Author's response to reviews

Title: The overmethylated genes in Helicobacter pylori-infected gastric mucosa are demethylated in gastric cancers

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Author's response to reviews: see over
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Dear BMC Gastroenterology Editor:

Thank you for your reply regarding our manuscript (Manuscript #:1097496650416394) entitled “The overmethylated genes in *Helicobacter pylori*-infected gastric mucosa are demethylated in gastric cancers”. The careful review and helpful comments by your reviewers were of great assistance. Accordingly, we have revised the relationship between the LOH events and the methylation alterations. The point-by-point corrections are listed below. The written English has been corrected with the assistance of a professional science writer (http://www.harrisco.net). We hope that the revised manuscript will now be suitable for publication. Thank you for your consideration and we look forward to your reply.

Sincerely yours,

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Point-by-point description of the changes made in response to the reviewers’ comments

The similar comments are discussed as follows;

1. **REVIEWER 1: Hiromu Suzuki, Point 2, Is there any correlation between the transitional**
CpG sites methylation and neighboring CpG island methylation? It is of interest to see whether CpG islands are methylated or not, when the neighboring transitional CpG sites are methylated by H. pylori infection:

And REVIEWER 3: Yong Sung Kim, Point 2, Introduction section: the concept of the transitional-CpG sites has to be defined for reader, though it has been described in the previous publications:

Most CpG-islands tend to be unmethylated in most tissue types when compared with the variable methylation of transitional-CpG sites. We have added new sentences to the Background section (lines 16-19 on page 6, “Meanwhile, most CpG-rich islands are weakly methylated or unmethylated in most tissue types, and the CpG-rich sites are not suitable for the analysis of dynamic methylation adjacent to gene-control regions. Therefore, the transitional-CpG sites, rather than CpG-rich islands, are likely to…”).

2. REVIEWER 1: Hiromu Suzuki, Point 4, Page 21: "This study suggests that the concurrent methylation of multiple CpG island genes initiates and sustains the adaptive differentiation of newly fixed stem cells in the gastric mucosa infected with H. pylori, and that the LOH-induced DNA demethylation and discordant methylation of stomach-specific genes may facilitate gastric carcinogenesis by reactivating a stem-cell intrinsic program." This study does not provide enough functional evidences to draw this conclusion.

And REVIEWER 2: Rommel Burbano, Point 4, The authors concluded that “concurrent methylation of multiple CpG-island genes initiates and sustains the adaptive differentiation of newly fixed stem cell in the gastric mucosa infected with H. pylori”. However, no comment about stem cells is made in the methods or results sections. How the authors conclude such information about stem cells? Is there any marker that was
used to check if the cells used in DNA extraction were newly fixed stem cells?:

We agree with the reviewer’s comment that the stem cell was not identified in this study. The Conclusion section was modified as follows: lines 6-10 on page 23, “This study suggests that the concurrent methylation of multiple CpG-island genes is initiated under the influence of nearby retroelement methylation in the gastric mucosa infected with *H. pylori*, and the LOH-induced DNA demethylation and discordant methylation of stomach-specific genes may facilitate gastric carcinogenesis by reactivating a stem-cell intrinsic program.”).

3. REVIEWER 2: Rommel Burbano. Point 5, The authors checked for *H. pylori* status of non-cancerous tissue samples. How is the *H. pylori* status of the gastric cancer tissue samples? Is there any difference in the methylation status between gastric cancer tissue samples *H. pylori* positive and *H. pylori* negative?:

And REVIEWER 3: Yong Sung Kim. Point 1, If the HP infection is examined in gastric cancer samples and the methylation status is compared between HP positive and negative, their opinion may be clearer. How do you think about it?:

We have added new sentences to the Discussion section (lines 14-17 on page 21, “Comparison of the *H. pylori*-positive and -negative gastric cancer tissue was not conducted, because the methylation status of the CpG-islands in the gastric cancerous and precancerous lesions has been found to be similar in the *H. pylori*-infected and non-infected patients.”, and line25 on page 21 and lines 1-2 on page 22, “Therefore, the dynamic methylation pattern of transitional-CpG sites in a given gastric cancer appears to be determined according to the level of LOH rather than *H. pylori* infection and the differentiation state of cancer progenitor cells.”).
Other comments are explained as follows;

REVIEWER 1: Hiromu Suzuki,

Point 1, Does methylation in the transitional CpG sites correlate with gene expression in the specimens used in this study? Only the SAGE tag results in normal stomach is shown, and this point is not mentioned:

