Prof. Jie Wang
Science Editor, Editorial Office
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World Journal of Gastroenterology
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RE: World Journal of Gastroenterology Manuscript NO: 51752 - Manuscript revision
Novel methylation panel genes in adjacent normal tissues predicts poor prognosis of colorectal cancer in Taiwan

Dear Professor Wang;
Thank you kindly for providing us with the opportunity to resubmit a revised manuscript. We have taken into account the reviewer's in-depth comments and have carefully and extensively revised our manuscript according to the reviewer's comments. We have highlighted amendments we made in red font. Our specific responses are as follows:

Special comments from the editor:
Please download the file of "51752-Manuscript-Edited" and revise the manuscript according to my reminders.

1 Make an audio file, you can record the context of "Core tip", such as .mp3 format.
Thank you for your comments. We have done and finished. Thank you very much.

2 Please provide the decomposable figure of all the figures, whose parts are all movable and editable, organize them into a PowerPoint file, and submit as "Manuscript No. - image files.ppt" on the system. Make sure that the layers in the PPT file are fully editable.
Thank you for your comments. We have done and finished. Thank you very much.

3 You need to provide the grant application form(s) or certificate of funding agency for every grant, or we will delete the part of "Supported by...".
Thank you for your comments. We have done and finished. Thank you

very much.

Reviewer #1: the topic of this issue is interesting, but some comments are highlighted below:

1-the number of cases is small.
We agreed with the reviewer's comments. We have acknowledged the results of this study should be carefully interpreted because of the small sample size. Please see the manuscript in Discussion on page 18.

2- it is a retrospective study.
It is a retrospective cohort study. Thank you very much.

3- still these biomarkers depend on human tissue.
Actually, these biomarkers depend on human tissues, including tumor and matched normal tissue samples. Thank you very much.

4- is the use of nearby normal tissue a feasible one for comparison i.e there may be a change in the genetic analysis in those patients.
Thank you for your comments. There was an increasing number of studies on carcinogenesis have demonstrated that molecular and microscopic changes in normal tissues surrounding tumors lead to cancer progression. Such changes are generally considered a result of the "field effect". Field effect theory postulates that repeated exposure to environmental carcinogens could lead to multiple epigenetic and genetic alterations in normal-appearing tissues. Several studies have shown that the aberrant methylation status of specific genes could be a potential marker of the CRC field effect, which is in line with our finding that compared with tumor tissues, aberrant DNA methylation in adjacent normal tissues is associated with poor prognosis after surgical resection. Please see the manuscript in Discussion on page 17.

Reviewer #2: I read the manuscript very carefully. The topic is very interesting. Many scientific studies try to identify risk factors and prognostic factors in patients with malignant colorectal cancer. The work is well conducted. The population object of the study is conspicuous. The results are interesting and well exposed.

Thank you very much for your comments.

Reviewer #3: The present paper by Hsu CH et al investigated the prognostic value of a panel of methylated genes in colorectal cancer. The study may facilitate approaching novel strategies to improve the prognosis of colorectal cancer. Several major points should be further addressed by the authors:

1. Represented results of methylation-specific PCR in tumor tissues and normal tissues should be shown.

We agreed with the reviewer's comments and added one sentence to represented results of methylation-specific PCR in tumor tissues and normal tissues. Please see the revised manuscript in Result on page 12.
"Although the six genes were methylated in both tumor and matched normal tissue samples, the percentage of methylation was higher in tumor tissues than normal tissues (*CDKN2A*, 67.3% vs 32.7%; *MGMT*, 76.3% vs 23.7%; *MLH1*, 51.6% vs 48.4%; *CSF2*, 51.6% vs 48.4%; *DIS3L2*, 55.1% vs 44.9%; *OAF*, 68.1% vs 31.9%)."

2. A recently published paper by the authors (Int J Mol Sci. 2019;20(19). pii: E4672) was quite similar in study design, but different methylated genes were selected for examination. The authors need to further address the rationale for candidate gene selection.

