Author's response to reviews

Title: DNA methylation analysis reveals distinct methylation signatures in pediatric germ cell tumors

Authors:

James F Amatruda (james.amatruda@utsouthwestern.edu)
Julie A Ross (rossx014@umn.edu)
Brock Christensen (Brock.Clarke.Christensen@dartmouth.edu)
Nicholas J Fustino (FustinNJ@ihs.org)
Kenneth S Chen (kenneth.chen@utsouthwestern.edu)
Anthony J Hooten (hoot0006@umn.edu)
Heather Nelson (hhnelson@umn.edu)
Jacquelyn K Kuriger (jkuriger@umn.edu)
Dinesh Rakheja (dinesh.rakheja@utsouthwestern.edu)
A Lindsay Frazier (Lindsay_Frazier@DFCI.HARVARD.EDU)
Jenny N Poynter (poynt006@umn.edu)

Version: 4 Date: 3 March 2013

Author's response to reviews: see over
February 28, 2013

Dr. Alistair Reid
Editorial Board
*BMC Cancer*

Dear Dr. Reid,

We would like to thank the reviewers for their careful review of our manuscript and many helpful suggestions. We have now prepared a revised manuscript in response to the reviewers’ comments. Below, we provide a detailed point-by-point response to the reviewers’ comments. Thank you very much.

Sincerely,

James F. Amatruda, MD, PhD

Reviewer’s report

**Title:** DNA methylation analysis reveals distinct methylation signatures in pediatric germ cell tumors

**Version:** 3  **Date:** 24 November 2012

**Reviewer:** Yasuhiko Kaneko

**Reviewer’s report:**
The authors studied methylation status of cancer-related genes and 5 imprinted genes in 51 pediatric germ cell tumors, and stated that they found distinct methylation signatures. Although their findings are mostly confirmatory, they usefully extend the previous findings on the epigenetic characteristics of pediatric germ cell tumors

Major Compulsory Revisions:
I have the following concerns:
The lengthy discussion should be shortened, and should focus on the subject relevant to their findings.

*We have shortened the discussion following the guidance of the reviewer.*

Examples of irrelevant discussion:
The fifth paragraph of discussion: “In a pathway analysis------for patients with GCTs.” The authors did not study expression of the stem cell marker genes in their GCTs, and the expression studies are mandatory to discuss the relationship between the aberrant expression of stem cell markers and germ cell tumorigenesis.

*As requested, we have deleted this paragraph.*

The sixth and eighth paragraphs: “Our results ------malignant teratoma.” And “Survival rates------that could be used.” The authors did not provide the data on survivals. Because greater than 95% of children with GCT are cured by the present therapy as they described in the discussion, it is very difficult to identify the prognostic markers. In addition, the fifth reference in this paper stated that methylation of tumor suppressor genes did not affect the outcomes of patients with yolk sac tumor. Thus, the paragraphs may be useless if they do not provide their own data on outcome and the methylation status of the genes.
As requested, we have deleted the sixth and eighth paragraphs.

Table 4: The numbers of YSTs and germinomas in Table 1 differ from the numbers of respective tumors in Table 4. The authors should describe the reason of the discrepancy.

The pyrosequencing analyses were conducted only on the samples from CHTN while the GoldenGate analyses included samples from CHTN and Children’s Medical Center. We have clarified this in both the methods and results sections of the paper.

Thirteen YSTs in Table 4 should be classified into male and female tumors, and the respective data should be provided, because the methylation status of the imprinted genes depends on the sex of the host in whom tumors develop.

As requested, we have stratified the analysis by sex. In addition to the YST, we also separated the extragonadal teratomas into male and female categories.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests: I declare that I have no competing interests.

Reviewer’s report

Title: DNA methylation analysis reveals distinct methylation signatures in pediatric germ cell tumors

Version: 3 Date: 23 January 2013

Reviewer: annie huang

Reviewer’s report:
Review of “DNA Methylation analysis reveals distinct methylation signatures in pediatric germ cell tumors” by Amatruda et al. Pediatric germ cell tumours are a heterogeneous group of tumours with a spectrum of clinical behavior. Survival of children with pure germinomas and teratomas treated with current approaches is excellent; however the behavior of mixed germ cell tumours including teratoma with immature features may be unpredictable. In this study, the authors apply global array based methylation profiling to examine 51 pediatric germ cell tumours which include germinomas, Teratomas and Yolk Sac Tumors. Using an informatics based approach they define 7 molecular sub-classes of GCT with distinct methylation signatures that largely segregate with tumour histology. They identified 233 CpGs with increased enriched methylation in YSTs and show through pathway analyses that these map to loci with functions in ESC differentiation and WNT signaling. They demonstrate using comparative methylation analyses of a small sub-group of tumours with matched normal tissue, that target loci identified in YSTs were tumour specific changes. They used a similar approach to examine methylation signatures in mature and immature teratomas. Although they did not find a correlation of methylation signature with teratoma histology, they identified a subset of differentially methylated CpGs that were significantly hypomethylated in mature versus immature teratoma. Notably, a number of pathways were related to stem cell biology.

