Author's response to reviews

Title: Hepatitis B virus X protein suppresses caveolin-1 expression in hepatocellular carcinoma through regulating DNA methylation

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Version: 2 Date: 1 June 2012

Author's response to reviews: see over
Dear Dr. Steenbergen

Thank you for your review of our manuscript (MS: 1027956701663771) entitled “Hepatitis B virus X protein suppresses caveolin-1 expression in hepatocellular carcinoma by regulating DNA methylation”. We appreciate the concerns and suggestions provided by the reviewers, and have revised our manuscript accordingly. Our point-by-point responses are provided below, and text that has been added or modified from the original text is shown in the revised manuscript in red font. We have had the language of this paper corrected by a professional language editing service that specializes in scientific manuscripts (Edanz).

We are apologized that there was something wrong for log on with the E-mail of Jiahong Dong, we have updated the E-mail address in the manuscript. And we recommend that Xiaowu Li as the co-corresponding author, he made the great contribute for conceiving the study, and he participated in its design and coordination and helped to draft the manuscript.

Upon review of our revised manuscript, we hope that you will find it acceptable for publication in *BMC Cancer* and we look forward to your response.

Sincerely,

Jiahong Dong, M.D, Ph.D

Professor of Hepatobiliary Surgery
Responses to Reviewer #1:

Version: 1 Date: 1 May 2012

Reviewer: Fengmin FL Lu

Reviewer's report:

The manuscript “Hepatitis B virus X protein suppresses caveolin-1 expression in hepatocellular carcinoma through regulating DNA methylation” by Yan and colleagues explored the role of HBx on the hypermethylation of caveolin-1 in HBV related HCC. This work is of importance to further the understanding of the molecular mechanism underlying the oncogenicity of HBx. I have some suggestions/comments:

1. Ethic issue. Has an informed consent been obtained from each of participants?

Response: Thank you for your comment. This study was approved by the Research Ethics Committee of the Southwest Hospital and informed consent from each patient was obtained preoperatively. The specific content has been added to the manuscript page 5, line 10-12. As follows: “This study meets the requirements of the Declaration of Helsinki, and was approved by the Research Ethics Committee of the Southwest Hospital. Informed consent was obtained from all participants.”

2. The authors suggested that HBx can induce caveolin-1 gene CpG island methylation, based on their in vitro study using a SMMC-7721 cell line. They also found that 28/33 HCC samples were positive for caveoline-1 gene CpG island methylation. How about the adjacent non-tumor tissues? Since HBx may commonly
expressed in the non-tumor tissue, has they seen any caveolin-1 CpG island methylation in specimen derived from the HBV positive non-tumor tissues? Also, it is of informative to compare the methylation statues of caveolin-1 gene CpG island between HBV-positive and HBV-negative HCC tissues.

Response: We appreciate your comments. In our previous study, we found that caveolin-1 expression is decreased significantly in HCC, compared with that in adjacent tissues, as demonstrated by immunohistochemistry (Figure 2I). We also performed an n-MSP analysis of the adjacent non-tumor tissue and HCC. We found that caveolin-1 promoter methylation was positive in HCC tissues, but was negative in adjacent non-tumor tissues in the same specimens (Figure 2J). The above result has been added to the manuscript. (The method of immunohistochemistry at page 5, line 14 to page 6, line 2, and the result at page 11, paragraph 1.)

Almost all HCC patients were associated with HBV infection. We hoped to perform a comparative study of HBV-negative HCC specimens, but unfortunately such specimens are too rare to meet the study requirement.
Figure 2I: Expression of caveolin-1 in HCC and adjacent tissue with HBV infection, as determined by immunohistochemistry. A: adjacent non-tumor tissue, B: HCC.

Figure 2J: DNA methylation status in adjacent non-tumor tissue and HCC was examined by n-MSP. U: unmethylated PCR products, M: methylated PCR products. 1: healthy liver tissue, 2: M.Sss I-treated healthy liver tissue, 3: adjacent non-tumor tissue, 4: HCC

3. Neither in the method nor the figure legends provides enough information about at what time point post adenovirus infection the cells were harvested for caveolin-1 expression level determination by RT-PCR and western blot. This is information of importance and the author should provide. Also, the method is not detailed enough for others to repeat the experiments.

Response: Thank you for your comments. We harvested cells after 48 hours post-adenovirus infection for RT-PCR and western blot analyses. We have added this information in the methods section and figure legends (page 6, line 13).
4. Only one cell line was used. Could same observation be made using different HCC cell lines?

