Author's response to reviews

Title: Epigenetic Alterations Differ in Phenotypically Distinct Human Neuroblastoma Cell Lines

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Author's response to reviews:

April 23, 2010

Nina Titmus
On behalf of Rachel Neilan
Scientific Editor
BMC-series Journals
RE: Revision of manuscript 1694332214342754

Dear Dr. Neilan,

As requested, we are submitting a revised version of our paper entitled, “Epigenetic Alterations Differ in Phenotypically Distinct Human Neuroblastoma Cell Lines” for consideration of publication in BMC Cancer. We have revised the paper according to the recommendation of the reviewer. Below I have outlined the specific modification that has been made in response to the request of the reviewer.

Response to the comments of reviewer: Debabrata Banerjee:

The mss is acceptable for publication provided the following minor revision is made prior to acceptance.

From the data provided for epigenetic effects following treatment with 5-aza-dC it is difficult to separate cytotoxic and or cytostatic effects from the observed effects. For example the methods section provides a range of doses and times for both 5-aza-dC and VPA. No rationale is provided for choice of drug dose of
4uM and a period of 24 h for many of the experiments/results presented. The Figures/legends for Fig 6 (where the treatment times are 7, 13 and 21 days at 10uM and 0.1uM 5-aza-dC) mention the particular doses and period of treatment for the various assays. The authors are encouraged to clearly justify and define treatment doses and period of treatment for all experiments.

Response: According to reviewer's suggestion, we added the sentences in the text on to clarify our rational for the doses and time periods that were selected for the experiments. Specifically:

On page 10, we have added the sentence, “This dose and time point have been shown to restore gene expression in NB cells [13].”

On page 12, we have added the sentences, “In a previous study, we showed that administration of the HDAC inhibitor valproic acid (VPA) changed gene expression in NB cells [6]. The cells were treated with 1 mM VPA for 2-48 h. Based on these results, we treated the tumorigenic LA1-55n cells with 5mM VPA for 1 day and investigated its effects on histone modifications and THBS-1 expression.”

On page 13, we have added the sentence, “We next assessed whether treatment with 5-Aza-dC would induce changes in the morphology of the N-type LA1-55n cells. For these studies, the cells were treated with 0.1 #M 5-Aza-dC, a dose that is not cytotoxic.” We also modified the sentence on page 13, “At a concentration of 10 #M, the ID50, the number of colonies was decreased by 95% compared to controls (p<0.001).”

Response to the comments of reviewer: Karin Klinga-Levan

Major compulsory revisions
1. The statistic methods used must be described. As it is now, the statistics is not possible to assess.

Response: We added the following sentences to the method section on page.9. Statistical analyses was performed using a two-tailed Student's t test. A p value of # 0.05 was considered statistically significant.

2. Table 1 must be revised. Even if it understood by most readers, why is Actin and MYOD1 included in the list? It would be a good idea to rearrange the table method wise.

Response: According to reviewer's suggestion, we have rearranged the table in an experimental order. We have added the following sentences on page 7 to clarify our rational for selecting actin and MYOD1 as controls. “Both #-actin and MYOD1 were used as internal reference genes. Primers of the #-actin and MYOD1 genes were located in an area without CpG nucleotides; thus, amplification of #-actin and MYOD1 by QMSP occurs independently of a CpG
island methylation status, whereas the amplification of THBS1, HIN1, TIG1, and CASP8 is proportional to the degree of cytosine methylation within their promoters.

3. The method part is confusing. It is difficult to follow in which experiment the different treatments are used. To add a table where the different treatments and experiments are described, may clarify the situation.

Response: We added the following new table (Table 1) on Page 24.

Table 1 Conditions for 5-Aza-dC and VPA treatments

<table>
<thead>
<tr>
<th>5-Aza-dC treatment</th>
<th>Experiment</th>
<th>5-Aza-dC (µM)</th>
<th>time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell proliferation</td>
<td>0.01, 0.1, 1, 10, 100</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>gene expression</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>methylation study</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ChIP assay</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>soft agar assay</td>
<td>0.5, 2.5, 10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>soft agar assay</td>
<td>0.1, 1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>morphology</td>
<td>0.1</td>
<td>14, 21</td>
<td></td>
</tr>
</tbody>
</table>

VPA treatment

<table>
<thead>
<tr>
<th>Experiment</th>
<th>VPA (mM)</th>
<th>time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChIP assay</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

4. As I understand, you are checking the expression of the genes in untreated cells. You have not described that in the method part.

Response: We have modified the leading sentence in the cDNA synthesis method section on page 5. The current sentence is, “RNA was isolated from untreated and 5-Aza-dC-treated LA1-55n and LA1-5s cells using Trizol reagent (Invitrogen).”

Minor essential revisions

1. Page 7 two rows below the heading "cDNA synthesis....." change to "reverse transcribe using........

Response: We made the suggested change.

2. Page 7 last row. The sentence that start with "data analysis.... should be changed to "In the data analysis the comparative method (##Ct) was used to

Response: We made the suggested change.
3. The table text must be more explanatory

Response: According to reviewer’s suggestion, we have rearranged the table in an experimental order. We also explained why we included actin and MYOD1 in the list.

Discretionary revisions
1. I think that the order in the methods description is a inconsistent compared the result part.

Response: We have rearranged the methods as suggested and have included cDNA synthesis methods prior to the methods describing DNA isolation.

2. It is very common to tell everything in the Background part. I do not like that, since it then will be less exiting to read the entire article. I would suggest to omit the text from "In the LA1-55n cells, we found high levels of....." until the end of the Background part.

Response: The sentences have been deleted according to reviewer’s suggestion.

Thank you for considering our manuscript for publication in BMC Cancer. We look forward to your review.

Sincerely,

Susan L. Cohn, MD
Professor and Director of Clinical Sciences
University of Chicago