Name of journal: World Journal of Gastroenterology
Manuscript NO: 34915
Title: Curcumin Inhibits Hepatitis B Virus Infection by Down-Regulating cccDNA-Bound Histone Acetylation

Reviewer's code: 00012216
Reviewer's country: Spain
Science editor: Ya-Juan Ma
Date sent for review: 2017-06-24
Date reviewed: 2017-07-02

Comments to authors 1: Authors should state the number of experiments performed for every analysis. This is referred neither in the result section nor in the figures. Obviously, the validity of the results would be low if it comes out from a few experiments.
Author reply: All experiments were repeated at least three times, ELISA and RT-PCR were performed in duplicate. In the Figure Legends section, the statements were added in each part of the study.

Comments to authors 2: Authors should include in material and methods a statistics section to describe the statistical methodology used for the different comparisons. They should state the test used for every single analysis and the level of significance.
Author reply: Statistical methods was removed from original location and added as an independent part at the end of Materials and Methods section.

Comments to authors 3: In the figures, authors should state the number of experiments performed in every analysis, the p value for all the comparisons and in the figure legend, they should state the statistical test used. For instance, in figure 1A we do not know the number of experiments carried out to show the decrease of HBsAg after curcumin treatment with different concentration and different incubation time. They do not show either whether the differences observed in the figure are statistically significant. Nevertheless, in figure 1C they show the p level by using asterisks. We assume that ** means a $p<0.01$, but they should explain that in the figure legend. Anyway, we can also consider that if they show the p level in this figure but not in the previous ones, this could be because of lack of statistical significance in those other figures.
Author reply: Statistical methods were added in the Materials and Methods section. And figure 1A was revised according to statistical methods.

4. In the references, authors show the complete Journal name but according to WJG guideline, they should use the reduced name.
Author reply: References were revised using the reduced name according to

WJG guideline.

Reviewer's code: 02942798
Reviewer's country: Slovakia
Science editor: Ya-Juan Ma
Date sent for review: 2017-07-01
Date reviewed: 2017-07-09

Comments to authors 1: Please describe the statistical methods used in this study.
Author reply: Statistical methods were added in the Materials and Methods section.

Comments to authors 2: Please add statistical significance in figure 1A, 1B and 4 and explain significance in other figures.
Author reply: Figure 1A, 1B and 4 were revised according to statistical methods.

Comments to authors 3: Please specify, how many experiments were performed in each part of the study.
Author reply: In the Figure Legends section, the statements were added in each part of the study and were revised as "All experiments were performed in duplicate and repeated at least three times".

Comments to authors 4: Please edit references.
Author reply: References were revised according to WJG guideline.

Reviewer's code: 00506552
Reviewer's country: South Korea
Science editor: Ya-Juan Ma
Date sent for review: 2017-06-24
Date reviewed: 2017-07-10

Comments to authors 1: In Fig 1, authors need to show cccDNA by Southern Blotting. The reference #15 also showed cccDNA by Southern blotting. After showing cccDNA by Southern blotting, they can show cccDNA by PCR (qPCR). Detection of cccDNA by PCR alone is highly likely to show the false result. It may be necessary to show the reduction of HBV RNA transcriptions by curcumin by Northern blotting (or RT-PCR or luciferase assay).
Author reply: The mean values of cccDNA in HepG2.2.15 cell is about 10 copies per cell. It is hard to be detected by Southern blotting, therefore, real-time PCR was used to quantify the cccDNA levels with cccDNA-specific primers, using a complex procedure for cccDNA preparation which including DNase digestion to degrade contaminating HBV inserted in cellular genomic DNA and OC (open circular) species.

The reduction of HBV RNA transcriptions has been evaluated and shown in Figure 1D.

Comments to authors 2: The curcumin concentrations for HBsAg and HBeAg were 5 to 30uM and cytotoxicity assay was at 20uM. Authors need to show or describe the cytotoxic effect at 30uM of curcumin. HBeAg was not reduced at 20uM of curcumin.
Author reply: The data of cytotoxicity of curcumin performed by CCK-8 assay was uploaded in the supplementary file (Supplementary Figure1).

And in the Results section part1, we revised the result as "Curcumin decreased HBsAg levels both dose- and time-dependently; HBsAg levels were reduced by up to 57.7%, 2 days after treatment with 20 μM curcumin (Fig. 1A). HBeAg was not reduced at 20uM of curcumin (Fig. 1B)".

Comments to authors 3: In Fig 4 lower panel, Southern blot result needs to be improved to clearly see the curcumin and siRNA effects. Also, levels of core particles by curcumin treatment through the core particle Western blotting through native agarose gel electrophoresis.
Author reply: Southern blot result was refined more clearly.
In the present study, curcumin was found to inhibit HBV through down-regulation of cccDNA-bound histone acetylation. Theoretically, the reduction in cccDNA and HBV mRNAs will decrease the levels of core particles. We did not detect the core particles by Western blotting, while investigate the core-associated HBV replicative intermediates instead. As shown in figure 1E, core-associated HBV replicative intermediates were significantly reduced upon curcumin treatment, suggesting the

down-regulation of core particles.

Comments to authors 4: In Fig 4, HBx siRNA inhibit HBV DNA replication more than HBs siRNA, however authors went too far to say that " …….that siRNAs may down-regulate the acetylation of cccDNA-bound histones H3/H4 by suppressing HBx expression." in pg 12 in Discussion without direct evidence.

Author reply: We agree with the reviewer's comments. The sentences were revised as "Furthermore, enhanced inhibition of intracellular HBV replication was revealed when curcumin was combined with siRNAs against HBV. Since HBx is pivotal for the steady state of cccDNA by regulating epigenetic modifiers of cccDNA-bound histones, the combination of curcumin with siRNAs, especially with siRNA targeting HBx may lead to a synergistic effect in modulation the steady state of cccDNA."