Response: We appreciate the reviewer’s suggestion. We had several liver cancer cell lines: SMMC-7721, HepG2, MHCC-97L, MHCC-97H and Hep3B. The infection efficiency of HepG2 was too low to meet the experimental requirements. MHCC-97L, MHCC-97H and Hep3B were not suitable for this study because of HBV infection. Hirasawa et al (2006) reported that hypermethylation of the promoter region of the caveolin-1 gene is also found in Hep3B. However, MHCC-97L and MHCC-97H have not been verified. Therefore, we selected SMMC-7721 cells for this study, and hoped that the results with this cell line could be verified in HCC tissues in further study.

5. The author claimed in the last paragraph of result part that “We found transfected HBx could significantly down-regulate activity from the caveolin-1 promoter”, Does the observation was made using HBx stably expressed or transiently infected SMMC-7721 cells? Need authors provide detailed information.

Response: We accept the reviewer’s suggestion. SMMC-7721 cells were transiently infected with adenovirus. The effect of transient infection could be maintained for 6 weeks. We have included this information in the manuscript (page 6, line 4).
Responses to Reviewer #2:

Reviewer: Naoshi Nishida

Reviewer's report:

“Hepatitis B virus X protein suppresses caveolin-1 expression in hepatocellular carcinoma through regulating DNA methylation” by Yan et al. The authors examined the DNA-methylation status of the caveolin-1 promoter by n-MSP in 33 HBV-infected HCC. Methylation of the caveolin-1 promoter was detected in 84.8% (28/33) of HBV-infected HCC samples. Expression of caveolin-1 was significantly. Transfection HBx significantly suppressed caveolin-1 promoter activity and induce methylation in HCC cell line. They concluded that HBx protein induces methylation of the caveolin-1 promoter region and suppresses its expression.

1. The authors examined the methylation status of HCC tissues using nested-MSP. However, quantitative analysis of methylation level should be better for this type of evaluation. More specifically, is there a correlation between methylation level and expression of corresponding mRNA or protein? In addition to the in vitro data of HCC cell line, analyses using MethyLight, COBRA, RT-real-time PCR and IHC are required to make the result more confident.

Response: Thank you very much for your kind advice. Methylation-specific PCR is a classic qualitative detective method for methylation. After our initial recognition of the methylation in these HCC tissues, unfortunately there was not a sufficient amount of HCC tissues for us to perform further experiments. We are very disappointed that
we could not provide further data. According to the reviewer’s comment, we will use more precise quantitative analysis methods in future studies.

2. Fig 2 D: For representation of bisulfite sequence analysis, which part of the targeted gene did the authors analyzed? The location of bisulfite sequencing should be shown in Fig 2 A. In addition, putative core promoter region of the caveolin-1 showed be indicated.

Response: We appreciate the reviewer’s suggestion. The targeted gene shown in Figure 2D was marked as “Gene of Interest” in Figure 2A. Caveolin-1 promoter DNA sequences were obtained from the NCBI GenBank (AF019742.1). We have included this information on page 9, line 11 and page 19, line 14 in the manuscript.

![Figure 2A](image)

**Figure 2A:** There is a CpG island in the promoter region of the caveolin-1 gene. Gene of Interest: The targeted gene that we studied.

3. The authors showed that methylation event of the caveolin-1 gene was unique in HBV-infected HCC and the role attributed to the action of HBx protein. Is there an association between HBx protein expression and methylation level and downregulation of the caveolin-1 gene? How is the methylation status in
HCV-infected or virus negative HCC?

Response: We appreciate your questions. In the previous study, we found that caveolin-1 expression is significantly decreased or even negative in HCC, compared with that in adjacent non-tumor tissues, as demonstrated by immunohistochemistry (Figure 2I). Moreover, caveolin-1 expression was inversely correlated with HBx expression in 65 human HCC tissues, as demonstrated by real-time PCR (P=0.034). Therefore, we suspected that HBx might inhibit the expression of caveolin-1. In this study, our main purpose was to verify this conjecture and to preliminary study its mechanism. Unfortunately there was not a sufficient amount of HCC tissues for us to perform a quantitative analysis of methylation, and therefore could not provide a detailed comparison between the expression of HBx and caveolin-1 methylation. We are currently performing these studies.

The vast majority of Chinese patients have HCC associated with HBV infection, and it is difficult to obtain HCV-infected or virus-negative HCC specimens. Therefore, the methylation status in HCV-infected or virus negative HCC has not been verified yet.
Figure 2I: Expression of caveolin-1 in HCC and adjacent tissue with HBV-infected, as determined by immunohistochemistry. A: adjacent non-tumor tissue, B: HCC.

4. Previously, the authors reported the association between caveolin-1 downregulation and HCC progression using human HCC tissues. Is the methylation level also increasing according to the tumor size, vascular invasion etc?

Response: We appreciate your questions. It is unfortunate that we did not conduct a quantitative analysis of methylation for the lack of sample volume. As a qualitative method, n-MSP could not meet the accuracy of analysis for the correlation between the methylation level and the biological characteristics of the tumor. Currently, we are collecting HCC specimens for such a study.