Peer Review File

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Reviewer A

Comment 1: Abstract: Gene abbreviations are sufficient in this section, and do not need to be written out in full, only in full text manuscript. These are also well-known genes in cancer.

Reply1: We deeply appreciate the reviewer's suggestion. We have rechecked well-known gene abbreviations in this section (see Page 2, line 43 -51).

Changes in the text: In total, there was no difference in the clinicopathologic characteristics of the three groups. *TP53, APC,* and *KRAS* genes were the top 3 alteration genes in sigmoid colon, rectosigmoid junction, and rectum cancer. The rates of the *KRAS, NRAS,* and *PIK3CA* increased as the location moved distally, while the rates of *APC* and *BRAF* decreased. Almost no significant molecular differences were found among the three groups. The prevalence of the FLT3, fms-related tyrosine kinase 1 (FLT1), and phosphoenolpyruvate carboxykinase 1 (PCK1) mutation was lower in the rectosigmoid junction group than the sigmoid colon and rectum groups (P>0.05).

Comment 2: Abstract: Results should be concise in this results section, and to add P value to all findings (remain consistent). The Myc signaling proportion was also much higher compared to other groups, why was nothing reported on this pathway?

Reply2: Thank you again for your positive comments and valuable suggestions to improve the quality of our manuscript. We have added P value to all findings in this section (see Page 2, line 54-59).

Changes in the text: The proportion of the transforming growth factor beta pathway was higher in the rectosigmoid junction and rectum groups than the sigmoid colon group (39.3% vs. 34.3% vs. 18.2%, respectively, P=0.121, P=0.067, P=0.682); a higher proportion of MYC pathway was also observed in the rectosigmoid junction than that in rectum and sigmoid colon (28.6% vs. 15.2% vs. 17.1%, P=0.278, P=0.202, P=0.171).

Comment 3: The Myc signaling proportion was also much higher compared to other groups, why was nothing reported on this pathway?

Reply3: We appreciate your positive evaluation of our work. We have added the Myc signaling proportion in the abstract and the main text (see Page 2, line 54-59 and Page 9-10, line 298-300).

Changes in the text: The proportion of the transforming growth factor beta pathway was higher in the rectosigmoid junction and rectum groups than the sigmoid colon group (39.3% vs. 34.3% vs. 18.2%, respectively, P=0.121, P=0.067, P=0.682); a higher proportion of MYC pathway was also observed in the rectosigmoid junction than that in rectum and sigmoid colon (28.6% vs. 15.2% vs. 17.1%, P=0.278, P=0.202, P=0.171).

Meanwhile, a higher proportion of MYC pathway was observed in the rectosigmoid junction than that in rectum and sigmoid colon (28.6% vs. 15.2% vs. 17.1%, P=0.278, P=0.202, P=0.171).

Comment 4: Introduction: Line 79- However, the treatment of colorectal tumors has become increasingly differentiated and individualized. - Needs a reference.

Reply4: Thank you for underlining this deficiency. PMID: 33842265 has been cited (see Page 4, line 109-110).

Changes in the text: The approach to treating colorectal cancers, however, has evolved to be more differentiated and individualized（PMID：33842265).

Comment 5: Methods: Line 108: The data of 96 CRC patients treated at the Wuxi Hospital Affiliated to the Nanjing University of Chinese Medicine from January 2017 to December 2021 were retrospectively analyzed.

- How were these 96 CRC patients selected? Need inclusion and exclusion criteria. And would be good to state how many CRC samples were diagnosed at this institution for this 5-year period, before selecting the 96.

Reply5: The reason that we chose these 96 CRC patients is all these patients underwent NGS by the 1021-gene panel at Geneplus-Beijing (Beijing, China) during the treatment. Besides around 2-3 CRC samples were diagnosed per week at this institution for this 5-year period.

Inclusion and exclusion criteria have been offered within the MDAR reporting checklist. We have added inclusion and exclusion criteria in our text as recommended (see Page 5, line 151-155).

Inclusion criteria: Patient was older than 18 years of age; Patient was diagnosed with carcinomas in the sigmoid colon, rectosigmoid junction or rectum; Genetic testing information of tissue was available or Tissue samples were available for genetic testing.

