Author’s response to reviews

Title: Comprehensive analyses of imprinted differentially methylated regions reveal epigenetic and genetic characteristics in hepatoblastoma.

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Author’s response to reviews: see over
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Professor Dafne Solera
Executive Editor
BMC Cancer

Dear Prof. Solera

Thank you very much for your consideration of our manuscript entitled, “Comprehensive analyses of imprinted differentially methylated regions reveal epigenetic and genetic characteristics in hepatoblastoma” by Rumbajan et al. (MS: 2038377211937522). We appreciate the helpful comments of the reviewers, which enabled us to substantially improve and revise our manuscript. Our responses are presented on the following pages, and we believe that we have addressed all the reviewers' comments.

The revised manuscript has been edited by a native English proofreader. The points changed from the first version are outlined as follows and indicated by red text in the manuscript. In addition, we added an author, Dr. Shigehisa Aoki, because he made a contribution to the pathological examination of hepatoblastoma samples.

Thank you for your consideration of our manuscript. We look forward to hearing from you.

Sincerely Yours,

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Reviewer #1

Major Comments

1. The authors conclude that hypermethylation occurred in gametic DMRs more frequently than in somatic DMRs (page 18, ll. 7-8). The P value should be clearly described here. More importantly, the authors conducted a lot of statistical tests in the study. Is the P value still significant even correcting this issue of multiple testing?

   We have described P values where significant differences are stated in the text (pp. 20-21) and presented in Figure 3. We have performed statistical analyses again and confirmed that the P values were still significant.

2. Figure 4. The authors analyzed methylation levels of four CpG sites of LINE-1 in the three sample groups. How did the authors select the four CpG sites? Are they shared by many variants of LINE-1? Also, were the methylation levels of the four CpG sites equal so that mixing-up the values could be justified?

   We have selected the four CpGs according to a personal communication with Prof. Yutaka Kondo, Aichi Cancer Center Research Institute, Japan. The CpG sites were located in the promoter region of LINE-1 and showed low mutation frequency in this region. The primers were designed to amplify the variants of LINE-1. We have added some references reporting the methylation analyses of these CpG sites to our citations. We again performed statistical analyses of the four individual CpG sites. Although CpG#1 demonstrated slight hypomethylation in tumors, other CpGs did not demonstrate such hypomethylation (bare hypermethylation in adjacent, normal liver tissue was seen at CpG#2 and #3). These results suggested that the methylation differences were not so large in the adjacent, normal liver tissue and the tumors. Based on this, we rewrote “Methylation status of LINE-1 in hepatoblastoma” in the Results and Discussion on pp. 21-23. We also updated Figure 4 accordingly.

3. The authors adopted a cut-off value of 0.15 for hyper- and hypo-methylation (page 11). This reviewer agrees that this value is mostly reasonable, but still depends on tumor cell contents. Did the authors confirm tumor cell contents in individual samples?
We agree that tumor cell contents in the samples are important. We have analyzed tumor cell contents by HE staining. All samples analyzed showed tumor tissues encased by a fibrous capsule. The average tumor cell content in the capsulized tumor tissues was approximately 70%. We think that this is sufficient for analyses. This result was described in “Samples” under the Methods section.

**Minor Comments**

1. The authors describe % values, but, since only 12 cases were analyzed, the values seem to have little meaning.

   We agree with your comment. We deleted the % values of 12 tumors, and, instead, we have reported such data as “7 of 12 tumors,” for example on p. 19.

2. Manuscript should be prepared more carefully. For example, the Abstract section starts from the bottom of page 4, and only a subsection title remains at the bottom of page 12. Gene names should be consistently italicized. A period after "Figure" should be removed throughout the manuscript.

   We prepared the revised manuscript very carefully according to the online instructions of BMC Cancer. All gene names were italicized and all the periods after “Figure” were removed.

3. Page 9. CL7 was obtained from a patient who died of cardiac arrest. If this was due to arrhythmia, it should be clearly described.

   We apologize for the wrong description of this patient’s cause of death. The cause of death was actually spinal muscular atrophy type I-C. We have corrected the cause of death of CL7 in “Samples” under the Methods section.

4. Page 14, l. 10. The authors state that eight regions were fully methylated and that five regions were fully unmethylated. Please specify the names of the loci.

