Peer Review File

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Reviewer A

1. The authors present a retrospective analysis of patients with locally advanced rectal cancer treated with neoadjuvant chemo-radiation aiming to evaluate the significance of CEA and CA19-9 as prognostic factors. Several studies have also shown that patients with high levels of CEA or/and CA19-9 have a worse prognosis (Zeng-Hong Huang, World J Gastroenterol. 2019; Lu-Ning Zhang, Medicine (Baltimore) 2015; Kai-Lin Yang, Radiat Oncol. 2013 and others …). Thus, I feel that the study presented here does not provide sufficient new data to justify a publication in Translational Cancer Research.

Reply: Thanks for your comment!

Neoadjuvant chemoradiotherapy (neo-CRT) combined with radical surgery and postoperative chemotherapy has been recommended as the standard treatment for patients with stage II/III rectal cancer (1-3). With the multimodality treatment, the 5-year overall survival (OS) rate exceeded 75% (4-6), local recurrence in about 5%, and distant metastasis in up to 30% in local advanced rectal (LARC) patients (7, 8). Besides, there is heterogeneity even in patients with locally advanced rectal cancer. CEA and CA19-9 are convenient and economic biomarkers in clinical practice, several studies have shown the prognostic value in rectal cancer (9-13). Therefore, we aim to investigate the prognostic factors and evaluate the role of CEA and CA19-9 in LARC patients who received neo-CRT, radical surgery, and adjuvant chemotherapy.

In addition to demonstrated clinicopathological factors that were predictive of prognosis and recurrence. We also found elevated CEA or/and CA19-9 were independent prognostic factors. LARC patients with high levels of CEA or/and CA19-9 at initial treatment have a worse prognosis, and patients with higher CA19-9 had

extremely poor survival, even standard treatment had been managed. Herein, we recommend CA19-9 should be routinely included in the initial assessment in addition to CEA for patients diagnosed with local advanced rectal cancer. These findings also suggest that this subset of patients require more intensive treatment or additional treatment strategies.

Several studies explored the prognostic role of CEA or/and CA19-9 in rectal cancer. Huang et al. assessed the prognostic impact of CEA/tumor size in stage I to III rectal cancer patients who underwent curative tumor resection, they found higher CEA/tumor size was associated with worse OS and DFS (Zeng-Hong Huang, World J Gastroenterol. 2019). However, patients who received neoadjuvant chemotherapy and/or radiotherapy were excluded from the study, which might reduce the survival and increase the risk of recurrence (9). Yang et al. investigated the prognostic value of CEA in patients receiving neo-CRT (Kai-Lin Yang, Radiat Oncol. 2013) (11). However, patients with low seated primary T2 disease (<6 cm from anal verge) were also treated with preoperative CRT followed by radical surgery in the research. In addition, adjuvant chemotherapy was implemented according to physicians' suggestions and patients'decisions. Only 71.8% of patients with pathologic stage III-IV and 23.2% of patients with pathologic stage 0-II received postoperative chemotherapy, irrespective of the preoperative stage. Zhang at al. reported CA19-9 was the most significant prognostic factor rather than CEA in LARC patients after receiving neo-CRT and radical surgery (13). However, 28% of patients without postoperative chemotherapy in that study (Lu-Ning Zhang, Medicine (Baltimore) 2015).

Reviewer B

1. How about the relationship between the level of pretreatment CEA or CA-199 and clinicopathological factors, such as the depth of tumor invasion, lymph node metastasis, stage, grade, PNI, LVI and CRM. Most important, as previous literatures reported, advanced or metastatic colorectal cancer patients more often had higher tumor marker and they had a worse survival than early stage patients. Confirm the multicollinearity and the interaction (reciprocal action) effects among stage and tumor markers CEA/CA 19-9.

Reply: Thanks for your input!

Previous studies have shown that advanced or metastatic colorectal cancer (CRC) patients more often had higher tumor markers and they had a worse survival than early-stage patients (12, 14). However, most investigations drew their conclusions in CRC. It is known that aside from embryological, anatomical, and physiological differences between the colon and rectum, colon and rectal cancer seem to differ in oncogenesis. According to the report from Zhang et al, which elucidated the clinicopathologic significance of elevated CEA and CA19-9, no relationship was found between clinicopathologic factors and both elevated CEA and CA19-9, including depth of tumor invasion, lymph node metastasis, and stage.

2. In your results, CA 19-9 was already revealed as an independent predictor for OS/DFS/LR/DM. Authors should clarify and discuss that combing CEA with CA19-9 is a better prognostic predictor.

Reply: Thanks very much for your kind hint! We added some data in the result section as advised (see page 11, line 230-244).

We found that combined CEA with CA19-9 was a stronger predictor of OS, DFS, LR, and DM. Kaplan-Meier analysis showed that patients with both elevated CEA and CA19-9 had significant reduced OS (5-year 44.9% vs. 91.3%), DFS (5-year 35.7% vs.