Exclusion criteria: Patient was younger than 18 years of age; No genetic testing information or tissue was available; Informed consent was declined.

Changes in the text: Patient older than 18 years of age was diagnosed with carcinomas in the sigmoid colon, rectosigmoid junction or rectum; and Genetic testing information

of tissue or Tissue samples available for genetic testing were included. Patient younger than 18 years of age, no genetic testing information or tissue available and informed consent declined were excluded.

Comment 6: Methods: Line 118: Comprehensive genomic profiling was performed using a custom-designed NGS panel containing 1,021 cancer-associated genes (Table S1). As previously described (16), the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used for the tissue sample extraction, and the genomic DNA sequencing libraries were prepared in accordance with the instructions of the KAPA DNA Library Preparation 123 Kit (Kapa Biosystems, Wilmington, MA, USA).

- Extraction information should precede NGS panel and library prep information.

Reply6: Our deepest gratitude goes to you for your careful work and thoughtful suggestions that have helped improve this paper substantially. Thank you for your suggestions. We have modified in this text (see Page 6, line 170-175).

Changes in the text: As previously described (23), the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used for the tissue sample extraction. Comprehensive genomic profiling was performed using a custom-designed NGS panel containing 1,021 cancer-associated genes (Table S1) and the genomic DNA sequencing libraries were prepared in accordance with the instructions of the KAPA DNA Library Preparation 123 Kit (Kapa Biosystems, Wilmington, MA, USA).

Comment 7: Results: Line 182: KRAS and 182 NRAS have been graphed together. The prevalence of the RAS mutation was 183 significantly higher in the rectum group than the other two groups (P=0.03).

- Does the author mean “grouped” together? And these 2 sentences should be combined.

Reply7: We were really sorry for our careless mistake. We have corrected this mistake (see Page 8, line254).

Changes in the text: KRAS and 182 NRAS have been grouped together.

Comment 8: Line 189: carried the germline mutations of PMS1 Homolog 2, Mismatch Repair System Component (PMS2) and MutS Homolog 6 (MSH6), and 1 patient had a family history of gastrointestinal tumors.

- The abbreviation should follow the full word. PMS1 Homolog 2 (PMS2)

Reply8: We are extremely grateful to reviewer for pointing out this problem. However, *PMS1* Homolog 2, Mismatch Repair System Component is the full word of gene *PMS2*.

Comment 9: Line 191: The median TMB was 6.76 mut/Mb, and the median TMBs were 6.96, 4.8, and 6 mut/Mb for sigmoid colon, rectosigmoid junction, and rectum cancer groups, respectively.

- This sentence does not read well, to rather only report on the medians for the groups separately and maintain the number format: The median TMBs were 6.96, 4.80, and 6.00 mut/Mb for sigmoid colon, rectosigmoid junction, and rectum cancer groups, respectively.

Reply9: Our deepest gratitude goes to you for your careful work and thoughtful suggestions that have helped improve this paper substantially. We have modified in our text according to your suggestions (see Page 8, line 264-266).

Changes in the text: The median TMBs were 6.96, 4.80, and 6.00 mut/Mb for sigmoid colon, rectosigmoid junction, and rectum cancer groups, respectively.

Comment10: Line 201: The results of the comparison showed that the prevalence of the fms-related tyrosine kinase 3 (FLT3), fms-related tyrosine kinase 1 (FLT1), and PCK1 202 mutations was significantly higher in the sigmoid colon than the rectosigmoid 203 junction.

- Need a P-value to show significantly higher evidence. Should consider combining the sentence in line 205.

Reply10: We feel great thanks for your professional work on our article. We have offered a P-value in our text as advised (see Page 9, line 276-279).

Changes in the text: The results of the comparison showed that the prevalence of the fms-related tyrosine kinase 3 (*FLT3*), fms-related tyrosine kinase 1 *(FLT1*), and *PCK1* mutations was significantly higher in the sigmoid colon than the rectosigmoid junction (P=0.0057, P=0.031, P=0.049).

Comment 11: Line 209: We also analyzed the mutations of FLT1, FLT3, and 209 PCK1 in 5050 CRC patients through cBioportal (https://www.cbioportal.org/), and the incidence rates of the FLT1, FLT3, and PCK1 mutations were 7%, 6%, and 5%, respectively.

