Peer Review File

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Reviewer A

Major comments:

Comment 1: the authors used rectal and rectosigmoid samples. These two diseases have lots of similarities but have different clinical outcomes; they are treated in different way also; please confirm if the model developed only on rectal cancer samples would look more less similar;

Reply 1: In response to the reviewer's questions, the following explanation is provided: The TCGA data used to establish the model in this study includes samples of rectal cancer and rectosigmoid junction cancer. However, the GSE103479 dataset used for validation consists entirely of rectal cancer samples. The validation results also demonstrate that this model has a good prognostic prediction effect for pure rectal cancer.

Changes in the text: NA

Comment 2: immunoscore assess the risk by testing both - cells in the tumor bed and tumor margin; the gene expression profile provides a single value for the whole sample; please discuss it thoroughly

Reply 2: Due to the similarity in names between the "Immunoscore" and "immune score" mentioned in this article, which may easily lead to misunderstandings, the following explanation is provided: The "Immunoscore" mentioned in the introduction section is a research method that assesses risk by examining cells in the tumor bed and at the tumor margin, and it serves to illustrate that previous studies have found that immune cells have good predictive power for tumor prognosis. In contrast, the "immune score" used in this study is derived from the analysis of immune cells within the entire tumor microenvironment, with the tumor as a whole being the subject of study. Therefore, the analysis focuses on the gene expression profiles of the entire sample.

Changes in the text: NA

Comment 3: immunoscore and other prognostication scales lead to similar separation of survival curves; could we estimate that these scales can have a

Reply 3: Due to the incomplete description of this issue, we were unable to understand the reviewer's confusion, and therefore did not provide a response.

Changes in the text: NA

Comment 4: entry data - the model uses gene expression profiling from microarrays, what is the risk that direct measurements of expression of these four genes can substitute microarrays which are rarely used in clinical practice? What normalization protocol should be used in a clinical test? Please discuss it or propose a validation study design

Reply 4: Thank you very much for raising this question, as it highlights an issue that needs to be addressed in our further research. The raw data for this study were sourced

from the microarray gene expression profiles in the TCGA database. Microarray technology provides a large amount of gene sequencing data, which is suitable for gene analysis but is less frequently used in clinical practice. Our plan for subsequent clinical applications is to use qPCR to obtain the expression levels of individual genes, and then standardize these expression levels using TPM (Transcripts Per Million), so that they can correspond to the data type used in this study.
Changes in the text: NA

Minor comments:
Comment 1: genes should be written in all capitalized letters - like FOXP3 not Foxp3; use italic font for gene and plain for its protein product (here majority should be in italic script unless the biological function of given gene is discussed).
Reply 1: We deeply apologize for the occurrence of such errors. We have reviewed the entire text and made modifications according to your suggestions.
Changes in the text: Page 2, line 29,35; Page 3, Highlight box; Page 7, line 148-149; Page 10, line 234,240,245,249,255; Page 12, line 303,304,310,313,315; Page 13, line 317-320,326,327,330;

Reviewer B
Comment 1: Abstract: Please revise the first sentence. It is too long and hard to understand. E.g. “Immunotherapy is playing an increasing role in the treatment of various cancers. However, its application in rectal cancer is very limited as only microsatellite-unstable bowel cancers with defective mismatch repair are found to benefit. The majority of rectal cancers belong to the microsatellite-stable phenotype. Thus, there is an urgent need to search for new biomarkers of response to immunotherapy.”
Reply 1: We have modified our text as advised.
Changes in the text: Page 2, line 17-20

Comment 2: Remove the sentence “It has a significant impact on…” in line 40-41 or add some well-established references to support this statement.
Reply 2: Thank you for the reviewer's suggestions. We have added corresponding references as the basis for our revisions.
Changes in the text: Page 4, line70

Comment 3: Rephrase the sentence “The Immunoscore…” in line 42-43. The sentence is too long with unnecessary confusion. Rather write something like: “The "Immunoscore," is prognostic tool based on the counts of two lymphocyte populations (CD3/CD45RO, CD3/CD8, or CD8/CD45RO) in the central and infiltrative margins of the tumor. The score has been shown to provide superior classification and prognostic capabilities compared to the current TNM staging system”.
Reply 3: We have modified our text as advised.
Changes in the text: Page 4, line72-78