Thank you for your comments. We have described the rationale for candidate gene selection in Introduction on page 6. Hypermethylation *CDKN2A*, *hMLH1*, and *MGMT* which were related to carcinogenesis pathways could be a potential prognostic marker of CRC.
We selected the other two candidate genes, *CSF2* and *DIS3L2,* from our previous study (Genet. Mol. Biol. 2013; 36(3): 323-328) which are involved in inhibitory effects on tumor growth. We use two databases, PRECOG (PREdiction of Clinical Outcomes from Genomic Profiles) of Stanford University and MethHC (a database for Human pan-cancer gene expression, methylation and microRNA expression) of National Chiao Tung University, to select a novel gene *OAF* which is related to small-cell lung carcinoma.

3. How was the cutoff number of aberrant methylated genes determined? Why

the patients were divided into two groups at 3 aberrancy?

Thank you for your comments. We have used the Kaplan–Meier method to plot the 5-year TTP and OS curves of the <3 aberrancy and ≥3 aberrancy groups and used the log-rank test to compare the difference between the two groups. The 5-year TTP survival curves showed a significant difference between the ≥3 aberrancy group and the <3 aberrancy group (*P* = 0.02 for normal tissue; *P* < 0.01 for tumor tissue). Furthermore, we have used the same way to compare the other compared groups, the ≥2 and <2 aberrancy groups and the ≥4 and <4 aberrancy groups. But there were statistically significant difference of survival curves among the ≥2 and the <2 aberrancy group only in normal tissue (*P* = 0.02) and the ≥4 and the <4 aberrancy group only in tumor tissue (*P*< 0.01).

In addition, the log-rank test revealed no significant differences among these three compared group, the ≥2 and the <2 aberrancy group, the ≥3 and the <3 aberrancy group and the ≥4 and the <4 aberrancy group in both types of tissues over the entire Kaplan–Meier curve.

Base on the above finding, we decided to divide the subjects into two groups at 3 aberrancy.

4. What are the area under the ROC curve (AUROC), positive predictive value (PPV) and negative predictive value (NPV) of the methylation panel in predicting the prognosis of colorectal cancer?

We agreed with the reviewer's comments and added one sentence to describe the positive predictive value (PPV), negative predictive value (NPV) and the area under the ROC curve. Please see the revised manuscript in Materials and Methods "The positive predictive value (PPV) and negative predictive value (NPV) of the ≥3 aberrancy group in predicting the prognosis of CRC were calculated. The area under the ROC curve was reported along its 95% CI."**on page 11 and in Result** "The PPV and NPV of the ≥3 aberrancy group in predicting the progression of CRC were 51.4% and 68.2% in normal tissues and 43.9% and 76.3% in tumor tissues. The PPV and NPV of the ≥3 aberrancy group in predicting the survival of CRC were 20.0% and 81.2% in normal tissues and 18.3% and 78.9% in tumor tissues." and "The area under the ROC curve of CRC progression and survival was 0.59 (95% CI=0.49–0.70, *P*=0.09) and 0.48 (95% CI=0.35–0.61, *P*=0.77) in tumor tissue, respectively. The area under the ROC curve of CRC progression and survival was 0.59 (95% CI=0.48–0.69, *P*=0.11)

and 0.51 (95% CI=0.38–0.64, *P*=0.91) in normal tissue, respectively." **on page 12 and 13.**

5. Was the prognostic performance of the methylation panel superior to the TNM staging system? The comparison should be made.

Thank you for your comments. We try to determine the effect of the methylation status of candidate genes on the relationship between the histological stage and prognosis of CRC in this study. We found that there was a significant joint effect between DNA methylation and clinical stage, especially in matched normal tissues. The methylation status of panel genes in adjacent normal tissues was significantly associated with poor prognosis. Hence, our findings can be used together with clinical staging to guide the re-evaluation of clinical management of cancer, and they can serve as suitable indicators to identify patients at a higher risk of recurrence and requiring intensive follow-up.

6. Subtitles are recommended for the Results section.

Thank you for your comments. We have added subtitles for the Results section. Please see the revised manuscript in Result on page 12-13.

We sincerely thanks for reviewer's comments and your editorial efforts on our manuscript. We believe that the revised manuscript is significantly improved for scientific merits.

Sincerely yours,

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