Author's response to reviews

Title: 3-D DNA methylation phenotypes correlate with cytotoxicity levels in prostate and liver cancer cell models

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Author's response to reviews: see over

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Author's response to reviews: see over

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BMC Pharmacology and Toxicology

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RE: AN ELECTRONIC RE-SUBMISSION TO *BMC Pharmacology & Toxicology*

Dear Dr. Morrey,

Thank you for conducting the review of our manuscript entitled ” 3-D DNA methylation phenotypes correlate with cytotoxicity levels in prostate and liver cancer cell models” for the scientific journal *BMC Pharmacology and Toxicology*. We have enriched the revised manuscript with additional results requested by Reviewer 1 and made changes to the wording as suggested by Reviewer 2. We would appreciate if all changes are found acceptable in the process of the revised manuscript evaluation.

We also would like to thank the reviewers for their constructive comments and suggestions. We hope, we addressed all highlighted areas of concern. A detailed response (original reviewers’ quotes are italicized) is presented below:

Overall Remarks: Our main goal with this initially more cell biological manuscript was to deliver a global topologic picture of hypomethylation and its nuclear progression in selected sample cell lines in the context of zebularine and comparatively of 5-azacytidine treatment. This way we also intended to probe, whether MeC and gDNA/DAPI topology can be utilized as signatures in the assessment of cytotoxicity. We appreciate the opportunity for additional quantitative molecular reference analysis that strengthens our image-derived results. We feel that the revised manuscript can present more data with an overall higher confidence. Our results show that said parameters, especially global MeC reduction (nuclear DNA hypomethylation), synonymous with an increase in LIM densities towards the interior of the cell nucleus, can be positively correlated with an increase in hypomethylation of repeat elements (revealed by the MethyLight assay), chromatin reorganization in the nuclear space and cytotoxicity. We believe that based

on the data presented, which builds on the conceptual foundation of DNA methylation topology and its exploitation by 3D-quantitative methylation imaging (3D-qDMI, reported in previous publications) high-throughput MeC phenotyping of cells could be considered as a future candidate assay in epigenetic drug toxicology.

Reviewer #1 (Dr. Hyang-Min Byun):

1) Comment: *The manuscript title made the reviewer to expect new approach of three-dimensional imaging to measure and monitor DNA methylation level with effects of DNA methylation inhibiting drugs. However observing DNA methylation with 5-methylcytosine antibody with DAPI staining is not novel, further more dose-dependent effects of Zebularine and Azacitidine in DNA methylation inhibition, growth reduction, and cytotoxicity have been widely studied in other papers.*

Answer: We would like to refer to the overall comment above. We have published the cytometric concept of our approach as proof-of-principle. In here we are probing our topologic approach/method in correlation with cytotoxicity and the first-time combination of two image analysis modalities for which we have developed the necessary algorithms for process automation: global MeC and gDNA colocalization, and LIM/LID density measurements. The correlation with the requested quantitative molecular analysis (MethyLight, below) has added innovative possibilities for cross-correlation of the two genres of data.

2) Comment: *Another problem with this work has to do with interpretation of the estimate of DNA methylation level. I'd be interested to know how 'Normalized MeC mean intensity' convert to global % methylation in other common techniques. If the authors named '3D quantitative DNA methylation imaging', then it needs to be validated the quantitation with other techniques.*

Answer: We appreciate the constructive comment of Dr. Byun. In this context, we have recruited a modified version of MethyLight, developed by and applied in collaboration with Dr. Daniel Weisenberger and colleagues at the USC Epigenome Center (Los Angeles). MethyLight is a validated quantitative method that robustly analyzes global DNA methylation status of the three most prevalent types of repeat elements in the human genome (Alu, Sat2, and Satα), which comprise the majority of heterochromatic and intergenic regions and are highly methylated in normal cells. Weisenberger et al. (2005, References #55 in revised) have demonstrated that this modified MethyLight assay (in combination with bisulfite conversion) relies only on real-time PCR and can provide surrogate markers for estimating global DNA methylation levels, validated by HPLC, conventionally used for said purpose. An advantage of MethyLight is its much less requirement of starting material compared with HPLC. The comparative analysis was made for Huh-7 cells treated with a series of different 5-azacytidine concentrations. The DNA hypomethylation trends with MethyLight were in agreement with results obtained with 3D-qDMI. High correlations were found between normalized nuclear MeC intensities of with the DNA methylation levels of all three repeat sequences: R = 0.96, 0.89, 0.86 for Alu, Sat2, and Satα, respectively, as displayed in the new Table 1 of the

revised manuscript. Dedicated text and references regarding this matter have been added to all relevant parts of the manuscript.

