Peer Review File

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Reviewer Comments

Comment 1: The telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer has been published previously (Clinical Cancer Research 1997; 3:1931-1941), the telomerase activity is suggested to be investigated simultaneously in addition to TFU and RTL.

Reply 1: We have seriously considered the Reviewer's suggestion. Yes, at the beginning, we also tried to investigate telomerase and telomere length simultaneously. But, at the end, we focused on telomere length alone, not including telomerase activity. The reasons were as follows: firstly, telomere length, in contrast to telomerase expression, regulated by a complex and dynamic process involving not only telomerase, but also other mechanisms, such as alternative lengthening of telomeres. [*1, Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet. 2010 May;11(5):319-30. doi: 10.1038/nrg2763. Epub 2010 Mar 30. PMID: 20351727; 2, Bojovic B, Booth RE, Jin Y, Zhou X, Crowe DL. Alternative lengthening of telomeres in cancer stem cells in vivo. Oncogene. 2015 Jan 29;34(5):611-20. doi: 10.1038/onc.2013.603. Epub 2014 Feb 17. PMID: 24531712; PMCID: PMC4135038.*]. Secondly, published studies suggested that telomere length were not correlated with telomerase activity [*1,Takagi S, Kinouchi Y, Hiwatashi N, Chida M, Nagashima F, Takahashi S, Negoro K, Shimosegawa T, Toyota T. Telomere shortening and the clinicopathologic characteristics of hu- man colorectal carcinomas. Cancer 1999; 86: 1431-1436 [PMID: 10526269 DOI: 10.1002/(SICI)1097-0142(19991015)86:8<1431: AID-CNCR7>3.0.CO;2-R];2, Rampazzo E, Bertorelle R, Serra L, Terrin L, Candiotto C, Pucciarelli S, Del Bianco P, Nitti D, De Rossi A. Relationship between telomere shortening, genetic instability, and site of tumour origin in colorectal cancers. Br J Cancer 2010; 102: 1300-1305 [PMID: 20386541 DOI: 10.1038/sj.bjc.6605644*]. In fact, we have investigated 30 patients' telomerase activity by immunohistochemistry and didn't find any association with telomere length. Finally, after document reading, we found that there seems a general agreement that high levels of TERT and/or telomerase activity were associated with poor prognosis, but most studies did not confirm the prognostic role of telomere length.

Changes in the text: None.

Comment 2: In Clinical characteristics of CRC patients paragraph: All the patients

were followed up for at least 2 years and the median follow-up time was 46 months. During the follow-up, 20 (20%) patients were dead and 5 (5%) patients were suffered from distant metastasis or local recurrence. The 1-year and 3-year OS rates of all the patients were estimated as 97% and 72%. Patients were enrolled between 2013 and 2014 and the last update of patient follow-up for this study was December 2020 and none was lost to follow up, but the median follow-up time was 46 months, and 3-year OS results were obtained?

Reply 2: We are very sorry for our serious mistake! Patients enrolled in this study were between 2015 and 2016, we have made serious mistake. We have changed in the text (see Page 6 Line 21). The last update of patient follow-up was correct.

Changes in the text: "2013 and 2014" corrected as "2015 and 2016" (Page 6 Line 21).

Comment 3: In Abstract section: When cut by the mean value of TFU of carcinoma cell and RTL; however, in Statistical analysis paragraph: The median values were used as cut-off. Inconsistent description between two parts.

Reply 3: We are very sorry for our incorrect writing, it has been corrected as "When cut by the median value of TFU of carcinoma cell and RTL" (see Page 3 Line 1).

Changes in the text: "the mean value" corrected as "the median value" (Page 3 Line 1).

Comment 4: In Figure 2, TFU and RTL changes in carcinoma, adenoma and cancer-associated fibroblast (CAF) cells. The above findings need to be clarified in the comparison with their normal counterparts to verify the role of TFU and RTL.

Reply 4: Yes, we can't agree more. We have added the date of TFU and RTL in adjacent mucosa (In the part of Results in Page 10 Line 17-18, Page 11 Line 10-17) and changed the Figure 2 A, C and E.

As for Figure 2B, we did discover an inverse relationship between TFU in CAF and age. According to the Reviewer's suggestion, we have associated TFU and RTL changes in CAF with gender, differentiation, T staging, lymph node metastasis, distant metastases or not and Dukes staging. However, no positive correlation was found.

Comment 5: In addition, Figure 2B, there was an inverse relationship between TFU in CAF and age; therefore, it seems that TFU and RTL changes in CAF would be associated the malignancy of CRC?

Reply 5: This is a good idea! Yes, the TFUs in CAF were found to be inversely

correlated with age, which was in line with previous studies found in normal mucosa tissue [ref. 16; ref. 18; ref. 31]. However, besides our study, these researches couldn't associate the malignancy of CRC with telomere changes.