General comments:
Although similar analyses have been reported (references 5 and 9), this study has the potential to corroborate and extend prior findings that may enable more precise distinction of GCT sub-types and predicate clinical behavior specifically of tumours classified as mature and immature teratomas. However, although the authors set out to investigate the molecular differences between mature and immature teratoma, they have not exploited their data to fully answer this important question which has the potential to make their study novel.

Major revisions:
The manuscript in its present form needs more detailed and robust data analyses as well as corroboration of methylation studies with gene or protein expression data.

We have added an additional figure describing the cluster analysis and we have added expression data for a select number of genes.

Specific comments:
1. Figure 1 – its not clear whether an unsupervised or supervised cluster analyses was performed on the tumour cohort. If the analyses is unsupervised then, a dendrogram in figure A would have been very helpful to assess the specific relationship of each tumour to the tumour molecular sub-groups. The authors indicate that they identified 8 sub-classes of GCT (only seven are shown in the Fig 1). They report an interesting sub-set of genes that define the YSTs, however the data could have been significantly strengthened by correlating significance of differential methylation with gene or protein expression data.

The analysis was performed using unsupervised clustering. This was included in the statistical methods section but we have also clarified this in the results section. We have added a figure showing the dendogram for the unsupervised clustering. The RPMM analysis (also using unsupervised clustering) has been moved to figure 2.

2. The authors observed discrepancies in molecular and histologic classification of the teratomas. This potentially very interesting finding is not fully explored.

Was tumour histology re-reviewed after the molecular analyses.

Tumor histology was reviewed prior to the molecular analyses by a pediatric pathologist with expertise in germ cell tumors. This has been clarified in the methods section.

The authors performed comparative analyses of methylation signatures of the mature versus immature teratomas – its not clear whether these were performed on histologic sub-groups or molecular sub-groups and may explain the lack of significance in their comparative analyses.

The tumors were classified into histologic subgroups based on pathology review for all analyses. We have clarified this in the text.

Finally, it is very interesting that Sox2 – a pluripotency gene is differentially enriched in pathways analyses of immature versus mature teratomas. Corroboration of these findings at the gene or protein expression level may potentially provide a diagnostic marker to distinguish or identify mature teratomas with foci of immature cells that may not be readily detected by routine morphology.

We thank the reviewer for bringing out this interesting point. We have added qPCR data for SOX2. We did see a difference in SOX2 expression by tumor histology overall, although the expression difference for the two groups of teratomas was not significant. Surprisingly, the mature teratomas had higher expression of SOX2 than the immature teratomas.

Minor Essential revisions:
The manuscript could also be significantly enhanced by careful editing. Several discrepancies in numbers of tumours or sub-groups analysed are noted.

We apologize for the errors and we have edited the paper more carefully.

The authors indicate that 5 CpG Loci with significant differences by tumor histology (q value <2.2E-16 and fold change >2.50) were selected for validation by pyrosequencing. Three of the loci selected appeared in table 2, which lists the top 23 loci with the most significant Q values and fold changes. The remaining loci are not among the top 23 listed in Table 2. Table 2 should include all loci with >2.50 fold change if they are referring to these values.
We chose to restrict Table 2 to loci with fold changes > 2.75 for brevity. Adding loci with fold changes > 2.5 would add an additional 24 CpG loci to the table. The two loci in the validation set with fold change between 2.5 and 2.75 were chosen for technical reasons as we had assays developed in our lab for these loci.

In the 4th paragraph, the authors are unclear as to the genes mentioned, whether they were found in the study or only in IPA. What is the significance of these genes are there promoters regions hyper or hypomethylated? If no analysis is being performed on this gene lists, perhaps it should be moved to the discussion section of this paper.

The IPA analysis comparing YST with other histologic subtypes included only CpG loci with upregulated methylation (>1.0 fold increase) based on the GoldenGate data. We have clarified this in the results section and indicated that 9/15 of the genes in the list had >2 fold increased methylation in YST.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Needs some language corrections before being published
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.