3) Comment: *Another thing to point out is that chromatin conformation with DAPI is not very convincible. I would suggest using co-localization with other heterochromatin markers, such as HP1#.*

Answer: Following this suggestion we have performed dual-color immunofluorescence with a series of 5-azacytidine-treated Huh-7 hepatocarcinoma cells, in which the cells were simultaneously stained for gDNA (DAPI), and H3K9me3 (histone 3 – lysine 9 –tri-methylated). H3K9me3 is associated with chromatin compaction in heterochromatin and therefore being recognized as a heterochromatin marker (Cowell et al., 2002) that recruits the heterochromatin protein 1 (HP1) in the process of heterochromatin formation/compaction (Lachner et al., 2001). Dedicated text and references regarding this matter have been added to all relevant parts of the manuscript.

Minor essential revisions: According to Dr. Byun's suggestion, we have made changes in the revised version also to render abbreviations more consistent throughout the manuscript.

Reviewer #2 (Dr. Samir Patra):

1) Comment: *The enthusiasm is dampened in many ways, may be due to lack of basic understanding of DNA-methylation epigenetics, reversible enzymatic reactions, and overall transparency of 3-D image processing tool.*

Thus starting from abstract there are many confusing sentences and erroneous, faulty statements.

For example the first sentence, "The effect of DNA methyltransferase inhibitors on higher-order genome organization and nuclear architecture is not well examined".

In fact there are many papers published with biochemical and biophysical evidences.

"Demethylation effects were modeled by comparatively dosing zebularine and 5-azacytidine".

This should be, "hypomethylation ---" Because zebularine and 5-azacytidine can't do any demethylation. Please see, Patra SK and Bettuzzi S (2009) Biochemistry (Moscow) paper for basic concept.

Answer: The term higher-order genome organization pertains to chromatin structure beyond the 30 nm fiber and its spatial distribution within the nucleus. With regard to that,

there hasn't really been published much sofar, especially not in conjunction with DNA methylation topology. And the most cited articles together with work done by our group had been already mentioned in the first submitted version of our manuscript. Here, we present a cell-by-cell topology-related study, in which we have extensively documented the concurrence of differential nuclear patterns of 5-methylcytosine and global DNA in response to two DNMTi, for which a number of publications that detail enzymatic mechanisms and molecular profiles already exist. Therefore our notion was not to repeat those experiments and present another biochemical study, but rather probe the application of 3D-qDMI – for which the concept has been comprehensively described and acknowledged by experts in epigenetics, image analysis and Cytometry (see Gertych et al. 2009, Gertych et al. 2010, and Tajbakhsh et al. 2011 in manuscript) – towards correlations of differential 3D codistribution patterns of MeC and gDNA and cytotoxicity in a more comprehensive fashion: with two different cell lines, with the combined battery of updated modules of the in-house developed algorithms. In this manuscript, we report the results of our comprehensive study for the first time.

Along these lines we would like to emphasize that generally, the application of 3D imaging and dedicated 3D image analysis is novel on this matter. There have been attempts made to describe nuclear patterns of MeC and gDNA in conjunction with epigenetic drugs (that we have referenced in the manuscript). However, these studies utilized 2D imaging and analysis at most. In our previous related publications we have demonstrated that spatial information regarding distribution of nucleic acid targets have an advantage over 2D measurements and provide more differential results for in situ topological measurements. In addition, the revised co-presentation and correlation of imaging-derived information on global MeC and DNA together with relevant sequence-based molecular data of repeat sequences introduces another novel aspect towards convergence of the two types of DNA methylation data.

We agree with Dr. Patra regarding the mechanisms of the DNMTi that we applied in our study. However, the term 'demethylation' can be used in combination with the word 'effect(s)' (as demethylation effect) without necessarily implying the nature of the underlying mechanism of the drug itself. In this sense, DNA demethylation can be interpreted as a downstream causal effect of drug application, which can lead to the status of DNA hypomethylation. Nevertheless, for the sake of solving semantic issues on the use of terms that may imply a more mechanistic background rather than sole observations, we have replaced the word "demethylation" with "hypomethylation" throughout the manuscript according to the reviewer's recommendation. Also, the word "modeled" (in the Abstract) was replaced by "studied" in conjunction with drug effects.