We used the SAGE data to represent the difference of transcription dose between the master-specific genes and the housekeeping genes in stomach tissue and bone marrow tissue. We have added sentences to the Material section (lines 20-23 on page 9, “Comparative analysis of the microarray and SAGE (Serial Analysis of Gene Expression) data has found that the number of transcripts counted in the SAGE data accurately represents a great difference in the gene activity between the stomach-specific genes and housekeeping genes.”) and the Discussion section (lines 5-8 on page 18, “In particular, there is a great difference in the number of SAGE transcripts between the highly expressed master-specific gene group and weakly active housekeeping gene group in the stomach (Table 1).”).

Point 3, Page 18: Are there any functional evidence for the statement "In cancer tissue that undergoes the LOH event that reduces a gene dose, the remaining gene copies have the increased possibility of using the nuclear proteins and this would lead to the demethylation of transitional-CpG segments"? If so, please include a citation for this statement. If not, please show functional evidence that hypomethylation is a consequence of LOH in cancer:

We have added references regarding the dosage compensation mechanism and the
methylation studies on the transitional-CpG sites (line 3 on page 20) [1-3].

Point 5, "Overmethylation" and "Undermethylation" are not commonly used terms to describe DNA methylation, and the authors have used "hypermethylation" and "hypomethylation" in their previous manuscript (Kim YH et al. BMC Cancer, 2006). Is there any specific reason to use these uncommon terms in this manuscript?

The transitional-CpG sites showed dynamic methylation changes in response to the transdifferentiation of bone marrow stromal cells and loss-of-heterozygosity events in gastric cancer. In order to distinguish the methylation status of transitional-CpG sites from that of CpG-islands that were usually unmethylated, we have analyzed the relative under- or overmethylation based on the common intermediate methylation of \textit{H. pylori} negative gastric mucosa.

REVIEWER 2: Rommel Burbano,

Point 1, The authors should clarify the number of gastric cancer samples used on the paper. In Methods section, they mention n=70 (48 males and 22 females). However, in page 9 of the manuscript they cited “40 duplicated DNAs of the same tissue, 48 pairs of 1-cm-adjacent tissues...”:

We have added new sentences to the Methods section about the number of tissue specimens used for the reproducibility of the transitional-CpG methylation analysis (lines 4-8 on page 11, “The comparative analysis of the paired samples was conducted using the duplicated DNAs of the 40 tissues and 48 pairs of 1-cm-adjacent tissues in addition to 100 pairs of antrum and body tissues that were obtained from the 50 \textit{H. pylori}-negative stomach tissues and the 50 \textit{H. pylori}-positive stomach tissues (Figure 2).”).
Point 2. There is no information regarding the histological type of the gastric cancer samples used in the manuscript. Is there any difference in the methylation status according to the histopathological type?

We have added new sentences regarding the methylation status according to the histologic type to the Discussion section (lines 17-25 on page 21 and lines 1-2 on page 22, “In this study, the transitional-CpG sites in diffuse-type gastric cancers tended to be overmethylated in the LOH-B cases and to be undermethylated in the LOH-H genotype cases depending on the level of chromosomal losses. Additionally, both the over- and under-methylation frequencies of individual and overall transitional-CpG sites were not significantly different between the distinct histologic types of the LOH-H cases (data not shown). When assuming that the methylation of transitional-CpG sites promotes cell differentiation, the LOH-induced demethylation may lead to the dedifferentiation of gastric cancer cells. Therefore, the dynamic methylation pattern of transitional-CpG sites in a given gastric cancer appears to be determined according to the level of LOH rather than *H. pylori* infection and the differentiation state of cancer progenitor cells.”).

Point 3. The authors classified one or no chromosome lost in diffuse-type of gastric cancer as LOH-B (baseline level) and as LOH-L (low level) in intestinal and mixed type. Why?

The diffuse type gastric cancers have shown a bimodal distribution for the number of chromosomal losses [4]. In a bimodal distribution, there are one and four chromosomal losses that occur frequently. The diffuse type gastric cancers with one or no chromosomal loss (LOH-B) showed early age of onset and poor prognosis compared with the intestinal- and mixed-type gastric cancers (LOH-L) [4, 5]. The relationship between the LOH-induced
cell adverse effect and the clinicopathologic features in the LOH-B genotype was described precisely in the other simultaneously submitted paper (“The gene-reduction effect of chromosomal losses detected in gastric cancers”). Please see the discussion on the Point 3 of REVIEWER 2 in the other paper.

