Format for ANSWERING REVIEWERS

February 3, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 14987-review.doc).

Title: PC/BCP mutants quantification throughout phases of HBV infection by Simpleprobe

Author: Wen-Hui Tu, Ying Lü, Yong-Mei Zhang, Wei Hou, Jin-Yu Wang, Yi-Jun Zhang, Hong-Yan Liu, Hao-Xiang Zhu, Yan-Li Qin, Ri-Cheng Mao and Ji-Ming Zhang

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

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

Part A (Reviewer #1): First of all, we sincerely thank the reviewer for all the valuable advices, and we have modified our manuscript according to the comments.

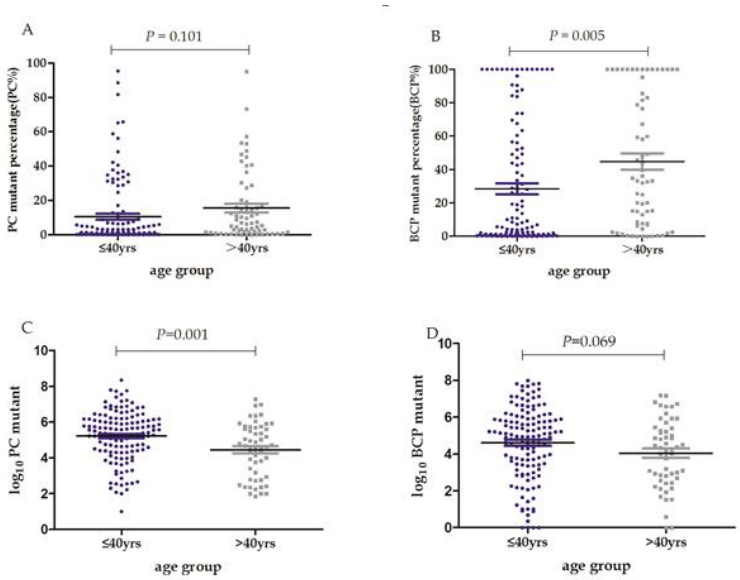
（1）The authors need to add the analysis results of the relationship between PC/BCP mutants and the clinical diseases in the patients.

Response: Thanks for the suggestion. With regards to clinical disease analysis, we have already analyzed the relationship among PC %, BCP % and age and among HBV, DNA and the status and titers of HBeAg in the early stage but we ignored ALT. Now we have already added the ALT analysis and modified the corresponding contents in Table 3. We have added the corresponding content **(line 28-30, page 15 and line 1-4, page 16)** as: Interestingly, our results showed elevated ALT significantly correlated with BCP%, indicating that BCP% reflects hepatic inflammatory degradation, which indicating that BCP% was an indicator of hepatic inflammation degradation. Nie et al（Nie H, Evans AA, London WT, et al. Quantitative dynamics of hepatitis B basal core promoter and precore mutants before and after HBeAg seroconversion. J. Hepatol 2012; 56: 795–802 [PMID: 22173170 DOI: 10.1016/j.jhep.2011.11.012]）reported that PC% (not BCP%) was positively correlated with ALT elevations among HBeAg(-) patients. There were several studies implied that PC/BCP mutants caused the hepatic inflammation, acute on chronic liver failure and liver cirrhosis. Our observation demonstrated that ALT elevation might also be a result of varied host immune responses.

（2）What is the basis on which the authors used age 35 to classify the patients into two age groups?

Response: Thank you for your advice, we have categorized the patients according to age 40 according to the guideline from AASLD2009 and APASL2012. Those patients at the age of over 40 may

suffer from inflammation and need treatment, thus liver biopsy is recommended for them. And the existence of variants in PC/BCP area is generally associated with inflammation, but liver biopsy may not be accepted by every patient. We were wondering whether we can consider variants in the PC/BCP area and their percentages as one of the substitution indexes for inflammation evaluation, so we chose the age of 40 as the basis for grouping, and re-conducted the statistical analysis, which has been listed in Figure. 2A to 2D as:



（3）There are some English grammatical problems which need to be amended, such as: ref. [25] "HBeAG"? Table 1 and Results, what does the "Baseline" mean? The meanings of Table 2. were not clear.