- The author should state if the 5050 CRC patients were differentiated by site for this analysis, or assessed as a CRC group regardless of site. It would explain the low frequencies obtained for each gene in this assessment, when compared to your findings.

Reply 11: We appreciate your positive evaluation of our work. We have added concerned indication throughout the text according to the comment (see Page 9, line284-288).

Changes in the text: We also analyzed the mutations of FLT1, FLT3, and PCK1 in 5050 CRC patients that assessed as a CRC group regardless of site through cBioportal (https://www.cbioportal.org/), and the incidence rates of the FLT1, FLT3, and PCK1 mutations were 7%, 6%, and 5%, respectively.

Comment 12: Line 222: pathway was also observed in the rectosigmoid junction, and rectum than the sigmoid 223 colon (39.3% vs. 34.3% vs. 18.2%, P=0.067, P=0.121).

- Should rather state pathway alterations.

Reply 12: We are grateful for the suggestion. The full sentence is "a higher proportion of TGF-β pathway was also observed in the rectosigmoid junction, and rectum than the sigmoid colon (39.3% vs. 34.3% vs. 18.2%, P=0.121, P=0.067, P=0.682).".

Comment 13: Discussion: Line 297: but the rectosigmoid junction cancer has different patterns of lymphatic spread compared to the sigmoid colon or rectum cancer and more frequently metastasizes to the pararectal nodes (7).

- Does the author not have histopathological data on lymphatic invasion status for this cohort as well. Would be interesting to see if similar findings observed.

Reply 13: Sadly, we don't have histopathological data on lymphatic invasion status for this cohort as well.

Comment 14: Line 327: We note that several therapies targeting the TGF-β pathway are already in clinical development, and we suggest that patients with metastatic CRC actively participate in such treatments to assess the efficacy of these novel targeted therapies in combination with anti-EGFR therapy (35). Given the lower incidence of RAS/FLT1/FLT3 mutations and the higher incidence of TGF-β pathway alterations, combination therapy with cetuximab should not be considered.

- These two sentences contradict each other. The author recommends combination therapy with TGF- β and anti-EGFR therapy. And Then in the following sentence says combination with cetuximab should not be considered. The author should base the latter sentence on the findings from this study, specific to the site (rectosigmoid tumours).

Reply 14: Thank you again for the suggestion. We have deleted the sentence of "Given the lower incidence of RAS/FLT1/FLT3 mutations and the higher incidence of TGF-β pathway alterations, combination therapy with cetuximab should not be considered." (See Page 14, line 448-450).

Comment 15: Line 168: avian myelocytomatosis viral oncogene homolog (MYC) (29%),

- Not much was said about the MYC alterations and signaling pathway for rectosigmoid tumors, despite its increased frequencies seen in this group. There is some literature on the epistasis interaction of c-Myc and SMAD 4. To include information on this in the discussion as well and recommend further studies to correlate this relationship.

Reply 15: Thank you for your precious comments and advice. We have quoted the recommended literature on the epistasis interaction of c-Myc and SMAD 4 (see Page 13, line 419-423). We would add relevant experimental studies in the future research.

Changes in the text: In kras-mutated/trp53-deleted murine colonocytes, either Myc activation or TGF-β inactivation increased tumor sizes, furthermore in human CRC, gain-of-function alterations in Myc and loss-of-function alterations in TGF-β exhibit, for example in SMAD2/3/4 genes, a masking epistatic interaction and are functionally redundant (PMID: 24627270).

Reviewer B

Comment 1: The title did not show detail marker of the study.

Reply 1: We feel great thanks for your work on our article. We could not show detail marker of the study because the title has its own word limit.

Comment 2: Methods

The method did not provide clear description of the study design. It used prospective instead of describe actual methodology. There was no description of data collection. There were no details about potential bias.

Reply 2: This study is a retrospective, unspecified screening cohort. Sampling bias occcurs because the concerned data collected from patients who have rececevied the next-generation sequencing (NGS). We have reduced bias by collecting data according to the inclusion and exclusion criteria and collecting the samples on the basis of an objective random sampling method.

