REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors: In this study, the authors introduced that a new prognostic biomarker that affects the prognosis and treatment decisions of stage III-IV colon cancer patients defined as tumor budding. Therefore, the purpose of this study was to analyze the relationship between tumor budding classification and clinicopathological characteristics. The authors concluded that tumor budding was an independent prognostic factor for progression-free survival and overall survival of stage III-IV colon cancer patients and recommended to apply the tumor budding report in the standard pathological report. Although the study is interesting, 1. it is recommended that consideration be given to the possibility that there may be differences between groups depending on the type of chemotherapy, such as FOLFOX vs. FOLFIRI. 2. Additionally, the comparative results of OS and PFS according to the invasion area (perineural and vascular invasions) in the analysis group must be clearly presented.

Response: Dear reviewer, we would like to thank you for your professional review work, constructive comments, and valuable suggestions on our manuscript. We have made a point-by-point response to each of the issues raised in the peer review report, and **highlighted the revised/added contents with yellow color in the revised manuscript**.

1. it is recommended that consideration be given to the possibility that there may be differences between groups depending on the type of chemotherapy, such as FOLFOX vs. FOLFIRI.

Response:

We think this is an excellent suggestion. Thank you for pointing this out.We enrolled a total of 547 patients, 319 of whom undergo chemotherapy in our study. However, 318 patients were treated with FOLFOX, and only one patient was treated with irinotecan. Regarding this problem, we also consulted professor of oncology in our hospital, and the reply was that the side effects of irinotecan are so great that the Chinese people generally do not tolerate it. Therefore, further classification of the type of chemotherapy, such as FOLFOX vs. FOLFIRI may not be statistically significant in this study.

2. Additionally, the comparative results of OS and PFS according to the invasion area (perineural and vascular invasions) in the analysis group must be clearly presented.

Response:

Thank you for pointing this out. The reviewer is correct, and we have added the revised text and figure 4 and figure 5.

Reviewer #2 (Remarks to the Author):

Scientific Quality: Grade B (Very good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Minor revision

Specific Comments to Authors: Congratulations on this manuscript. Tumour budding is an interesting topic. It is not new and a consensus of 2016 has been released. However, this work informs the significant of tumour budding in Stage III and IV colorectal cancer patients and how Bd2-3 are significant prognostic markers.

Response: Dear reviewer, thank you very much for finding interest in our findings and pointing out the flaws in the analyses. We have addressed your concerns in a point-by-point manner below and **highlighted the revised/added contents with yellow color in the revised manuscript**. **We hope that you will find the added information suitable and sufficient for publication.**

Abstract

1.In the last statement of the 'Patients and methods' section, authors mentioned 'counted according to the 2016 International Tumor Budding Conference'. Suggest to add the word 'guidelines' at the end of the statement.

Response:

Thank you for pointing this out.We have added the word 'guidelines' at the end of the statement.

2.Within the result section of abstract, Bd should be introduced at abstract level to allow readers to understand abstract without referring to manuscript.

Response:

Thank you for pointing this out. The reviewer is correct, and we have added the revised text reads as follows. The TB scoring categories were Bd1 (0-4 buds: low), Bd2 (5-9 buds: intermediate), and Bd3 (≥10 buds: high).

Introduction

3.The last sentence of first paragraph – Please change to "expected to increase".

Response:

We were really sorry for our careless mistakes. Thank you for careful reading. As suggested by the reviewer, we have corrected the "expected to increased" into "expected to increase"**.**

4.Please delete the space after AJCC.

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

5.Please add space after tumor and before [4], and after The.

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

6.The definition of tumour budding can be further refined. It says "single cancer cell of up to four cancer cells at the tumour invasive margin". Please refine this statement further.

Response:

We think this is an excellent suggestion. We have explained the change made, including the exact location where the change can be found in the revised manuscript. Tumor budding (TB), defined as **a single cancer cell or a cell cluster of up to four cancer cells** at the tumor invasive margin, has emerged as a promising independent prognostic biomarker in CRC

7.Please leave a space between metastasis and [8], and between model and [9].

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

8.Methods and Patients – Please change to Patient samples and methods OR Methodology

Response:

We were really sorry for our careless mistakes. Thank you for careful reading. As suggested by the reviewer, we have corrected the "Methods and Patients" into "Methodology".

9.Please remove : after methods and patients. This can be rephrased as methodology too.

Response:

We were really sorry for our careless mistakes. Thank you for careful reading. As suggested by the reviewer, we have removed"after methods and patients".

10.Please give space between enrolled, and and for this statement "A total of 547 patients with CRC were enrolled, and". Please state the reasons why only these 547 patients were enrolled, and why 291 were excluded.

Response:

We think this is an excellent suggestion. We retrospectively analyzed the medical records and clinical data of 838 patients with stage III-IV resectable CRC pathologically diagnosed at Union Hospital, Tongji Medical College of Huazhong University of Science and Technology from January 1, 2015, to December 31, 2018. A total of 547 patients with CRC were enrolled.