Comment 4: Please add at least one sentence regarding the role of Immunoscore for specifically rectal cancer or CRC in general, as the manuscript is about rectal cancer and not several cancers.
Reply 4: We have incorporated a description of the role of "Immunoscore" in CRC within the text, along with the corresponding references.
Changes in the text: Page 4, line76-78

Comment 5: The whole introduction section is very chaotic and confusing. It should be more systematically written. E.g., start with 2-3 sentences regarding the alarming tendencies and statistics in rectal cancer. Then write about the TME and include the sentences in lines 46-50 shortly after the part regarding the TME here. Then you can discuss the immunoscore but remember to add some sentences about its relevance in rectal cancer please.
Reply 5: We have added an analysis of the current status of rectal cancer as requested. This study focuses on analyzing immune cells and stromal cells within the tumor microenvironment, therefore, when introducing the tumor microenvironment, we have separately presented existing research related to immune cells and stromal cells. Additionally, we have incorporated the relevant role of the immunoscore in rectal cancer as per the request.
Changes in the text: Page 3-4, line48-65

Comment 6: Please state the aim and focus of the manuscript in the end of the introduction. E.g. “The aim of this study was to investigate…” or “In this paper we aim at establishing new biomarkers…” etc. etc.
Reply 6: We have added the purpose and focus of this paper at the end of the introduction section
Changes in the text: Page 5, line98-91

Comment 7: Methods: Please define overall survival, as this is the primary endpoint.
Reply 7: We have improved the definition of overall survival in the Methods section.
Changes in the text: Page 6, line134-135

Comment 8: Results: I find this statement problematic: “Due to the limited number of rectal cancer patients with survival data in the GEO database, we ultimately chose to analyze 23 cases with comprehensive clinical and follow-up information from the GSE103479 dataset”. It is a lot to go from 122 patients to 23, it’s almost 100 patients that were not included in the analysis.
Do we know if any of the patients received neoadjuvant treatment? Neoadjuvant treatment is very common in non-metastatic rectal cancer. The stromal scores indication better prognosis seems a bit interesting – may be due to receiving neoadjuvant therapy.
Reply 8: The establishment of the model and the screening of core genes in this study were based on data from 122 patients sourced from TCGA, while the 23 cases of data from the GSE103479 dataset were used to validate the predictive performance of the

model. Therefore, it is not accurate to say that the study was only conducted using 23 cases of data, as the reviewer had suggested.
The data for these 23 samples can be accessed in the GEO database, revealing that all patients had not undergone neoadjuvant therapy.
Changes in the text: NA

Comment 9: Discussion: The first section of the discussion, meaning from line 253 to 267 should be moved to the introduction section – already in the beginning of the introduction. The discussion should start with the first part from line 268 summarizing the findings of the study.
Reply 9: We have modified our text as advised.
Changes in the text: Page 3-4, line 48-65

Comment 10: Please correct the whole discussion section for grammatical errors e.g., line 285 don't say reduced but rather to significantly reduce.
Reply 10: We believe that the use of "reduced" here indicates a completed action or state, which is appropriate in the context and does not contain any grammatical errors.
Changes in the text: NA

Comment 11: There are no references to all the statements between line 298-303.
Reply 11: We have reviewed the article once again and added relevant literature citations.
Changes in the text: Page 13, line 320-328

Comment 12: The discussion on FOXP3 is interesting, but should be more in-depth as previous to explaining the impact of FOXP3, the authors emphasize that Tregs are associated with immune-suppression and that CCR4 is involved in recruiting Tregs. Tregs also express FOXP3.
Reply 12: We have added a discussion on the relevant role of FOXP3+ Tregs in CRC.
Changes in the text: Page 13, line 321-323

Comment 13: The discussion is very superficial. There need to be more reflective comments from the authors. All the many limitations of this study should be stated, especially the fact that all the results are based on data from only 22 cases. This is a huge limitation, please emphasize.
At the end of the discussion the authors are just repeating the findings once again. Instead, the limitations and strength of the study should be stated together with a more in-depth discussion regarding the future directions.
Reply 13: In the response to comment 8, it has been clarified that the results of this study are based on information from 122 samples, rather than just 22. At the end of the discussion section, we have included statements outlining the limitations of this study, along with a discussion on future directions.
Changes in the text: Page 13, line 340-344