Comment 3: There was no explanation about sampling strategy, and data requirements. There was no information about whether the sample was naive or post treatment.

Reply 3: Thank you for your careful review. We have added inclusion and exclusion criteria in our text as recommended (see Page 5, line 151-155).

Changes in the text: Patient older than 18 years of age was diagnosed with carcinomas in the sigmoid colon, rectosigmoid junction or rectum; and Genetic testing information of tissue or Tissue samples available for genetic testing were included. Patient younger than 18 years of age, no genetic testing information or tissue available and informed consent declined were excluded.

Comment 4: They did not give detail about the border between sigmoid, rectosigmoid junction and rectum. No detail about sample collection whether from surgical or endoscopy biopsy.

Reply 4: Thank you again for your positive comments and valuable suggestions toimprove the quality of our manuscript. We have added concerned content in our text as recommended (see Page 3, line 80-85 and Page 5, line 155-159). Sadly, we don't have further more details about sample collection whether from surgical or endoscopy biopsy.

Changes in the text: There used to be no international consensus definition for the rectum. The most commonly definitions of the proximal extent of the rectum were 15 cm from the anal verge and the sacral promontory. The ‘‘sigmoid take-off’’ as a more consistent and accurate classification of rectal versus sigmoid cancers - an anatomic, image-based definition of the junction of the mesorectum and mesocolon - has emerged as the consensus of international experts (PMID: 30973385).

Classification of tumors would be based on their anatomical location: Sigmoid: distal sigmoid tumors that arise above the sigmoid take-off; Rectosigmoid: tumors that straddle the take-off; Rectal: high/upper third rectal tumors which are located below the sigmoid take-off, but above the peritoneal reflection.

Comment 5: Results: There was no reason for non-participant. Line 147 mention "cohort" Meanwhile data was collected retrospectively. The conclusion should not mention:" we showed the distinctive molecular profile of the sigmoid colon, recto sigmoid Junction and rectum ". It was not correct. Actually, no significant difference among three groups. The result only showed the proportion of the molecular profile gradually change, but not significantly. The result showed mutation was overlapping among three groups.

Reply 5: With our great thanks, we have modified our text as advised (see Page 15, line 473-474).

Changes in the text: We showed the characterized molecular profiles of the sigmoid colon, rectosigmoid junction, and rectum.

Reviewer C

1. Please check all abbreviations in the main text, such as below. All abbreviated terms should be full when they first appear.

19 with an absolute 19% reduced risk of death due to that RCCs more commonly are diploid and
20 characterized by mucinous histology, high microsatellite instability, CpG island methylation,
21 and BRAF mutations, while LCCs were found to have frequently p53 and KRAS mutations
22 (10). For example, BRAF- V600E mutant CRLM (mutation in a specific BRAF locus V600E)

Reply: We are extremely grateful to editors for pointing out this problem. We have rechecked all abbreviations in the main text to make sure that all abbreviated terms should be full when they first appear.

2. Please check whether the below C19 is correct.

2 Classification of Diseases for Oncology (ICD-O), 3rd Edition of the World Health
3 Organization (www.who.int), the rectosigmoid junction (C19) is encoded as an

Reply: Thank you again for your positive comments and valuable suggestions toimprove the quality of our manuscript. We have corrected this mistake based on your suggestions (see page 3, line 1-4).

Changes in the text: The rectosigmoid junction (ICD-O; C-19) is encoded as a separate segment of the large intestine under the Classification of Disorders for Oncology, International Classification of Diseases for Oncology (ICD-O), 3rd Edition of the World Health Organization (www.who.int).

3. Please check if any more references need to be added in the below 3 sentences since you mentioned "Studies", but only one reference was cited. If not, "studies" should be changed to "a study/a previous study".