86.9%), and higher risks of LR (14.3% vs. 5.6%) and DM (64.3% vs. 10.9%) than those with normal CEA and CA19-9. Besides, the Cox regression model also showed that patients with both elevated CEA and CA19-9 had the poorest DFS (HR: 8.157, 95% CI: 3.232-20.591, $p<0.001$) and the highest risk of DM (HR: 8.790, 95% CI: 3.324-23.248, $p<0.001$).

Changes in the text: Section Results (line 243-257), Section Discussion (line 279-292).

3. CEA and CA19-9 are representative blood markers in patients with rectal cancer in many previous study (For example, adjuvant radiochemotherapy of stage II and III rectal adenocarcinoma: role of CEA and CA 19-9, Anticancer Res. May-Jun 2005;25(3A):1787-93). Please comment on how your results compare with those from previous studies and clarify which is the novelty of your work in discussion.

Reply: Thank you very much for your constructive comments again! We added some comparison in Discussion (see page 13, line 275-292).

There have been several studies that explored the prognostic role of CEA or/and CA19-9 in rectal cancer (9-11). For example, Weissenberger C et al. evaluated the impact of CEA and CA 19-9 values on clinical outcome in locally advanced rectal cancer patients, the authors aimed at patients who received post-operative or pre-operative radiochemotherapy. They found the prognostic value of CEA/CA19-9 in this subset of patients (10). Huang et al. assessed the prognostic impact of CEA/tumor size in stage I to III rectal cancer patients who underwent curative tumor resection, they found higher CEA/tumor size was associated with worse OS and DFS. However, patients who received neoadjuvant chemotherapy and/or radiotherapy were excluded from the study, which might reduce the survival and increase the risk of recurrence (9).

Neoadjuvant chemoradiotherapy (neo-CRT) combined with radical surgery and postoperative chemotherapy has been recommended as the standard treatment for patients with stage II/III rectal cancer (1-3). With the multimodality treatment, the 5-year overall survival (OS) rate exceeded 75% (4-6), local recurrence in about 5%, and distant metastasis in up to 30% in local advanced rectal (LARC) patients (7, 8). Besides, there is heterogeneity even in patients with locally advanced rectal cancer. CEA and CA19-9 are convenient and economic biomarkers in clinical practice, several studies have shown the prognostic value in rectal cancer (9-13). Therefore, we aim to investigate the prognostic factors and evaluate the role of CEA and CA19-9 in LARC patients who received standard first-line treatment.

In addition to demonstrated clinicopathological factors that were predictive of prognosis and recurrence. We also found elevated CEA or/and CA19-9 were independent prognostic factors. LARC patients with high levels of CEA or/and CA19-9 at initial treatment have a worse prognosis, and patients with higher CA19-9 had extremely poor survival, even standard treatment had been managed. Herein, we recommend CA19-9 should be routinely included in the initial assessment in addition to CEA for patients diagnosed with local advanced rectal cancer. These findings also suggest that this subset of patients requires more intensive treatment or additional treatment strategies.

4. Was the KRAS, BRAF and MMR status of the tumors known? If yes, was there an association between tumor mutational status, tumor markers and oncologic outcomes?

Reply: Thanks for your comments very much!

We conducted KRAS/NRAS/BRAF mutation, and MMR status testing for all patients diagnosed with stage IV rectal cancer and used to guide the management in our center. However, it is not mandatory for non-metastatic rectal cancer patients owing to the high testing cost.

In the study, we found LARC patients with high levels of CEA or/and CA19-9 at initial treatment have a worse prognosis, even standard treatment had been managed. More intensive treatment or additional treatment strategies were required for the subset of patients, including KRAS/NRAS/BRAF mutation and MMR status testing to select suitable treatment options.

5. The present study indicated that patients with high CEA/CA-199 had poor prognosis. How do the authors discuss about the pre-therapeutic or adjuvant management of these patients with rectal cancer receiving CCRT?

Reply: The study suggested LARC patients with high levels of CEA or/and CA19-9 at initial treatment have a worse prognosis, despite receiving neoadjuvant chemoradiotherapy, subsequent radical resection, and adjuvant chemotherapy. More intensive treatment or additional treatment strategies were required for the subset of patients. Such as intensifying preoperative chemotherapy, adding targeted therapy to preoperative treatment, adjusting postoperative chemotherapy to preoperative to strengthen systemic control, or KRAS/NRAS/BRAF mutation and MMR status testing to select suitable treatment options. Additional studies are warranted to further determine the clinical significance of CEA and CA19-9 in LARC patients.

6. Tables: Downstage, it means clinical or pathological stage? Please clarify it more clearly. And how you evaluate it, by MRI or sonography or CT scan or others?

Reply: Thanks again for your kind reminder!

Downstage in the study means the clinical stage, which referred to the clinical restaging after receiving neo-adjuvant chemoradiotherapy and before radical surgery. Enhanced pelvic magnetic resonance imaging (MRI) was performed to restaging. Chest computed tomography (CT) and abdominal CT or MRI were performed before surgery to detect distant metastasis.

Changes in the text: Table 1-3, Supplementary Table S1.

7. Figure's quality should be improved.

Reply: We are very sorry for the Figure's quality. We have revised the figures in the manuscript. Please allow us to express our deep thanks again for your kind hint!

Changes in the text: Figure 1-3 (A.B.C.D).