   According to the suggestion, we have specified the names of the DMRs that were fully methylated or fully unmethylated on p. 16.
5. Page 15, l. 8. "LOH, UPD, or copy number abnormality". Local copy number abnormality includes LOH, and this phrase needs re-writing.

   According to the suggestion, we have changed “LOH, UPD, or copy number abnormality” to “…chromosome abnormalities, such as UPD or copy number abnormality” on p. 17.

6. Page 16, l. 8. "fifty-five percent" should be corrected to "55%" as in elsewhere.

   We have made the correction on p. 19

7. Page 16, l. 8. "of thirty-three DMRs" -> "of 33 DMRs"

   We have made the correction on p. 19.

8. Page 17, ll. 6-7. Although the authors describe that they compared hypomethylation and hypermethylation, they compared adjacent normal and tumors first and then compared the ways of occurrence between hypomethylation and hypermethylation. The sentence needs rephrasing.

   As the reviewer pointed out, this part (first paragraph in “Comparison of aberrantly methylated DMRs,” p. 20) was not well described. We have rewritten this part for clarity.

9. Page 18, l. 15. The authors should consider to cite the original paper, Takai et al, 2000.

   We have added the citation of Takai et al., 2000, on p. 21.

10. The discussion section is too long. It should be limited to topics directly related to the authors’ data.

    We have rewritten the Discussion section to make it simpler and shorter according to the reviewer’s suggestion.
11. The legend to Figure 2. AxC, TxC, and TxA should be explained not only in page 13 but also here.

According to this suggestion, we have described AxC, TxC, and TxA in the legend of Figure 2.
Reviewer #2

Minor Essential Revisions

1. The use of two well recognised methods to quantify methylation (MALDI-TOF and Pyrosequencing) results in high quality methods and quantification of methylation. The results for normal liver were surprisingly close to 50% for most DMRs. Are these the raw data or has it been normalised in some way? If it has been normalised this should be stated: if not congratulations on the precise data.

These are the raw data and no normalization was implemented.

2. I would have liked some more discussion around the hypomethylation that was detected in adjacent liver. Could this be a constitutional aberration as seen for H19 in Wilms tumor (Okamoto K et al PNAS 1997).

Thank you very much for pointing out the similarity between the result of Okamoto et al. (1997) and ours. We have added some discussion by referring to the result of Okamoto et al. on p. 23.

3. Could the authors summarise (or detail in full) the range of differences seen between the TxA, TxC and AxC pairs. I have no sense of whether there was a lot of technical variability, with a few pairs (as shown) exceeding the 0.15, or whether the 0.15 cutoff was a large difference relative to most pairs.

It is very difficult to summarize our methylation difference data in a consistent way for display in one simple figure or table because we used a specific definition for aberrant methylation as described in the manuscript. The methylation level of each CpG site or CpG unit within a DMR is usually not the same. Some sites/units demonstrate higher levels of methylation, while others demonstrate lower levels. The methylation data of a certain CpG site or CpG unit within a DMR is sometimes lacking probably due to DNA quality, insufficient amplification of bisulfite-PCR, or slight incompatibility of machines. This phenomenon appears on a case by case basis. These are reasons why we explicitly defined methylation, which was not a simple average of methylation. In addition, some samples had two kinds of methylation data, which were obtained by both MALDI-TOF MS and pyrosequencing; however, others
had one dataset obtained by MALDI-TOF MS alone. It was also difficult to summarize methylation data due to this situation.

To answer the reviewer’s comment, we prepared a Figure R1 for Reviewer #2. In this figure, we presented the methylation data of seven hepatoblastoma samples, which did not show aberrant methylation at \( RB1 \)-DMR, as a representative DMR (Figure 2 in the manuscript). According to our definition, the methylation data in HB02, HB03, HB04, HB06, HB07, and HB09 obtained by MALDI-TOF MS did not show aberrant methylation (Figure R1A). Therefore, these samples were not subjected to pyrosequencing, and, as such, we concluded an absence of aberrant methylation. As for HB08, MALDI-TOF MS data was assigned as hypomethylation in the TxA comparison. However, the pyrosequencing analysis did not confirm the hypomethylation (Figure R1B). Thus, this sample was categorized as normally methylated in Figure 2.

We are very confident that it is reasonable to use 0.15 as a cut-off value to define aberrant methylation for the reason described in “MALDI-TOF MS analysis” under the Methods section on p. 12. Reviewer #1 also agreed with our definition.