Response: Thanks for pointing out our mistake, which has been corrected **(line 25, page 21). We have modified the titles of "Table 1 and Results" as "Baseline characteristics of patients with chronic HBV infection" (line 2, page 11 and table 1). We have aslo amended the Table 2, We hope you find it more straight forward to understand.**

Part B (Reviewer #2): First of all, we thank the reviewer for providing valuable advices, and we have modified our manuscript according to the reviewer's comments.

（1）The difference between the number of patients in group ENH (HBeAg-negative) with 20 patients and the overall detected HBeAg-negative 69 patients is not really clear to me.

Response: Thanks for the suggestion. We enrolled 69 HBeAg-negative patients as two groups: (Low-replicative (LR) = 49, and HBeAg-negative hepatitis (ENH) = 20).

（2）Do the authors have any idea how PC/BCP and PC%/BCP% status could be applied in clinical practice? In my opinion such test assays are only possible in specialist laboratories.

Response: Actually, we do think this method can be applied to the clinic, since the equipment and

reagents required in this examination is routinely available in most of the laboratory department in teaching hospitals.

（3） The authors described the exclusion criteria, e.g., of other viral co-infections. However, there is no description concerning the detection methods for other hepatitis viruses and HIV.

Response: Thanks for bringing out this question. We have added the corresponding content **(line 29-30, page 7 and line 1-3, page 8)** as: anti-HCV, and anti-HDV were assessed using commercial AxSYM MEI kits (Abbott Laboratories, North Chicago, IL). anti-HIV assayed by Diagnostic Kit for Antibody to Human Immunodeficiency Virus Type 1 and/or 2 and HIV-1 Antigen(bioMérieux, France).

（4）Quantification of PC/BCP mutants. Did the authors evaluate their SimpleProbe method? Should be shown in detail because their results based on this single method.

Response: Thanks for the suggestion. We have added the corresponding content **(line 27-30, page 8, line 1-25, page 9)**.

（5）The authors described that 96.86% and 84.82% of PC and BCP could be detected by their method in the patient collective. This statement implies that the PC/BCP mutants might be predominant in all tested patients. That is obviously not the case and has been also described. A more detailed description concerning the prevalence and percentage of these mutants would be helpful.

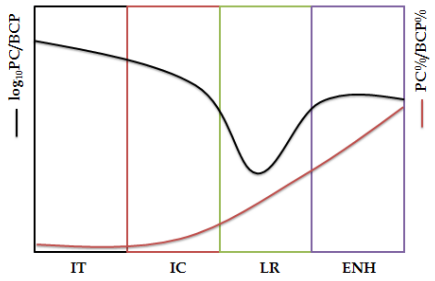
Response: Thanks for pointing it out. BCP mutation was detected in 96.86% and PC mutation in 84.82% of a population of 191 patients, but not 96.86% and 84.82% of HBV isolates in one patient. PC/BCP mutants were predominant in HBeAg-negative but not in HBeAg-positive patients. **(line 17-20, page 11)**.

（6）Discussion section. What is the rational for dividing the patients in aged >< 35 years?

Response: Thank you for your advice, we have categorized the patients according to age 40 according to the guideline from AASLD2009 and APASL2012. Those patients at the age of over 40 may suffer from inflammation and need treatment, thus liver biopsy is recommended for them. And the existence of variants in PC/BCP area is generally associated with inflammation, but liver biopsy may not be accepted by every patient. We were wondering whether we can consider variants in the PC/BCP area and their percentages as one of the substitution indexes for inflammation evaluation, so we chose the age of 40 as the basis for grouping, and re-conducted the statistical analysis, which has been listed in Figure. 2A to 2D.

（7） Discussion section. The authors provided an interesting model of their results how to classify the PC/BCP distribution and course of the HBV-infection. However, for me it is slightly confusing. A schematic graph would be very helpful.

Response: Thank you for the advice. We have drawn a diagram (Fiure.1E) to show the dynamic variation of log_{10}PC, log_{10}BCP, PC% and BCP%. We hope you find it more straight forward to understand:



（8） There are some minor English spelling and grammar errors which should be polished.

Response: Thanks for pointing out our mistakes，which have been corrected according to the comment.

Part C**(Reviewer #3):** First of all, we sincerely thank the reviewer for all the valuable advices, and we have modified our manuscript according to the comments.