Changes in the text: In the part of Statistical analysis, we added "or adjacent mucosa" in Page 8 Line 21. In Page 9 Line 11, we added "adjacent mucosa cell". In Page 10 Line 17, Page 11 Line 4 and Page 11 Line 11, we added "and in adjacent mucosa cell". In Page 10 Line 18, we added date "3425 [1890-4727], P=0.0079". In Page 11 Line 5, we added date "r=0.1650, P=0.1009" and "r=-0.043, P=0.6737". In Page 11 Line 12, we added date "2810 [1916-3602]". In Page 11 Line 12- 17, "TFU and the RTL were statistically significantly lower in adenoma cells than in carcinoma (607 [246–1413] vs. 1968 [572–5519], P < 0.0001; 0.826[0.26-1.97] vs. 0.362[0.11-0.81] vs. 1.353[1.003-1.588], P < 0.0001, respectively. Fig. 2, D-E)" have been changed as "TFU in adenoma cells were statistically significantly lower than in carcinoma (607 [246–1413] vs. 1968 [572–5519], P < 0.0001. Fig. 2, D). RTLs in carcinoma, adenoma and adjacent mucosa cell were also compared and RTL in adenoma and adjacent mucosa cell were significantly lower (0.826[0.26-1.97] vs. 0.362[0.11-0.81] vs. 1.353[1.003-1.588], P < 0.0001, respectively. Fig. 2, E)". The above date has added the Figure 2 A, C and E.

Comment 6: In Figure 3, Lower TFU and RTL were associated with poor prognosis. As OS is often affected by various treatment strategies, the PFS or DFS should be included in the current study. In addition, please add the number of patients in each subcategory.

Reply 6: Yes, we can't agree more! We analyzed our date again and added DFS analysis in the current study. We accepted Reviewer's advises and added add the number of patients in in each subcategory. **Changes in the text:** In the section of "Lower TFU and RTL were associated with poor prognosis", firstly, we added a figure (Fig. 4) and a table (Table.3); secondly, we added TFU and RTL survival analysis for DFS (see Page 13 Line 6-11), as well as the survival analysis without the Dukes D stage patients (see Page 13 Line 12-17). In multivariate Cox proportional hazards analysis, the independent prognostic factors for DFS were also added (see Page 14 Line 6-8).

Comment 7: The data was doubtable, apparently, TFU of tumor was higher in Dukes C compared to Dukes A and B?

Reply 7: There are very good questions. We have to emphasize that, because of non-normal distribution, TFUs were expressed as median [interquartile range]. Actually, TFU of tumor in Dukes C didn't high than Dukes A and B (1909[721-4968]

vs. 2405[557-77598], P=0.6838). What's more, there was no significantly differ between Dukes A, B and C when Dukes D was taken off (P=0.9167).

Comment 8: And TFU and RTL was not associated with T and N stage, and was just correlated to Dukes D stage?
Reply 8: This was a good question. As previous researchers reported, telomere length in cancer tissue was statistically significant correlated with UICC stage (Gertler R, Rosenberg R, Stricker D, Friederichs J, Hoos A, Werner M, Ulm K, Holzmann B, Nekarda H, Siewert JR. Telomere length and human telomerase reverse transcriptase expression as markers for progression and prognosis of colorectal carcinoma. J Clin Oncol 2004; 22: 1807-1814 [PMID: 15143073 DOI: 10.1200/JCO.2004.09.160]). Our results were similar as Gertler R et al.'s.

Comment 9: Moreover, tumor located in rectum should be categorized into left colon CRC.
Reply 9: We have made correction according to the Reviewer's comments (see table 1).

Comment 10: Several important parameters were missing, for example, perineural invasion, lymphovascular invasion and adjuvant chemotherapy.
Reply 10: According to the Reviewer's comments, date of perineural invasion, lymphovascular invasion have added in Table 1 and no patients received (neo)adjuvant radiotherapy or chemotherapy before operation, so this date can't be added.
Changes in the text: In Table 1, date of tumor located, rectum have been categorized into left colon CRC and P values and date of Left colon have been changed in the Table (Table1, Line 7-8). Date of perineural invasion, lymphovascular invasion have added in Table 1(Table1, Line 15-18) and in the text (see Page 12 Line 4-79).

Comment 11: Lower TFU and RTL were associated with poor prognosis paragraph. Authors have to clarify what was their analyzed end point here. Likewise, Table 2. Univariate and Multivariate Cox Regression Analysis for Univariate and Multivariate needs to indicate its end point. No Dukes stage parameter here?
Reply 11: We have to say we have defined OS in the section of "Statistical analysis" (see Page 8 Line 3-4). Of course, Reviewer's comments reminded us to clarify the analyzed end point of DFS and we have added. We are so grateful! As for prognosis

analysis, we excluded Dukes stage parameter because Dukes stage was the sum of T stage, Lymph node metastasis, distant metastases and it could finally influence Multivariate Cox Regression Analysis.

Changes in the text: we have added the definition of DFS in in the section of "Statistical analysis" (see Page 9 Line 7-9).