletions in the preS region are not the only predictors for HCC, mutations in the basal core promoter, precore mutations are also predictive for HCC (Jang et al 2012, Cancer Sci 103: 296-304; Tatsukawa et al. 2011, BMC Cancer 11: 458; Liu et al. 2009, J Natl Cancer Inst 101:1066-82).***

Response: We agree and now the mutations in HBV BCP and pre-C are mentioned in the Introduction section (page 4, line 8).

2. ***The establishment of the CGE method is followed by screening for HBV preS mutants in HCC and non-HCC patients with the result that the presence of pre-S deletions is associated with a higher risk for HCC. But is unclear whether this result can be generalised as no information regarding the HBV genotypes is given. The information will have to be included and the HBV genotypes in the HCC patient group and non-HCC patient group will have to be presented.***

Response: This is indeed a very good suggestion. Now the information about the HBV

genotype is added into the Methods section (pate 5, line 28). In consistent with the previous reports from our group (Jin Y *et al*, Zhonghua Gan Zang Bing Za Zhi 2010, 18:511) and the others (Qu L *et al*, PLoS One 2013, 8:e59583), genotype C is highly prevalent in Qidong area. In this study, only 5 cases (3.2%) in HCC group and 6 (3.8%) in control group were found to be infected with genotype B virus. Therefore, it is unclear from this study whether HBV pre-S deletion can be served as a predictive marker for HCC in patients infected with other viral genotypes. Pre-S deletion has been reported to occur more frequently in HBV carriers with genotype C than in those with genotype B (Chen BF *et al*, Gastroenterology 2006, 130:1153; Chen CH *et al*. Gastroenterology 2007, 133:1466), but very few study has been focused on the relationship between pre-S deletion and HCC in genotype B-infected patients. This intriguing issue awaits further investigation in the future. We have added this discussion in our revised ms (Page 11, line 1).

3. ***Introduction ? Please avoid "overlapping" in the sentence "…. three overlapping envelope genes containing within a single ORF". Overlapping is confusing. Maybe better " three different in-frame start codons to express …." ?***

Response: We agree. In the revised manuscript, we have changed the statement to "HBV envelope proteins are encoded by three different in-frame start codons." (page 4, line 4).

4. Change "The pre-S region mediates the attachment …" to "The preS1 region mediates …." ? It is unclear why "the preS region …. regulates immune responses by its B or T cell epitopes". The majority of B cell epitopes are located in the 'a'-determinant region, and hot spots of T cell epitopes are located in the C-terminal region of HBsAgS. There are epitopes located in the preS1 and S2 domains, but I disagree that they regulate the immune response.

Response: We thank the reviewer for pointing out that it is the preS1 region mediates the attachment of the virus to hepatocytes. We have now changed the statement in the Introduction (page 4, line 8). We agree that the majority of B- and T-cell epitopes are located in the small S protein, but noted that several studies have reported that pre-S region also

contained B- and T-cell epitopes which would be helpful for the design of improved and more immunogenic hepatitis B vaccines (Chisari FV1 *et al*, Annu Rev Immunol 1995, 13:29; Ferrari C *et al*, Gastroenterology 1992, 103:255; Maeng CY *et al*, Virology 2000, 270:9). Since the reviewer thought this expression is not appropriate, we now remove the saying "pre-S region regulates immune responses by its B or T cell epitopes" from the text.

5. Materials & Methods + Results ? Cohort - 807 hepatitis B surface antigen (HBsAg) positive individuals. Please provide information about the HBV genotypes. The quality of the manuscript will be strengthened if information regarding the association of HCC versus deletion in the preS1/preS2 region versus HBV genotype can be provided. Liu, Zhang et al (2009) reported that the preS mutations have a stronger association regarding an increased HCC risk in the presence of HBV genotype C. ?

Response: HBV genotype has been indicated into the Materials and Methods section (page 5, line 28). And the related discussion in added in Page 11, line 1.

6. M&M indicates that the samples are derived from a cohort (started in 1992) with 807 HBsAg positive and 761 HBsAg negative individuals. In the result section, the authors stated that serum samples form 157 HCC patients and 160 non-HCC were used. What were the selection criteria for the 317 samples?

Response: The HCC cases and HBV-infected non-HCC controls were obtained from a prospective cohort started in 1992 that included 807 hepatitis B surface antigen (HBsAg)-positive and 761HBsAg-negative individuals from 7 towns in Qidong. Till the end of 2012, a total of 181 HCC were diagnosed, of which 169 were from HBsAg-positive group and 12 were from HBsAg-negative group. The inclusion criteria for HCC case group were: 1. Diagnosed as HCC; 2. Positive in HBV pre-S gene amplification. The inclusion criteria for non-HCC control group were: 1. HBV-infected individual without HCC; 2. Positive in HBV pre-S gene amplification; 3. Age, sex, and living location matched with the subjects in HCC group. A more detailed description about the patients and samples of this study is provided in Material & Methods section (page5, line 22)

7. ***M&M: "…. to align the validated sequences against the wild-type genotype C HBV DNA reference sequence." Were the 19 serum samples pre-selected because they contain HBV genotype C? What is the HBV genotype distribution of the complete cohort?***

Response: Yes, the 19 serum samples used for clone sequencing were from patients infected with HBV genotype C. The genotype distribution of the complete cohort is not fully determined, but we have analyzed the viral genotype for the subjects used this study. Consistent with the previous reports (Jin Y *et al*, Zhonghua Gan Zang Bing Za Zhi 2010, 18:511; Qu L *et al,* PLoS One 2013, 8:e59583), genotype C is highly prevalent in Qidong area (~95%). We now provide this information in Material & Methods section (page 5, line 28)