（1）Although the methodology employed by Wen-Hui for quantification of these specific mutations have been previously reported by several studies Nie et al (J Clin Virol 2011, J Clin Microbiol 2011), explicit data about primers, probes an blockers, used for must be included in the manuscript.

Response: Thanks for the suggestion. We have added the corresponding content **(line 27-30, page 8, line 1-25, page 9)**.

（2）Morever, a short study of mutations in the presence of non-mutated forms in different proportions and and its confirmation, at least qualitatively, by Sanger Sequencing must be also provided: e.g: analysis of at least five of processed samples with relative values of mutated form (eg in BCP) and unmutated form in proportions 100: 0, 90:10, 80:20, 60:40 and 0: 100 must be includeded in the manuscript as an additional figure in order to confirm the specificity and sensitivity of the method.

Response: Thanks for the suggestion. We have added the content (supplementary material) as:

1） Quantitation of PC mutation：

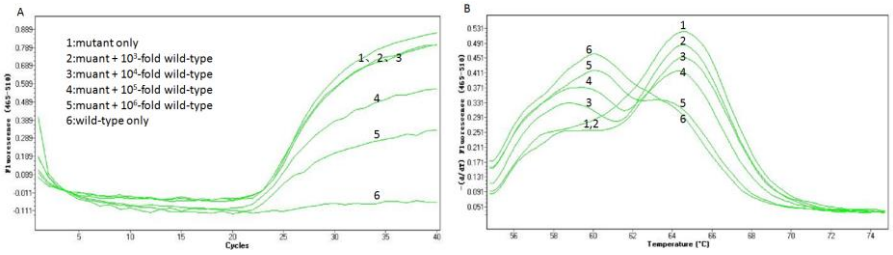


Figure S1：Templates containing 60 IU G1896A mutant at different ratios were first amplified in the WT-selective PCR blocker for 20 cycles and then amplified in a Simpleprobe PCR. The amplification curves are shown in panel A, and the corresponding melting curves are in panel B.

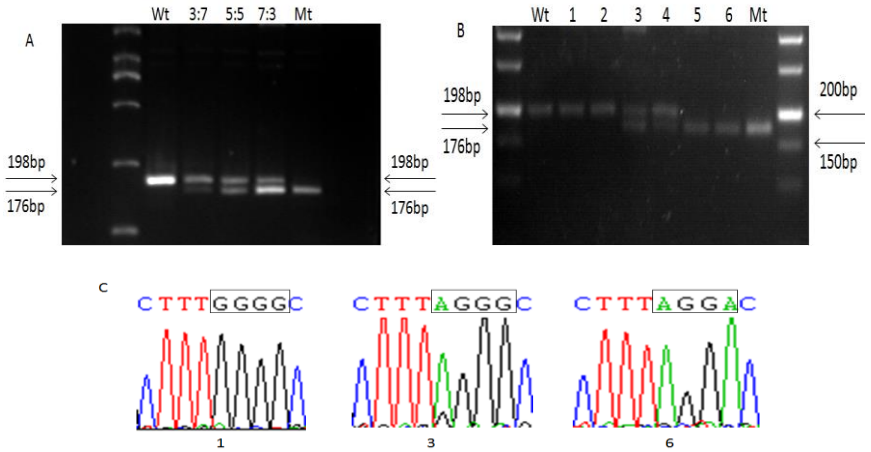


Figure S2: Confirmation of the ultrasensitive quantification of the PC mutation by RFLP assay and direct sequencing. Assay results of six samples are shown in Table 1. For the RFLP assay, the PCR product was digested with XagI and separated by 3% agarose gel electrophoresis. The wild-type band was 198 bp and the mutant band was 176 bp (as shown in panel A). The six samples were detected by RFLP, samples 1 and 2 were wild-type, samples 3 and 4 were mixed strains, samples 5 and 6 were mutants (as shown in panel B). We selected samples 1, 3, and 6 for direct sequencing; the sequence inside the box represents nt1896–1899 (as shown in panel C). Wt: PC wild-type; Mt: PC mutant-type; ratios: Mt: Wt