2) <u>Comment:</u> *I am confused how one can directly correlate the following results and conclusions??*

Results
Treated and untreated (control) human prostate and liver cancer cells (DU145, Huh-7) were analyzed by confocal scanning microscopy and dedicated 3D analysis software for the following phenotypic features: differential nuclear MeC/DAPI load and codistribution patterns, cell similarity based on these patterns, and corresponding differences in the

topology of low-intensity MeC (LIM) and low in intensity DAPI (LID) sites. Both agents generated a high fraction of similar MeC phenotypes across applied concentration ranges. Zebularine exerted similar effects at 10-100-fold lower concentrations than its analogue: concentration-dependent progression of global cytosine demethylation and the concurrent increase in nuclear LIM densities correlated with cellular growth reduction and cytotoxicity.

Conclusions
3D-qDMI is capable of monitoring dose-dependent topological demethylation of drugs in cell nuclei. MeC/DAPI patterns are in contrast to individual nuclear MeC and DAPI load measurements largely independent from interphase cell cycle stages. The results indicate towards DNA methylation topology possibly serving as a robust sentinel for demethylating drug impacts on chromatin conformation with a conceivable application in epigenetic drug toxicology.

Answer: The additional molecular data on differential drug-induced levels of repetitive DNA hypomethylation also support the imaging-derived evidences for 3D-qDMI being capable of reporting drug-induced and dose-dependent global DNA demethylation in a quantitative fashion. If the conclusions seem to be a bit strong, we have made changes to make them sound less rigid, without changing the above fact. Besides the reviewer is citing the Abstract, which is an ultra-succinct version of the main text body, so the causal flow appears abrupt. The new conclusion part reads: *'3D-qDMI demonstrated the capability of monitoring dose-dependent drug-induced spatial progression of DNA hypomethylation in cell nuclei, independent from interphase cell- cycle stages and in conjunction with cytotoxicity. The results support the notion of DNA methylation topology being considered as a potential indicator of causal impacts on chromatin organization with a conceivable application in epigenetic drug toxicology.'*

3) Comment: *Main body of the manuscript: Background section: Second sentence is not correct. and so on ---------- It needs a major modification and thorough revision for comprehensive reading Referencing: many original works on prostate cancer are not cited*

1. Patra A, Deb M, Dahiya R and Patra SK (2011) 5-Aza-2'-deoxycytidine stress response and apoptosis in prostate cancer. Clin Epigenet, 2: 339-348

2. Patra SK and Bettuzzi S (2009) Epigenetic DNA-(Cytosine-5-Carbon) Modifications: 5-Aza-2'-Deoxycytidine and DNA-Demethylation. Biochemistry (Moscow), 74 (6): 613-619.

3. Patra SK, Patra A, Zhao H and Dahiya R (2002) DNA methyltransferase and demethylase in human prostate cancer. Molecular Carcinogenesis, 33(3): 163-171.

4. Patra, S. K and Szyf, M. (2008) DNA methylation mediated nucleosome dynamics and oncogenic Ras signaling: insights from FAS, FASL and RASSF1A FEBS J, 275:5217-5235

5. Patra SK, Patra A, Rizi F, GhoshTC and Bettuzzi S (2008) Demethylation of (cytosine-5-C-methyl) DNA and regulation of transcription in the epigenetic pathways of cancer development. Cancer Metast. Rev. 27(2): 315-334

Answers: a) We changed the second sentence to: "Cancer cells frequently exhibit abnormally high levels of DNA methylation in gene-specific CpG-rich promoter regions [2–5]". b) Our manuscript does not aim at presenting a prostate cancer-specific topic. We only used DU145 prostate cancer cells along with the Huh-7 hepatocarcinoma cell line as cellular models in our study, as we are familiar with those types of cells. We apologize that we may have overseen relevant art in this explosion of publications in epigenetics. We have added a more recent research article of Dr. Patra (from the above list) with relevance to our manuscript in the revised version: Patra A, Deb M, Dahiya R and Patra SK (2011) 5-Aza-2'-deoxycytidine stress response and apoptosis in prostate cancer. Clin Epigenet, 2: 339-348.

We hope that the answers above, together with the changes made in the revised version of the manuscript, could provide clarification to the raised concerns.

Sincerely,

Jian Tajbakhsh (Senior author)