Inclusion criteria: 1. Patients undergone radical CRC surgery; 2. The hematoxylin and eosin (H&E) stained tissue sample slides were well preserved, without staining and fading to ensure that the assessment of tumor budding was not compromised under the microscope; 3. The pathological diagnosis was primary colorectal adenocarcinoma.

Exclusion criteria: 1. Patients without radical surgery; 2. In some special histological subtypes of colorectal cancer such as signet-ring cell, medullary, low adhesion cancer and neuroendocrine cancer that TB cannot be assessed.

11.Please delete the space between (PFS) and , .

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

12.Please explain how did researchers define or establish 0.785mm2. Explain single hotspot field area.

Response:

We are grateful for the suggestion. To be more clear and in accordance with the reviewer concerns, ,we were based on the literature **Lugli A, Kirsch R, Ajioka Y, Bosman F, Cathomas G, Dawson H, El Zimaity H, Fléjou JF, Hansen TP, Hartmann A et al: Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. Mod Pathol 2017, 30(9):1299-1311.[PMID:28548122 DOI:10.1038/modpathol.2017.46].** Tumor budding is assessed in one hotspot (in a field measuring 0.785 mm2) at the invasive front. To ensure standardization of field size, the ITBCC group recommends reporting by area (ie, mm2) rather than objective lens (eg, 20x), as the field of vision varies widely between different microscopes. The field area selected by the ITBCC group is 0.785 mm2, which corresponds to the field area (20x objective lens with a 20 mm eyepiece field number diameter). A conversion table has been developed to normalize bud counts to 0.785 mm2 for microscopes with ocular lenses associated withdifferent fields of vision (as shown in the following Figures).

Objective magnification: 20		
Eyepiece FN Diameter (mm)	Specimen Area (mm2)	Normalization Factor
18	0.636	0.810
19	0.709	0.903
20	0.785	1.000
21	0.866	1.103
22	0.950	1.210
23	1.039	1.323
24	1.131	1.440
25	1.227	1.563
26	1.327	1.690

Figure 2 Conversion table to adjust and standardize the tumor bud count for different microscope types.

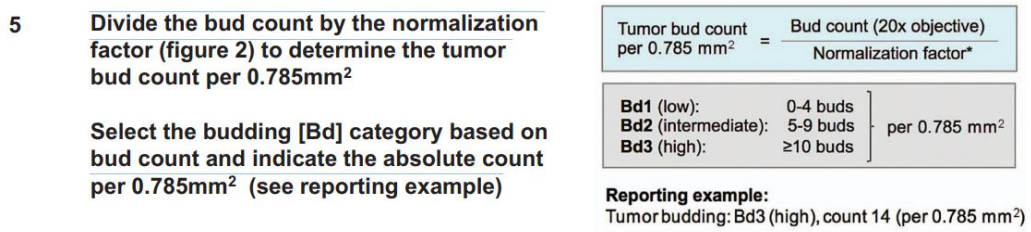
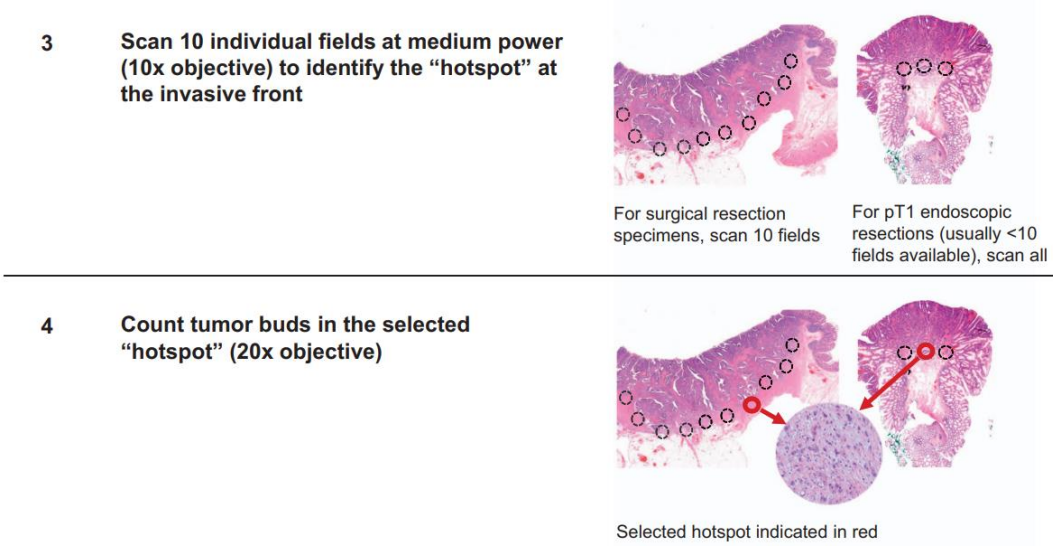


Figure 4 Procedure proposed by the ITBCC 2016 for reporting tumor budding in colorectal cancer in daily diagnostic practice.