Table S1：Ultrasensitive quantification of the PC mutation from 6 HBV patients

Sample no	Mutant type	Mutant titer (IU/mL)	Total HBV DNA (IU/mL)	%Mutant
1	G1899A	7.24×10^{4}	5.54×10^{7}	0.131
2	G1896A	9.58×10^{4}	1.35×10^{7}	0.712
3	G1896A	2.96×10^{6}	4.54×10^{6}	65.198
4	G1896A	2.54×10^{5}	6.26×10^{5}	40.575
5	G1896A	3.80×10^{6}	4.74×10^{6}	80.169
6	G1896A/G1899A	1.38×10^{6}	1.69×10^{6}	81.678

2） Quantitation of BCP mutation：

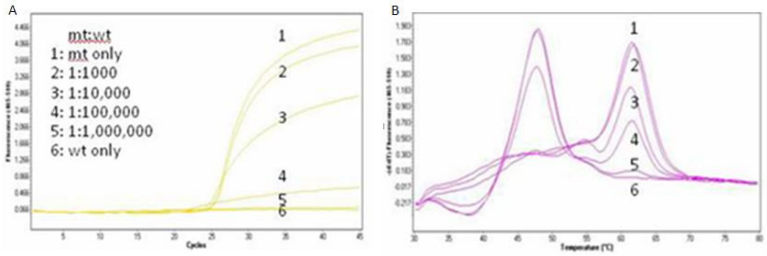


Figure S3: Templates containing 60 IU of A1762T/G1764A mutants were amplified in the WT-selective PCR blocker for 20 cycles and then amplified in a Simpleprobe PCR. The amplification curves are shown in panel A and the corresponding melting curves are in panel B. wt: BCP wild-type; mt: BCP mutant-type

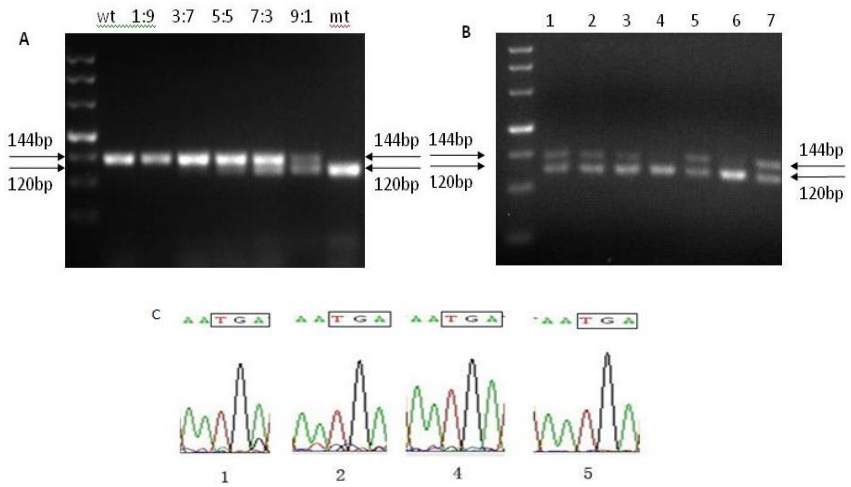


Figure S4: Confirmation of utrasensitive quantification of the BCP mutation by RFLP assay and direct sequencing. The quantification assay results of seven samples are shown in Table 2. For the RFLP assay, the PCR product was digested with BclI and separated by 3% agarose gel electrophoresis. The wild-type band was 144 bp and the mutant band was 120 bp (as shown in panel A). The seven samples were detected by RFLP: samples 1, 2, 3, 5, and 7 were mixed strains, samples 4 and 6 were mutants (as shown in panel B). We selected samples 1, 2, 4, and 5 for direct sequencing; the sequence inside the box represents nt1762–1764 (as shown in panel C). wt: BCP wild-type; mt: BCP mutant-type; ratios: mt: wt

Table S2：Ultrasensitive quantification of the BCP mutants from 7 HBV patients

Sample no	Mutant titer (IU/mL)	Total HBV DNA (IU/mL)	% Mutant	ALT (U/L)
1	1.88×10^3	2.70×10^3	69.556	<40
2	5.82×10^3	9.72×10^3	59.877	667
3	1.20×10^3	2.50×10^3	48.000	505
4	4.82×10^6	5.02×10^6	96.016	115
5	1.30×10^2	2.64×10^2	49.091	<40
6	1.96×10^4	2.06×10^4	95.340	<40
7	6.04×10^2	1.22×10^3	49.346	<40

（3）The method used to quantify HBV-DNA it is not too sensitive (500 copies/ mL) and can allow a bias in the selection and study of inactive carriers.

