

## Cover Letter

Dear Editors and Reviewers,

Thank you very much for your efficient work and thank you for your letter and comments. Accordingly, we have revised the manuscript entitled *"Identification of Differentially-Expressed Genes Regulated by Methylation in Colon Cancer Based on Bioinformatics Analysis"* (Manuscript NO:47414), and would like to resubmit it for your consideration. We have addressed the instructive comments raised by the reviewers, and the amendments are highlighted in yellow in the revised manuscript. Point by point responses to the reviewers' comments are listed for your consideration. We would like to express our sincere thanks to the editors and reviewers for the constructive and meaningful comments.

We are so grateful that you have offered us this opportunity to resubmit our manuscript. We hope that the revised version of the manuscript is now acceptable for publication in your journal.

**Please address correspondence to:** Dong-Qiu Dai, Professor, Chief Physician. Department of Gastrointestinal Surgery and Cancer Center, the Fourth Affiliated Hospital of China Medical University & Cancer Research Institute of China Medical University, Shenyang 110032, Liaoning, China. E-mail: daidq63@163.com.

We shall look forward to hearing from you at your earliest convenience.

Yours sincerely,

Dong-Qiu Dai

## Response to Reviewers

### Reviewer # 1 (Number ID: 03001816)

**Comment 1:** This is an excellent study. I find no significant problems. There are a few minor text formatting that can be dealt with in copy editing. I therefore recommend acceptance of this manuscript, which provides new information on the role of methylation in color cancer.

Response 1: Thank you very much for your meaningful comments and we are fully appreciative for your understanding and approval to our research. Hopefully, this may address your concerns.

### Reviewer # 2 (Number ID: 00044333)

**Comment 1:** The authors presented well-organized analysis on differentially-expressed genes regulated by methylation in colon cancer by utilizing bioinformatics tools like GO and KEGG pathway, GSEA and PPI, using TCGA public data. Especially, detailed analysis were performed on GDNF and RELN, including survival analysis. Minor typo: In 'MeDEGs related to the prognosis of GC patients' of Results, (Figure 5A, B) --> (Figure 4A, B) In legend of Figure 4, gastric cancer --> colon cancer

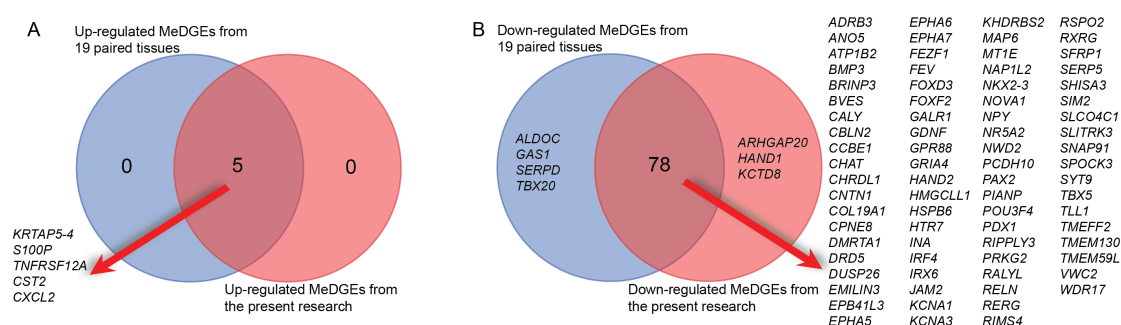
Response 1: We are so grateful for your meaningful comments, and we fully agree with you and apologize for the mistakes. Accordingly, we have corrected them in the manuscript. Hopefully, this may address your concerns.

### Reviewer # 3 (Number ID: 00070916)

**Comment 1:** It is of highest importance to compare the presented data from the "303 tumor tissues and 19 normal tissues" with the results of the subgroup analysis of the pairs of the 19 normal tissues + matching tumor tissues from the very same patients. Only if there is no major difference, the overall results can be considered as reliable. Alternatively, (or preferably in addition), the strategy should be used to analyse a second independent set of data (for example from the clinic of the authors).

Response 1: We are grateful for your meaningful comments, and we fully understand and agree with you. According to your opinion, we collected and constructed an RNA matrix and methylation data matrix that including 19 normal tissues and corresponding colon cancer

tissues using PERL software. Methylation-regulated differentially-expressed genes (MeDEGs) were identified in the same method as described in our research. As shown in the figure below, the up-regulated MeDEGs that obtained from two comparisons are totally overlapped. In addition, most of down-regulated MeDEGs were also overlapped. Therefore, we believe that the analysis results from our research are reliable and valid. Currently, the application of Illumina Human Methylation 450K BeadChip is still in exploration phase and we would perform further research to analyze the samples from our hospital in the future. We are fully appreciative for your understanding and approval to our study. Hopefully, this may address your concerns.