As you mentioned, there should be technical variability in our methylation analyses, although the two techniques used in this study are the most reliable methods for methylation analysis at present (Tost J, et al. Biotechniques, 2003; Ehrich M et al., PNAS, 2005; Claus R et al., Epigenetics, 2012). This intrinsic technical variability is the reason why we used two analytical methods to define the aberrant methylation of a sample.

We hope this is an appropriate response to this comment.

4. In the methods section, this sentence could be modified by adding in ‘which ranged from’. “Epityper software analysis of the signals gave the methylation index which ranged from 0 (no methylation) to 1 (full methylation)”

We made the suggested correction on p. 12.

Discretionary Revisions

5. in the abstract the statement “Hypomethylation was observed at certain DMRs not only in tumors but in adjacent normal liver tissue” is too vague for my liking. It implies widespread hypomethylation in adjacent liver tissue (which is clearly not normal based
on this data). It could be replaced with something like “Hypomethylation was observed at certain DMRs not only in tumors but also in a small number of adjacent histologically normal liver tissue samples”

We have made the suggested correction on p. 5.

6. In the abstract “Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry” could be abbreviated to the more familiar MALDI-TOF MS.

We have made the suggested correction on p. 4.

7. In the methods section the description of MALDI-TOF could be shortened by use of an appropriate reference.

In accordance with the above suggestion, we have shortened the description of MALDI-TOF on p. 12.
Reviewer #3

Minor Essential Revisions

1) Results; comparisons of aberrantly methylated DMRs; please add a comment regarding the BWS case; inclusion of this sample in the analysis of frequency of 11p15 epigenetic change in adjacent normal would bias the results as it would be known before commencement of the study that germline epigenetic alterations would be very likely therefore this case should be omitted from the 11p15 analyses.

   We absolutely agree with this comment. In fact, BWS109 was not included in the 11p15 analyses because the sample showed chromosomal abnormalities in this chromosomal region. All the samples with chromosomal abnormalities were omitted from the analyses of comparisons of aberrantly methylated DMRs. We described this on p. 17.

2) Figure 4 It would be more reasonable to plot the average MI for each control at each CpG (possibly including an error bar) rather than plotting each of the three measurements which gives the impression that more than 3 separate samples were used

   We have revised Figure 4 according to your comment and the comment of Reviewer #1.

3) Determination of genetic alterations (mostly described in legend to supplementary figure S4). Assignment of genetic alteration based on microsatellite marker allelic ratio is completely dependent on the assumption that the cell population was homogeneous (which in a non-microdissected, and chemotherapeutic treated tumour is rather unlikely). Unless the authors can demonstrate homogeneity an alternative (probably more likely) cause is a mixed cell population. For example the allelic ratio of 3 could be due to 3 paternal and one maternal copy (the 4 copies described by the authors), but could instead be due to a mixture of cells 1/3 of which have a normal karyotype and 2/3 of which have lost the maternal copy. Correct quantification of copy number requires a technique such as MLPA or Q-PCR. Unless the authors can provide a convincing evidence for this quantitation, they must remove these data or add qualifying comments to all of their descriptions of the genetic aberrations.
We agree with the reviewer’s comment. We do not have any data that supports three copies or four copies. We rewrote this part (see the first paragraph on p. 18) by using “abnormal allelic copy number” instead of “three copies or four copies.” In either case, we excluded these samples from further analyses because of their chromosomal abnormalities.

**Discretionary Revisions**

**Results; Analyses of aberrant methylation; last paragraph; please make some comment regarding the DMRs where the authors observed methylation not ~50%**

We suppose that this comment is not for the last paragraph but for the first paragraph. We believe that these regions were tissue-specific and/or developmental stage-specific DMRs because most of the regions were also analyzed by pyrosequencing and their full or no methylation were confirmed (Figure S1). We added this comment on p. 16.

**Figure2; I feel that it would aid the overall picture of gene dysregulation if the chromosomal rearrangements were also coloured to indicate the effect on methylation ie red with the TC label where the three copies caused an increase in methylation.**

Based on the following two reasons, we do not think that it is acceptable to present methylation data of samples with chromosomal rearrangements in Figure 2.

Firstly, for such samples, we analyzed their methylation status only by MALDI-TOF-MS because of their chromosomal rearrangements. Therefore, their methylation statuses were not confirmed by pyrosequencing. Secondly, we excluded such samples from the statistical analyses of aberrant methylation shown in Figure 3 because we focused on aberrant methylation caused by mechanisms other than chromosomal rearrangement.

