Author’s response to reviews

Title: The silence of MUC2 mRNA induced by promoter hypermethylation associated with HBV in Hepatocellular Carcinoma

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Author’s response to reviews: see over
Dear Journal Editorial Office,

Thank you for arranging a timely review for our manuscript. We have carefully evaluated the reviewers’ critical comments and thoughtful suggestions, responded to these suggestions point-by-point, and revised the manuscript accordingly.

Reviewer: 1 Norishige Yamada

Major Compulsory Revisions:

1. In figure 2B, No.159 and Non-HCC in No.185, which expressed high levels of MUC2 clearly showed methylation; Non-HCC in No.51 and No.53, which expressed low levels of MUC2 showed unmethylation band. Moreover, methylation band was obtained in almost all samples including Non-HCC. The authors need to show the methylation ratio in each sample. Otherwise it might be difficult to conclude that MUC2 promoter methylation is associated with MUC2 mRNA expression in each sample.

Reply: In Materials and methods: The methylation index (MI) of MUC2 was calculated by the following formula: 100 x methylated reaction/(unmethylated reaction + methylated reaction). We have calculated the MI of each sample. We revised the figure 2B and added MI accordingly.

2. In figure 5, AZA+TSA in Huh7 showed methylation regardless of MUC2 mRNA high expression. The authors should speculate on the reason.

Reply: AZA+TSA in Huh7 showed a little demethylation. It could be due to individual differences of cancer cells by incubated with 5-aza and TSA together. The inhibitors of histone deacetylation and DNA
methylation could have a different synergistic effect of MUC2 mRNA on cancer cells. We will pay more attention for it. We revised the Discussion accordingly.

Minor Essential Revisions:
3. The authors need to define the name of the company, city, states (in the case of United States), and the nations the authors got the materials from should appear first, followed by the company name, thereafter.
Reply: We revised accordingly in Materials and methods.

4. Figure 2B was not referred to in the text.
Reply: We revised the Results accordingly.

5. The quality of the photograph for bands in Fig.2B is not fine enough.
Reply: The bands of MSP have a little difference with normal PCR. We have done the best as far as possible.

6., line 6 of 2nd paragraph and line 7 of last paragraph: “MUC4” should be “MUC2”.
Reply: We revised the Discussion accordingly.

Reviewer: 2  Yujing Zhang

1. In Table 1. it shows the correlation between HBV status and MUC2 mRNA levels. But there is no data on the relationship between HBV status and MUC2 promoter hypermethylation shown in Results.
Reply: We will investigate the role of HBV in MUC2 hypermethylation with cells and animals model for the next.

2. Authors tested MCU2 mRNA levels and promoter hypermethylation status in 7721, Huh7 and HepG2 three HCC cell lines, but not show the HBV infection status in those cell lines. So it lacks the data on HCC cell lines to support the point: “HBV could play an important role for the loss of MUC2 gene expression in HCC.”
Reply: We will investigate the role of HBV in MUC2 hypermethylation with cells and animals model for the next.

3. For detecting MUC2 methylation status by using MSP, did author use positive and negative controls? It didn’t mention in Materials and methods, not show in figures in Results either. For MSP in Figure 2, the marker (bp) also needs to be shown.

Reply: Distilled water was used as negative control, DNA methylated by SssI methylase (Sss DNA) was used as positive control (pos). The band of methylation is 217 bp, unmethylation is 216 bp. We revised accordingly in Materials and methods.

4. For detecting MUC2 expression, if author tested its protein levels by using immunohistochemical method or Western blot it could make mRNA levels data more solid.

Reply: We will investigate the role of HBV in MUC2 hypermethylation with cells and animals model for the next. Then the protein levels must be detected.

5. Two references should be cited either in Background or in Discussion:

Reply: We have added two references in Background.

6. Authors should explain the purpose to run DMSO in Materials and Methods.

Reply: DMSO was being a blank control. We revised accordingly in Materials and methods.

The manuscript contains a number of typos/errors that need to be corrected or edited. The followings are few examples:
1. Background page: line 10 from bottom, Cholangiocarcinoma should be cholangiocarcinoma; line 9 from bottom, Extrahepatic should be extrahepatic.

Reply: We revised accordingly in Background.
2. Hcc should be HCC (in Abstract page and other pages).
   Reply: We revised accordingly.

3. In Materials and Methods, under Cell Culture and Treatment, The HCC cancer lines should be HCC cancer cell lines (top line).
   Reply: We revised accordingly.

4. In Figure 5., G2 should be HepG2.
   Reply: We revised accordingly.

5. In “Association of MUC2 mRNA with clinicopathologic features (Results), last sentence “This implicated…..” should be “These results implicated……”.
   Reply: We revised accordingly.

6. Abstract, Materials and Methods and Discussion need to be re-written and English edited.
   Reply: We revised accordingly. Reply: We revised accordingly.