Response: We agree with your statement. The limit of HBV DNA detection is 500 copies/ mL In most hospitals of China. Our method may have caused some bias in selection.

（4）Detail the HBeAg quantification methodology.

Response: Thanks for the suggestion. We have added the corresponding content **(line 3-6, page 8)** as: HBeAg values were determined using a microparticle enzyme immunoassay, and was expressed as signal/cutoff (S/CO), HBeAg value>1S/CO was considered as positive (Abbott Architect i2000SR, USA).

（5）Prevalence of PC / BCP mutants (page 9) quantification ranges of each group must be included along with the values of qualitative prevalence. It is understood that is considered "the presence of a mutation" from any value of quantitative signal detected.

Response: Thank you for your advice, and we have rephrased this part as: The PC mutation detection rate was 84.82% (162/191, PC mutant quantification range: 60-60 million IU/ml). We identified 96.86% (185/191, BCP mutant quantification range: 60-60 million IU/ml) cases as carriers of the BCP (A1762T/G1764A) mutation. **(line 17-20, page 11)**

Part D(Reviewer #4): First of all, we sincerely thank the reviewer for all the valuable advices, and we have modified our manuscript according to the comments.

（1）First of all, it is necessary to examine the setting criteria of four groups; immune tolerance, immune complex, low replication, and ENH.

Response: Thanks for the suggestion. We have added the corresponding content **(line 15-25, page 7)** as: The IT phase is characterized by high HBeAg titers, high HBV DNA levels, but normal ALT, and normal liver histology; The IC phase is characterized by presence of HBeAg, high HBV DNA levels (over 20,000IU/ml), elevated ALT, necroinflammation of the liver; The LR phase is characterized by HBeAg negativity and anti-HBe positivity, low or undetectable HBV DNA (below 2000IU/ml), persistently normal ALT, no histologically active inflammation, with mild fibrosis; The ENH phase is characterized by negative HBeAg, positive anti-HBe, detectable HBV DNA levels (2000-20 million IU/ml), elevated ALT, and moderate to severe necroinflammation with variable amounts of fibrosis.

（2）It is well known that natural course of HBV infection is depend on HBV genotype. What differences in PC/BCP mutants were there between genotype B and C?

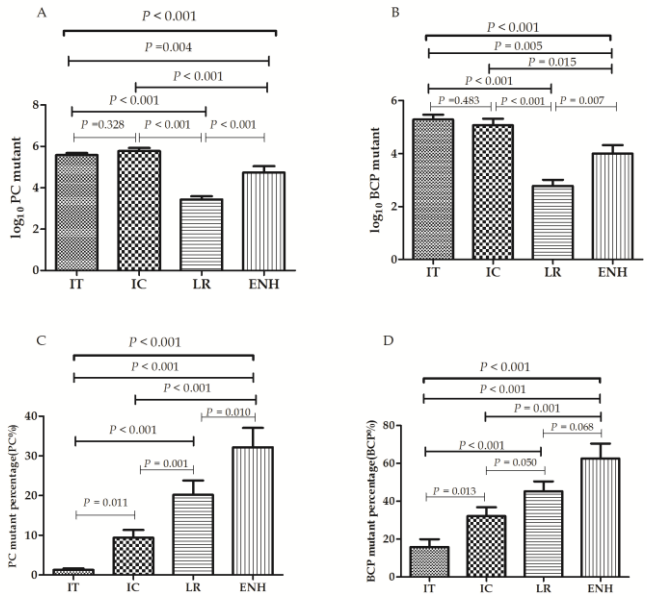
Response: Thank you for your advice, We have added the corresponding content **(line 4-7, page 16)** as: Previous reports suggest the PC mutation is more commonly seen in genotype B patients, while the BCP mutation predominates in genotype C patients[30]. (Chen CH, Lee CM, Hung CH, et al. Clinical significance and evolution of core promoter and precore mutations in HBeAg-positive patients with HBV genotype B and C: a longitudinal study. Liver Int. 2007; 27: 806-815 [PMID: 17617124 DOI: 10.1111/j.1478-3231.2007.01505.x]) Our results showed no significant difference in the prevalence of PC/BCP mutation between the two genotypes.