8. ***"Fig 1B demonstrates the profiles of CGE separation for the DNA marker and 5 PCR products of the pre-S gene." Five different samples were used. I assume that the samples were well characterized by PCR, cloning and sequencing of a statistically relevant number of clones. Are the results obtained by measuring the peak areas consistent with PCR, cloning and sequencing regarding % mutants versus % wildtype?***

Response: The 5 samples demonstrated in Fig.1B had been characterized by clone sequencing. For each sample, five clones were randomly selected and sent for sequencing. The results determined by CGE were identical to those determined by clone sequencing for sample a, b, and c. But for sample d, while clone sequencing method suggested a 33.3% (2/6) deletion mutants in viral population, CGE showed wild-type HBV co-existed with 40.1% deletion mutants. In patient e, CGE profile demonstrated that the wild-type virus was mixed with 14.7% type I mutants (-21bp) and 34.1% type II mutants (-54bp). However, by using clone sequencing method, we found that the wild-type virus was mixed with 16.7% (1/6) type I mutants and 16.7% (1/6) type II mutants. These data revealed that compared with the semi-quantitative clone sequencing method, CGE is more accurate in quantification of the mutants distribution.

(2)Answer to Reviewer 02449415

We really thank the reviewer for his/her highly-relevant questions.

1. This m/s presents data to show that a rapid PCR – CGE method is able to accurately determine Pre-S deletions associated with HCC and that it has greater utility than sequencing based approaches. In Fig1 some, presumable representative, samples of 114 clones show peaks corresponding to Pre-S deletions. However origins of these samples are not clear. In the Methods Section 807 HBsAg +ve and 761 HBsAg –ve individuals are mentioned. Later 19 serum samples are described as the source of 114 clones that were selected for sequence analysis. Although I assume that the 114 clones used to test the accuracy of the PCR-CGE method are the same as the ones previously mentioned this is not made explicit. In addition there is no indication as to which clones came from which of the 19 serum samples.

Response: We are sorry not indicating the sample sauce clearly in the ms. Now, a more detailed description about the patients and samples of this study is provided in Material & Methods section (page5, line 22). Figure 1B showed the representative samples of 5 sera, not 5 clones. We make a clearer statement in Fig.1B figure legend. Meanwhile, we add one supplementary table 1 to demonstrate the sample sources of the 114 clones. The 5 serum samples a-e used in Fig.1B are correspondent to case 486, 440, 207, 70 and 356 in supplementary table 1.

2. The second objective i.e. the analysis of Pre-S in HCC patients mentions 157 HCC and 160 non-HCC. Where did these come from e.g. the 807 HBsAg +ve and 761 HBsAg –ve individuals? If so how were they selected?

Response: The HCC cases and HBV-infected non-HCC controls were obtained from a prospective cohort started in 1992 that included 807 hepatitis B surface antigen (HBsAg)-positive and 761HBsAg-negative individuals from 7 towns in Qidong. Till the end of 2012, a total of 181 HCC were diagnosed, of which 169 were from HBsAg-positive group and 12 were from HBsAg-negative group. The 157 HCC patients who showed the positive PCR results for pre-S gene amplification were selected as the case group. The inclusion criteria for non-HCC control group were: 1. HBV-infected individual without HCC; 2. Positive in HBV pre-S gene amplification; 3. Age, sex, and living location matched with the

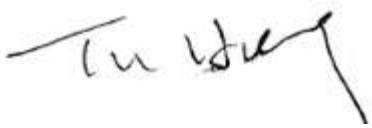
subjects in HCC group. A more detailed description about the patients and samples of this study is provided in Material & Methods section (page5, line 22)

3、*It does appear however that small Pre-S deletions occur more frequently in HCC than in non-HCC patients. Similarly the longitudinal observation indicates that there is an increase the prevalence of Pre-S mutants as the disease progresses. However a relatively small number of samples were examined and it is not clear if there is a temporal increase in number of small Pre-S deletions in these samples.*

Response: We thank the reviewer's for his astute comments. This is indeed a very important issue. Of the 9 HCC cases we selected to conduct the longitudinal observation, 5 (6-177, 252, 7-002, 3-073, 5-001) cases contained pre-S deletion ≤ 99bp, while 4 (372, 1-117, 86, 570) cases contained pre-S deletion ＞99bp. The type of the deletant in HCC stage was identical to that in the chronic hepatitis stage. One case (5-001) exhibited a mixed infection with wild-type viruses (39.7%), 168bp deletion mutants (27.6%) and 87bp deletion mutants (32.7%) 3 years prior to HCC. At the year of HCC diagnosis, the viral population consisted of 20% wild-type viruses, 30.3% 168bp deletion mutants, 35.8% 87bp deletion mutants and 13.9% newly-appeared 120bp deletion mutants. We did not find an obvious selection of small pre-S deletion mutants during the development of HCC. The evolution of pre-S deletion with different sizes and their relationship with HCC needs a further well-designed prospective cohort study to reveal.

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely



Hong Tu, M.D., Ph.D.

Professor

State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute,

Renji Hospital, Shanghai Jiao Tong University School of Medicine

Shanghai 200032

China

Fax: 86-21-64437196

E-mail: tuhong@shsci.org