Point 6. The authors should explain the 5-level and 10-level classification (including the intermediary status) of methylation status. In a previous paper (Hong et al., 2009), they explain the 5-level. I believe that a better explanation should improve the manuscript:

We have modified the sentence about the validation of the semiquantitative assay to the Methods section (lines 1-2 on page 11, “To validate the reproducibility of the variable methylation density using a semiquantitative analysis,…”).

Point 7. The first paragraph of the Results section is about results of a previous paper. Please, transfer it to the Discussion section. However, if they are results of the current manuscript (although similar of the obtained previously), please cite the methodology used in the Methods section:

We have transferred sentences about the gene expression data to the Discussion section (lines 13-14 on page 20, “The two stomach-specific genes (PGA5 and TFF2) were most highly expressed in the gastric mucosa (Table 1).”).

Point 8. The last paragraph of page 13 should be transferred to Methods section:

We have added sentences about the overmethylation rate associated with H. pylori infection to the Methods section (line 16 on page 10, “Semiquantitative evaluation of transitional-CpG methylation variation” and line 9-15 on page 11 “The overmethylation rate of each transitional-CpG site in the H. pylori-positive and -negative
gastric mucosa was calculated by the relative proportion of overmethylated cases to the total sum of the over- and under-methylated cases. The *H. pylori*-associated overmethylation rate was calculated using the *H. pylori*-positive-to-negative ratio of each transitional-CpG-overmethylation rate. This was used to evaluate the relationship between the methylation of transitional-CpG sites and the distance between the transcription start site and the nearest retroelement (Figure 3).”). Also we have modified the sentence of the Results section (lines 17-23 on page 15, “The relationship between the transitional-CpG methylation and the distance to the nearest retroelement was evaluated using the *H. pylori*-associated overmethylation rate (Figure 3). In the CpG-island genes, the *H. pylori*-associated overmethylation rate was higher as the distance of the nearest retroelement became shorter. The CpG-island-lacking genes that showed no significant methylation difference between the *H. pylori*-positive and -negative mucosa were methylated irrespective of the distance of the nearest retroelement.”

Point 9, In page 13, 1st and 2nd lines, the authors can substitute 20 percent for 20%:

We have replaced ’20 percent’ with ‘20%’ (line 24 on page 14).

Point 10, In page 14, lines 14-17: The authors say that PGC and TFF1 are stomach-specific genes weakly active when compared with the two master stomach-specific genes. In which tissue? The authors also say that those genes are densely methylated in bone marrow. And in the stomach?:

We have modified the sentence (lines 7-8 on page 16, “The *PGC* and *TFF1* stomach-specific genes, which were weakly active in the gastric mucosa…”).
Point 11. Is there any microsatellite in which the authors observed LOH more frequently?
And how about LOH and histologic type?:

The relationship between the LOH frequency and the clinicopathologic features in gastric cancers using the same 40 microsatellite marker set on 8 chromosomes were previously published [4, 5]. The relationship between the level of chromosomal losses and the clinicopathologic features in the gastric cancer was described more precisely in the other simultaneously submitted paper (“The gene-reduction effect of chromosomal losses detected in gastric cancers”). Please see the discussion on the Point 1 of REVIEWER 2 in the other paper.

Point 12. In page 18, please change H2O2 for H2O2 (there is a zero instead of an “O”):

We changed “H₂O₂” to “H₂O₂” (line 20 on page 19).

REVIEWER 3: Yong Sung Kim,

Point 3. In subsection of collection of tissue samples, HP-positive or –negative cases tested were described as each 50 cases. But actually authors analyzed with 100 HP-positive and 100 HP-negative cases to examine the methylation status, respectively. Correction is needed:

We used the antrum and body pairs for the methylation analysis in the *H. pylori*-positive and *H. pylori*-negative normal gastric mucosa. To clarify the sample numbers, we have corrected the sentence regarding the collection of tissue samples (lines 11-13 on page 8, “This study included 50 antrum and body pairs in *H. pylori*-negative cases with a mean age of 53.2, and 50 antrum and body pairs in *H. pylori*-positive cases…”).

Point 4. ‘1,040’ µl may be a misprint. Correction is needed:
We have substituted ‘1.04 mL’ for ‘1,040 µL’ (line 13 on page 9).

References