（3）Author should show the cut off index of HBeAg. What of titer of HBeAg meant HBeAg negative? Also, authors had better omit the sample below cut off of HBeAg, in comparison of HBeAg titer and PC/BCP mutant% or log. Because the samples with below of cut off were not quantified.

Response: Thanks for bringing out this question. We have added the corresponding content **(line 3-6, page 8)** as: HBeAg values were determined using a microparticle enzyme immunoassay; results are expressed as signal/cutoff (S/CO), with HBeAg values >1S/CO considered positive (Abbott Architect i2000SR, USA).

（4）What did authors speculate that Log PC/BCP was associated with? It is necessary to examine the differences between PC/BCP% and Log PC/BCP in HBV four groups (Figure 1).

Response: Thanks for bringing out this question. Our assumption is that HBV isolates with PC or BCP mutation have higher fitness under the pressure from host immune reaction. And we've added the data as Figure. 1A to 1D:



（5）Minor comment: 1. Some mistakes should be revised; line 2, page 10, log10 PC. Line 26, page 10 The PC5 and. 2. B and P should be explained the meaning of the abbreviations in Table 2, and 3. For example, P; p-value.

Response:Thanks for pointing out our mistake，the content have been adjusted according to the comment. HBeAg: hepatitis B early antigen; HBV: hepatitis B virus; ALT: alanine aminotransferase; PC: precore; PC%: PC mutant quantity per total viral load; BCP: basal core promoter; BCP%: BCP mutant quantity per total viral load. PC: precore; BCP: basal core promoter; PC%: PC mutant quantity per total viral load; BCP%: BCP mutant quantity per total viral load; HBV: hepatitis B virus; ALT: alanine aminotransferase; HBeAg: hepatitis B early antigen. **(line 2, page 13; line 4-7, page 24 and line 3-6, page 25)**

Part E (Reviewer #5): First of all, we sincerely thank the reviewer for all the valuable advices, and we have modified our manuscript according to the comments.

（1）What criteria was used to define each stage of HBV infection? Please add definitions in Methods.

Response: Thanks for the suggestion. We have added the corresponding content **(line 15-25, page 7)** as: The IT phase is characterized by high HBeAg titers, high HBV DNA levels, but normal ALT, and normal liver histology; The IC phase is characterized by presence of HBeAg, high HBV DNA levels

(over 20,000IU/ml), elevated ALT, necroinflammation of the liver; The LR phase is characterized by HBeAg negativity and anti-HBe positivity, low or undetectable HBV DNA (below 2000IU/ml), persistently normal ALT, no histologically active inflammation, with mild fibrosis; The ENH phase is characterized by negative HBeAg, positive anti-HBe, detectable HBV DNA levels (2000-20 million IU/ml), elevated ALT, and moderate to severe necroinflammation with variable amounts of fibrosis.

（2）Prefertially HBV-DNA load should be expressed as IU/mL instead of copies/mL to comply with international guidelines.

Response: Thanks for the suggestion. We have replace the copies/mL with IU/mL according to your advice. (1 IU/mL≈5copies/mL).

（3）It would be interesting that the authors comment on the fact that these mutations were prevalent regardless of genotype (B or C) and its implications in other genotypes that have low replication rates such as HBV genotype H.

Response: Thanks for bringing out this question. We have added the description in discussion part **(line 4-7, page 16)** as: Previous reports suggest the PC mutation is more commonly seen in genotype B patients, while the BCP mutation predominates in genotype C patients[30] (Chen CH, Lee CM, Hung CH, et al. Clinical significance and evolution of core promoter and precore mutations in HBeAg-positive patients with HBV genotype B and C: a longitudinal study. Liver Int. 2007; 27: 806-815 [PMID: 17617124 DOI: 10.1111/j.1478-3231.2007.01505.x]). Our results showed no significant difference in the prevalence of PC/BCP mutation between the two genotypes. Unfortunately, we have no access to other genotype HBV infected patients, otherwise we would love to give some data or discussion on other genotypes.