We are concerned that it confuses readers to present such aberrant methylation data in Figure 2.

**Minor issues not for publication**

**Comment 1**

Background; paragraph 1 “are mutated” would be better as “are frequently mutated”
We have made the suggested correction on p. 8.

Comment 2
Background; paragraph 2, second part describing ICRs and DMRs is long and unclear and should if possible be rephrased

We rewrote this part (pp. 8-9) according to this suggestion; however, it could not be excessively shortened in the rewrite. We need to describe maternal and paternal DMRs and also gametic and somatic DMRs because we compared aberrant methylation between these DMRs.

Comment 3
Background penultimate sentence “We found” would be better “We therefore describe”

We have made the suggested correction on p. 10.

Comment 4
Abstract; Background “was reported” would read better “has been reported”

We have made the suggested correction on p. 4.

Comment 5
Methods; samples; first sentence; normal is duplicated, remove 1st occurrence

We have corrected this mistake on p. 10.

Comment 6
Methods; samples; 3rd sentence; could be rephrased for clarity to become Ten of the patients were treated......protocol (HB08 and HB09 were not).”

We made the suggested correction on p. 11.

Comment 7
Methods; MALDI-TOF; 5th sentence; “difference of methylation indexes in the two samples” (page 12, L. 2) would read better “difference of methylation indexes between the tumour and matched normal”
Because we compared the methylation indexes not only between tumor and matched normal tissue, but also between tumor and normal control tissue and between matched normal and normal control tissue, we could not change the sentence according to the comment. However, we changed “difference of methylation indexes in the two samples” to “difference of methylation indexes between the two samples” (p. 12) as per the reviewer’s comment.

Comment 8
Results; Analyses of aberrant methylation; paragraph 3 could be made clearer and more concise by appending copy numbers in brackets after the sample ID eg “four copies of 11p13-p15.5 in HB01 (3Pat and 1 Mat)”, and removing the following 3 sentences that describe the proposed parental origins in text.

We rewrote this part (p. 18) by deleting “four copies of 11p13-p15.5” and “three copies of 19q13 and 20q13” according to minor essential revision comment 3 of this reviewer.

Comment 9
Results; Analyses of aberrant methylation; last paragraph “aberrant met hylations” should read “aberrant methylation” “such as for copies, LOH and UPD” could be omitted.

We have made the suggested correction on p. 19.

Comment 10
Discussion; paragraph 2; last sentence “would be” would read better as “could be”

This sentence has been deleted because we have rewritten this paragraph to make the discussion shorter according to minor comment 10 of Reviewer #1.

Comment 11
Discussion; paragraph 3; 1st sentence; “showed frequently” would read better as “frequently showed”

We have deleted this paragraph in order to shorten the discussion in response to minor comment 10 of Reviewer #1.
Comment 12
Discussion 4th paragraph; 4th sentence; LOI should be omitted as it is a restatement of the preceding clause “biallelic expression”

We have made the suggested correction in the Discussion section (4th paragraph, 4th sentence) on p. 25 of the revised manuscript.
Reviewer #4

1. The sample set is on the small side and I would have liked to have seen more precision in the description of how hypo and hypermethylation is defined. Particularly in the figure legend for fig 1.

In addition to the description of the definition in the Methods section under “MALDI-TOF MS Analysis,” we also described the definition in the legend of Figure 1.

2. The conclusion that aberrant methylation profiles observed in adjacent normal liver tissue indicate that this implies that the methylation changes are therefore present prior to tumour formation is not supported by the data. Other reasons - eg. field effects such that the tumour may induce methylation changes in the adjacent tissue.

We agree with your comment. We have rewritten the first paragraph in the discussion concerning the hypomethylation in adjacent liver tissue according to your opinion.

3. Another comment is the genetic abnormalities observed are not causative of the epigenetic alterations, as the authors suggest. Change this sentence on p15 to "associated with".

We have made the suggested correction on p. 17.

4. The manuscript would be more readable by restructuring the introduction to give more information about what is meant by fetal and embryonal types of hepatoblastoma - this is pertinent to the data set and would help the reader understand why these data were collected and their relevance to the study.

We have added a description of fetal and embryonal types of tumor in the Introduction.