**Comment 2:** A short look into PubMed makes clear that this analysis is not the first of his kind (see also minor point 3). Thus, it would definitely help to put a little effort into "making a difference" - for example is the pure listing of enriched pathways not of relevance for most researchers. Identifying genes with roles in several cancer-related pathways would for example be of higher interest.

Response 2: We are grateful for your meaningful comments. In the present research, we aimed to identify the MeDEGs and confirm the role of methylation in colon cancer. Base on KEGG pathways analyses, multiple cancer-related pathways including “transcriptional misregulation in cancer”, “cAMP signal pathway” and “cGMP-PKG signaling pathway” were enriched. However, several MeDEGs that involved in cancer-related pathways have not been reported regulated by methylation and we would perform further research in the future. Hopefully, this may address your concerns.

**Comment 3:** The part of the discussion dealing with MLH1 methylation is really bad. It is clear that this is a prerequisite for microsatellite-instability also associated with aberrant general methylation - among other features. Together with the non-MSI CpG island methylated CRCs, the MSI+ CRCs will be most likely represent the hypermethylated cases. This point has to be made clear to the readers showing the molecular features of the CRC cases included into the final analysis.

Response 3: Thank you for your meaningful comments, and we fully understand and agree with you. We have revised it according to your suggestion (See in the “DISCUSSION” section). We hope this may address your concerns.

**Comment 4:** In line with this argument goes my comment on the overall results concerning GDNF and RELN: if my understanding is correct, a big part of the better prognosis for the CRC cases with hypermethylation might be contributable to the fact that this is a side-effect of MSI - and there, it has been clearly shown that the reason of better prognosis lies in the hypermutated state which has not much and not directly to do with the hypermethylation - a co-incidence and not a correlation. This might be wrong - thus, the authors should adjust for MSI when performing the survival analysis (Figure 4).

Response 4: Thank you for your meaningful comments. We performed Kaplan–Meier’s method and the log-rank test only to analyze the relationship between methylation and overall survival. As shown in Figure 4, hypermethylation group and hypermethylation low-expression MeDEG group was related to poorer prognosis. However, Illumina Human Methylation 450K BeadChip that we analyzed in the present research did not supply the data of MSI. Thus, it is difficult to reveal the relationship between MSI and prognosis in our research. This is the limitation of our study and we will focus on the relationship between MSI and methylation of GDNF and RELN in future research. We hope this may address your concerns.

**Comment 5:** The discussion would clearly benefit from a reduction in the amplitude and more focus. For example: "Vodenkova et al. [50] indicated that “base excision repair” capacity is a potential prognostic biomarker, applicable for prediction of therapy response. The “cell cycle” pathway is a critical mechanism in regulating cell proliferation." is not really giving information to the reader.

Response 5: Thank you for your meaningful comments, and we fully understand and agree with you. We have revised it according to your suggestion (See in the “DISCUSSION” section). We hope this may address your concerns.

**Comment 6:** In the abstract, MeDEG should be introduced properly - "Functional analysis of MeDEGs" is misleading, since there are no experiments described. Maybe "In silico functional analysis of MeDEGs" fits already better.

Response 6: Thank you for your meaningful comments, and we fully understand and agree with you. Accordingly, we have revised it according to your suggestion (See in the “MATERIALS AND METHODS” section). We hope this may address your concerns.

**Comment 7:** In the results part, "MeDEGs related to the prognosis of GC patients" must be replaced - a little bit to sloppy copy- and-pasting is here obvious again.

Response 7: We are so grateful for your meaningful comments, and we fully agree with you and feel sorry for the elementary mistakes. Accordingly, we have corrected it in the manuscript. (See in the "RESULTS" Section). Hopefully, this may address your concerns.

**Reviewer # 4 (Number ID: 02856239)**

**Comment 1:** Overall, this is an interesting study. Analyses were generally well performed. The authors should improve the paper on following points: The authors should emphasize the need for a larger validation cohort to replicate findings. This study does not address tumor microenvironment or macro-environment surrounding patients. In this context, it is worth discussing molecular pathological epidemiology (MPE), integration of molecular pathology and data science, which can use tumor markers as surrogate of disease pathologies. MPE deeply studies environmental exposures, intermediate variables (such as plasma markers), and molecular changes in cancer. MPE helps precision medicine. These epigenomic and epigenetic markers can be useful. MPE has been discussed previously. Eg, I can see Gut 2011; Mod Pathol 2013; Annu Rev Pathol 2019, etc in the website.

Response 1: Thank you for your meaningful comments, and we fully understand and agree with you. We have revised it according to your suggestion (See in the "DISCUSSION" section). We hope this may address your concerns.