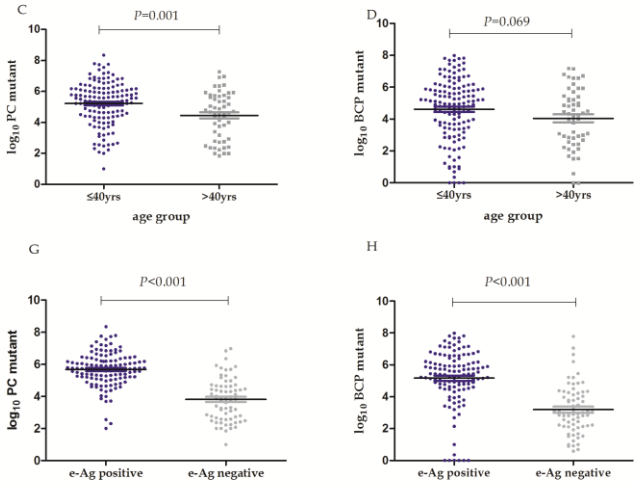
Part F (Another comments): First of all, we sincerely thank the reviewer for all the valuable advices, and we have modified our manuscript according to the comments.

（1）Please describe the meaning of log_{10}PC, log_{10}BCP, log_{10} PC/BCP, PC/BCP mutation, PC%, BCP% and PC/BCP% because it is quite difficult to conceive the results.

Response: Thanks for the suggestion. We have added the corresponding content **(line 29-30, page 9 and line 1-3, page 10)** as: **Definitions,** G1896A and G1896A/G1899A variants are defined as PC mutations; A1762T/G1764A variants are defined as BCP mutations. We used the log_{10} PC and log_{10} BCP values to represent PC and BCP mutant quantities; PC% and BCP% represent PC and BCP mutant quantities per total viral load.

（2）For the results in topic of PC/BCP mutant distribution by phase, age, and HBeAg status, why did the authors presented graph only PC% and BCP% compared with age groups and with HBeAg status in figure 2? Because authors also described the association of log_{10}PC and log_{10}BCP with age group and with HBeAg status which some results were positive finding. Thus the authors should add graph of log_{10}PC and log_{10}BCP distribution compared with the age group as well as HBeAg status to complete the results.

Response: Thanks for the suggestion. We have adjust our figure according to the comment as Fig2C, Fig2D, Fig2G, Fig2H:



（3） Authors please describe abbreviation of manuscript after correspondence and in each table for more clearly understandable.

Response: Thanks for the suggestion. We have added the corresponding description. **(line 2-6, page 23; line 4-7, page 24 and line 3-6, page 25)**

（4）The authors did not give the page number and line in each page thus it's quite difficult to show the directly point of editing.

Response: Thanks for your advice, and we have labelled the page and line, hoping you find it more easy to edit.

（5）Authors should explain the characteristics of each patient group: how are they different?

Response: Thanks for the suggestion. We have added the corresponding content **(line 15-25, page 7)** as: The IT phase is characterized by high HBeAg titers, high HBV DNA levels, but normal ALT, and normal liver histology; The IC phase is characterized by presence of HBeAg, high HBV DNA levels (over 20,000IU/ml), elevated ALT, necroinflammation of the liver; The LR phase is characterized by HBeAg negativity and anti-HBe positivity, low or undetectable HBV DNA (below 2000IU/ml), persistently normal ALT, no histologically active inflammation, with mild fibrosis; The ENH phase is characterized by negative HBeAg, positive anti-HBe, detectable HBV DNA levels (2000-20 million IU/ml), elevated ALT, and moderate to severe necroinflammation with variable amounts of fibrosis.

（6）Authors should change or clarify details of table2 and 3 for more easily understanding and please organize data in the table in the same pattern.

Response: Thanks for the suggestion. We have added the corresponding description. **(line 4-7, page 24 and line 3-6, page 25).** We have also adjusted the content according to the comment in Table 2 and Table 3 **(line 10-28, page 13)**.

（7）At page 10, 2nd – 3rd sentence as following "the log_{10} PC distribution did differ between groups (t = 2.07, P = 0.04)." Please add "age" between these words "between" and "groups".

Response: Thanks for pointing out our mistake，we have added the word "age" between these words "between" and "groups". **(line 8, page 12)**

（8）At page 10, the last paragraph and 2nd sentence, as the sentence below "The PC5 and BCP% significantly differed between HBeAg" Please change word from "PC5" to be "PC%".

Response: Thanks for pointing out our mistake, which has been corrected according to the comment. **(line 2, page 13)**

3 References and typesetting were corrected

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

Sincerely yours,

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