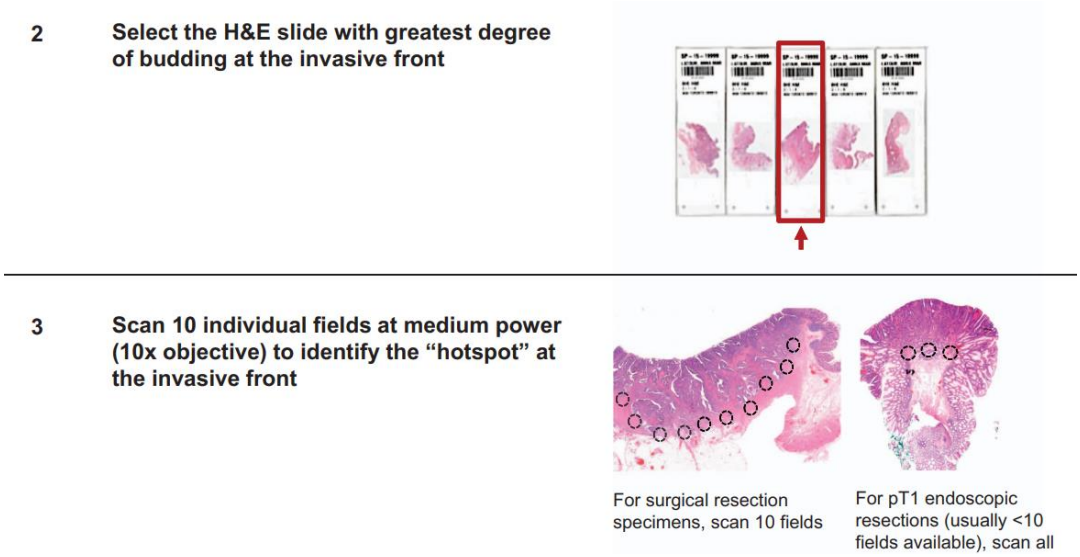
For tumor budding assessment in colorectal cancer, the hotspot method is recommended. Most studies have performed tumor bud counts in a single field with the highest density of tumor buds ('hotspot' method), whereas others have used multiple fields (eg, '5 high power field' and '10 high power field' methods). Counting across multiple fields has the advantage of being more representative of the entire invasive front, and there is also some evidence of improved inter-observer agreement using this approach. On the other hand, counting multiple fields may 'dilute' the final (mean) tumor bud count in cases with focally many tumor buds. The 'hotspot' method therefore better reflects the maximal extent of tumor budding at the invasive front. The ITBCC group recommends the use of the 'hotspot' method, as this is the method used in the vast majority of outcome based studies, and interobserver agreement using this method is quite acceptable. However, to ensure that the field with the highest tumor budding is selected, it is recommended that 10 separate fields (20x objective) along the invasive front are scanned before counting of tumor buds in the single selected 'hotspot'. **(as shown in the following Figures).**



13.Please summarise the 2016 ITBCC definition of invasive front here.

Response:

Thank you for pointing this out. Unfortunately, we searched a large number of relevant literature and did not find clear definition of invasive front on the basis of 2016 ITBCC. Our understanding of this definition is the cancer cells infiltrated the outermost layer in a high-power field **(as shown in the following Figures according to 2016 ITBCC).**



14.Please add scale bar.

Response:

Thank you for pointing this out. We have added scale bar to figure 1.

15.The definition of TB is even more confusing here: TB was defined as a single cancer cell or a cell cluster of up to four tumor cells or less at the invasive margin. Is it between 1 and 4 cells? Is there a better and simpler way of defining?

Response:

We were really sorry for our careless mistakes. Thank you for careful reading. As suggested by the reviewer, we have removed"or less". TB was defined as a single cancer cell or a cell cluster of up to four tumor cells at the invasive margin according to 2016 ITBCC. It is between 1 and 4 cells.

16.Please give space between pathologists and (Figure 2). Please also spell out Figure instead of leaving it as Fig. Please do the same for Figure 1.

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

17.Please ensure that font sizes are uniformly used for texts and sub-titles.

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

Results

18.On Page 6 – it states 410. Is it not 547? Please explain.

Response:

We sincerely thank the reviewer for careful reading. The reviewer is correct, we have corrected the "410" into "547". We feel sorry for our carelessness. In our resubmitted manuscript, this mistake is revised. Thanks for your correction.

19.Please space between Table 1. And TB.

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

20.Tables 2 to 5 are overlaid with Hazard ratios. Please improve and refine the tables further.

Response:

Thank you for pointing this out. We have improved and refine the tables 2 to 5 further .

21.Please change Fig to Figure.

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

22.Please add (%) to probability of survival.

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

Discussion

23.Please edit immunoscore (uncap the I please)

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.

24.Please check through the paragraphs for space and punctuation.

Response:

We were really sorry for our careless mistakes. Thank you for your reminder. As suggested by the reviewer, we have revised.