ANSWERING REVIEWERS

July 30, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 11884-review.doc).

Title: Occult infection related hepatitis B surface antigen variants showing lowered secretion capacity

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Name of Journal: *World Journal of Gastroenterology*

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1. We have attached the language certificate letter by professional English language company on top of manuscript.

2. The manuscript has been improved according to the suggestions of reviewers.

Reviewer 1)

Major comments:

A key conclusion of this study is that occult infection may be attributed to defects in HBsAg secretion. The data behind this conclusion rely on the ability of the HBsAg assays used in the study to detect the secreted/intracellular HBsAg in the expression system. In samples classified into groups I, II, or V, the results suggest that the assays were sufficient for detection of the variant HBsAg forms. However, for groups III and IV, particularly samples ALK and KD, there is the question of whether the HBsAg assays could detect the variant HBsAg if it was expressed. Although ALK and KD have mutations outside the 'a' determinant, this is not sufficient to conclude that the variants would be detected by the HBsAg assay used. The detection of recombinant HBsAg samples with commercially available HBsAg assays can be highly dependent on the combination of mutations, expression system, post-translational modification, and other variables associated with generating recombinants. The authors need to demonstrate independently that the HBsAg assays are capable of detecting such variants if they are present.

- ALK and KD mutations are classified as group II according to the secretion level of HBsAg and viral DNA formation capacity. The levels of HBsAg from all mutants including ALK and KD after transiently co-transfection in HuH-7 cell line were measured using three types of commercial HBsAg ELISA assay kit as mentioned Materials and Methods. The commercial HBsAg ELISA assay kit used in this study is as follows; Bioelisa HBsAg color ELISA Kit (BIOKIT, Barcelona, Spain), MONOLISA HBsAg ULTRA (BIO-RAD, CA, USA), and ETI-MAK-4 HBsAg Enzyme Immunoassay Kit (DiaSorin, Saluggia, Italy). All kits shows similar results in HBsAg detection of both extracellular and intracellular level, irrespective of their types.

Minor comments:

1) Methods section: Sentence two is unclear and needs revision.

- We could not find any mark which indicates unclear sentence in edited manuscript.

2) Introduction: The references cited linking occult HBV infection to severe forms of liver disease date from over 30 years ago. The authors should include more recent references that support this link.

- We have replaced the recent articles which have suggested that occult HBV infection may cause severe liver disease (ref. 23-24) and updated the papers which have shown that HBV DNA from HBsAg negative individuals could have been detected using molecular-based technology, such as PCR-based methods (ref. 20-22).

3) Discussion: It would be helpful if the authors could discuss the ratios of HBsAg to HBV DNA/virions among the ten cloned samples relative to the ratios observed In chronic HBsAg-positive HBV infection.

- Ten cloned samples having novel mutations in this study were related to occult HBV infection, not chronic HBsAg-positive HBV infection. We have checked the ratio of HBsAg to HBV DNA/virions among the ten cloned samples. There was no great difference of the secretion ratio between comparative sAg (Figure 2 and Table 3) and sAg/viral DNA from extracellular and intracellular conditions (Data not shown).

Reviewer 2)

Major comments:

Kim et al studied the secretion capacity and intracellular expression of the hepatitis B virus (HBV) virions and HBsAgs of 10 HBsAg variants derived from patients with occult genotype C HBV infection in a transient co-transfection system. They found that all variants exhibited lower levels of HBsAg secretion compared with the wild type, and that most variants exhibited normal virion secretion capacities comparable with, or even higher than, the wild type. Furthermore, most variants generated higher reactive oxidative species (ROS) production than the wild type. Their findings are interesting and provide a mechanistic explanation for occult HBV infection of genotype C and its potential link to liver disease progression.

Two major comments are:

1. Does the extracellular HBV DNA level of the HBsAg variants in the transient transfection system (Table 3 and Figure 2) correlate with the serum HBV DNA level of the individual patient from whom the HBsAg variant was cloned? Please include the serum HBV DNA level of each patient in Table 2.

- We have measured the HBV DNA level of serum from individual patient using VERSANT HBV bDNA 3.0 assays in Bayer 340 bDNA Analyzer systems (SIEMENS, USA). The concentration of HBV DNA in wild type was 1 x 10^6 copies/ml, but viral DNA level from patients having ten types of mutation was lower than 2,000 copies/ml using positive cut-off value of HBV bDNA assay system. So, we cannot compare DNA levels between our transfection system and patients serum. According to reviewer's comment, we include HBV DNA level of patients in revised Table 2.

2. The Group III HBsAg variants (CNR, LL and PAHS) showed negative or weakly positive HBsAg expression or secretion yet increased levels of viral DNA production and secretion as compared to the wild type. If protein stability was the reason for reduced HBsAg expression, how can the HBV virion be formed without HBsAg? Please provide an explanation.

- One likely answer is that HBsAg made by these variants may be just only used for the viron formation, not for void HBsAg particles. Another may be difference in protein solubility between mutants and wild type due to disparity in the subcellular localization, which could affect HBsAg ELISA results. To address these issues, in the future, we have the plan to elucidate the reason why the HBV virion was formed without HBsAg under the reduced expression condition.

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

Sincerely yours,

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