Author’s response to reviews

Title: Genome-wide analysis reveals the association between alternative splicing and DNA methylation across human solid tumors

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Author’s response to reviews:

Dear Editors,

Thanks a lot for having reviewed our revised manuscript (Manuscript ID: MGNM-D-19-00004R1, Title: "Genome-wide analysis reveals the association between alternative splicing and DNA methylation across human solid tumors"). We would like to express our sincere thanks to the Editorial Office and the reviewers for the constructive and positive instructions and comments. We are delighted to resubmit a recently revised version of the manuscript which was strictly revised according to the comments.

Attached please find following files.
1. Point-by-point response.
2. Revised manuscript text-highlighted in yellow.

Thank you for your further consideration of this manuscript. We look forward to your response.

Yours sincerely,
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Point-by-point response
Thomas Fleissher (Reviewer 1): The authors have addressed all of my comments in a satisfactory manner. I think the manuscript has improved a lot, and I hope the authors agree too.
Response: Thanks a lot for your constructive and positive instructions and comments.
Reviewer 2 (Reviewer 2):
1. The response is not clearly described. For most concerns/comments, the authors simply replied they did or revised. It is common that the authors should describe what the methods they used and the similarity or difference of the new results after revising.
Response: Thanks and we have depicted more detailly in the following responses.

2. For my point 1, review your response: "we performed permutation tests in revised manuscript, and we found similar results with the previous one." Please specify what permutation tests you used and what "similar results" they are. And your Table 2 seems to report both splicing and methylation on one gene in specific cancer type. If so, my comment in the previous version is that whether the survival results from your joint analysis of splicing and methylation are novel findings, rather than that such survival significance can be reached by using splicing or methylation data only.
Response: Thanks. In present study, we carried out permutation test on the obtained correlation coefficients to infer their statistical significance. The number of permutations was 1,000 and FDR<0.05 was considered as statistically significant. The aovperm() function in R package “permuco” and spearman_test() function in package “coin” were used for permutation test. We have depicted it in the Method section (Page 8, paragraph1). After permutation test, 869 CpGs were found to be highly correlated with 465 cancer-specific AS events (FDR <0.05)(Additional file 1:Table S4). Before constructing the permutation test, we found 710 CpG sites and 442 cancer-specific AS events showed significant associations.

The survival significance can be reached by using splicing or methylation data separately. After adjusting for age, gender, TNM stage and adjuvant therapy, results showed that 44 AS events in 41 genes could significantly distinguish patients with longer versus shorter survival prognoses (Table 2). We then examined the genes containing cancer-specific AS events, and found the majority of them (75.6%, 31/41) were not significantly associated with survival, suggesting that these AS events are partially influenced by their gene expression (Table 2). (Page13, paragraph3)

3. For my point 2, please specify "what the most splicing event were not biased by their genes themselves." For scientific writing, be accurate and be specific.
Response: Thanks for your comments. Because splicing of some introns is regulated under a co-transcriptional mechanism, an alteration in gene expression may affect alternative splicing of corresponding genes. Thus, we examined the genes containing cancer-specific AS events, and found that majority of them (75.6%, 31/41) were not significantly associated with survival, suggesting that these AS events are partially influenced by their gene expression (Table 2). (Page13, paragraph3)

4. For my point 3, please at least briefly summarize the splicing analysis from previous work, like how many splicing events reported, and how they are clinically relevant.
Response: Thanks a lot. The TCGASpliceSeq database has loaded 33 tumor types, and contained PSI values for 80,000+ splice events on 19,036 genes for TCGA samples [1]. However, in the previous work, the authors did not evaluate the clinical usage of alternative splicing events, such as the potential prognostic signature for cancers.

5. Point 4, thanks for the explanation. Then, please add such information in the manuscript for justification of the parameter. This is common way to describe the scientific analysis.
Response: Thanks for your valuable comments. We have added this information in the discussion section as “we analyzed the association between cancer-specific AS events and CpGs at their exon boundaries. The parameter selected in present study was based on previous reports[36-38], especially Castle’s study, in which they examined the exon neighborhoods in the size of 200 nucleotides for intronic regions and 39 nucleotides for exonic regions to identify splicing cis regulatory elements in sequences [29]. ”(page 15, paragraph 1)

6. The authors need to enhance the manuscript in more scientific report style. It has to be accurate, not general difference. Like my comments above, and a few more examples here: "the hypomethylation was more frequently observed in tumor tissues”. - you need to specify how "more frequently”? just one case here. And if you used the data from other sources with parameter setting, please report how many you obtained. For example, the number of AS events on page 6.
Response: Thanks. We have added the accurate number as your suggestion in Abstract section “the hypomethylation was more frequently observed in tumor tissues (51.3%)” (Page 3, line 9). We also revised other sentences in a more scientific style and highlighted in yellow. For the alternative splicing events, we have also added the parameters we used as “In present study, AS events (379,749 in total) that have PSI values in more than 75% samples were included.”(Page 6, paragraph 2)

7. English writing still needs enhancement. Just some examples in one page: "We then extracted data of methylation levels on CpGs harboring boundaries of these alternative spliced exons from TCGA data, and compared DNA methylation in 641 matched tissues." "Data collections". "Information of somatic mutations, copy number variations and DNA methylations was downloaded from …"
Response: Thanks for your valuable comments. Revised accordingly after a careful proofreading. We have revised the sentence “We then extracted data ….” Into “Then, the methylation levels of CpGs in the boundaries of alternative spliced exons were compared between 641 normal and tumor samples from TCGA.”. “Data collections” into “Data Acquisition” "Information of somatic mutations, copy number variations and DNA methylations was downloaded from ….” into “Information of somatic mutations, copy number variations and DNA methylations were downloaded from ….”(Page 6). We have also proofread other parts of our manuscript and highlighted in yellow.

8. Page 8, please specify the source of "ClusterProfiler" package in R software."
Response: Thanks. We added a reference (references 30) in page8, last paragraph for “ClusterProfiler” package.

9. Survival analysis: do you need to consider covariate of "drug history”? Methylation alternation may be associated with drug or diet treatment.
Response: Thank you so much for your suggestion. We have adjusted for the “adjuvant therapy (including chemotherapy and radiotherapy)” in survival analysis. The results showed that 44 AS events in 41 genes could significantly distinguish patients with longer versus shorter survival prognoses (Table 2). We also evaluated CpGs at the 44 survival-related AS exon boundaries and found 16 CpGs that had statistically significant differences (Table 2).
10. Figures are barely readable. For Figure 5, it is not sure how important to be included as the main findings. The PPI analysis seems premature.
Response: Thank you for your valuable comments. We tended to use the PPI analysis to explore connectivity network for the full understanding of biological phenomena for methylation-associated cancer-specific AS events. However, we only found statistically significant networks in COAD and LUSC. According to your suggestion, we moved the results of PPI analysis into Additional file 3: Figure S2 and GO analysis into Additional file 1: Table S5.

11. For KIRC, it has been well known many variants have survival outcome, when compared to other cancer types. You may add some discussion.
Response: Thanks. We have added “Renal carcinoma has various histological and molecular subtypes, and is characterized by poor prognosis and high recurrence. Until now, several molecular biomarkers have been investigated for renal carcinoma, while none of them have been used in clinical practice. Therefore, identification of novel and effective prognostic biomarker is important for patients suffering from renal carcinoma. The signatures identified in present study in renal cancers were not well reported previously, and could provide the basis for further study into the pathomechanisms and served as potential therapeutic targets.” in the Discussion section (Page 16, paragraph 3).