2 Recent studies have reported that the frequencies of the CpG island methylator
3 phenotype (CIMP), microsatellite instability-high (MSI-H), and *BRAF* mutations in
4 cancer increase gradually along the colorectum subsites from the rectum to the
5 ascending colon (18). A previous study also revealed that the sigmoid-rectal region

8 RNAs of the rectosigmoid junction cancer have been reported (20,21). Multi-omics
9 studies of gastric cancer have also demonstrated the heterogeneity of molecular
10 features (22). Thus, differences in disease prognosis and progression urgently need to

24 is significantly increased in urothelial cancer, breast cancer, and others (27,28). Thus,
25 to investigate the correlations between the different tumor locations, gene mutations
26 were also assigned to immune-related signaling pathways as defined by previous
27 studies (29). With the exception of a higher proportion of activated dendritic cells

Reply: We feel great thanks for your professional review work on our article. According to your nice suggestions, we have made corrections to our previous draft. “studies” have been changed to “a study/a previous study” in the concerned 3 sentences (see page 4, line 5-8,11-13 and page 8, line 31-32).

Changes in the text:

In a recent study, it was shown that the frequencies of the CpG island methylator phenotype (CIMP), microsatellite instability-high (MSI-H), and *BRAF* mutations in cancer progressively increase from the rectum to the ascending colon along the colorectum subsites (18).

A multi-omics study of gastric cancer has also demonstrated the heterogeneity of molecular features (22).

Thus, to investigate the correlations between the different tumor locations, gene mutations were also assigned to immune-related signaling pathways as defined by a previous study (29).

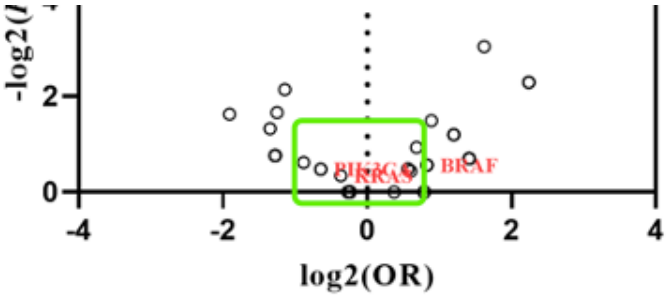
4. Table 1:

Please indicate how the data are presented in Table 1 footnote. For example, Data are presented as No. (%) or Median [range].

Reply: We appreciate your positive evaluation of our work. We have added concerned indication throughout the text according to the comment (see page 19, line 7).

5. Figure 2:

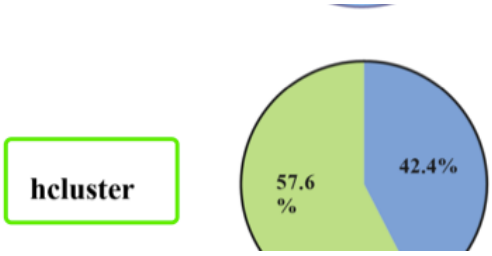
The below words are too close and not clear. Please modify and resubmit Figure 2.



Reply: We are grateful for the suggestion. We have modified Figure 2 and replaced the original one in the main manuscript. Please see the attached file named as Figure 2-revised.

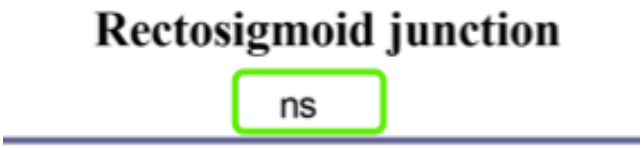
6. Figure 4:

1) Please check whether the below word should be modified to “Ward.D of hcluster”.



Reply 1): Thank you again for the suggestion. We have modified Figure 4 and replaced the original one in the main manuscript. Please see the attached file named as Figure 4-revised.

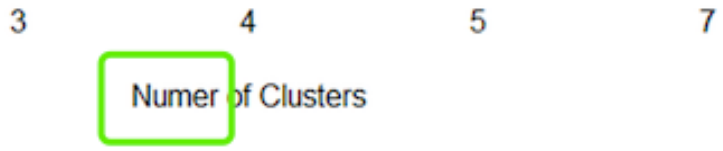
2) Please indicate the meaning of ns in the legend.



Reply 2): Thank you again for your kind reminding. We have indicated the meaning of ns in the legend of main manuscript (see page 23, line 4). “ns” indicates not significant.

7. Figure S2:

There is a spelling mistake. Please revise.



Reply: We were really sorry for our careless mistake. Thank you again for your reminding. We have modified Figure S2 and replaced the original one in the main manuscript. Please see the attached file named as Figure S2-revised.