Peer Review File

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Response to Reviewer A

We thank the reviewer for the insightful comments.

Major points:

1. In introduction, the chapter about PD-L1 is insufficient as it lacks background information about PD-L1 expression in tumor tissue (not in TILs) as scored in this work and as references are not cited correctly (e.g. ref 17). Please rewrite.

We thank the Reviewer for this comment. As a response to the Reviewer's comment, we have added below sentence have revised references in the introduction section.

"Programmed death ligand-1 (PD-L1), a ligand for programmed death receptor-1 (PD-1), is expressed in tumor infiltrating immune cells and tumor cells. PD-1 is an inhibitory receptor expressed on activated T-cells. When PD-L1 expressed on tumor cells binds to its receptor, PD-1, it suppresses T cell function, downregulating the immune response. Targeting the PD-1/PD-L1 blockade has been proposed as a promising breakthrough in cancer treatment including CRC.

Previous studies showed that genetic alterations within tumors may influence immune system engagement; EGFR mutations or EML4-ALK fusions activate the PD-1/PD-L1 pathway via PD-L1 upregulation. *PIK3CA* mutations are associated with PD-L1 overexpression in gastric cancer." (Introduction see Page 7, line 137-148)

2. In M&M please add the following:

We thank the Reviewer for this comment.

- cut-off values of CA19-9 and CEA

In response, the cutoff values for CA 19-9 and CEA are described in the M&M section and also added to the footnotes in Table 1.

"Serum tumor markers, including carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9), were collected preoperatively, and their cut-off levels were 5.0 ng/mL and 37 kU/L, respectively." (Methods see Page 8, line 165-168)

"Serum CEA elevation; defined as > 5.0 ng/mL. Serum CA19-9 elevation defined as > 37 kU/L." (Table 1 see Page 28, line 579-580)

- cut-off value of minimal % of tumor cells used for DNA extraction

In this study, surgical resection samples were used. For the NGS study, H&E-stained slides were reviewed, tumor areas with sufficient viable tumor cells were marked, and then macrodissection was performed. In this way, sites with more than 50% of the tumor volume were used for examination.

We have added below sentence in the methods section.

"Areas with more than 50% tumor volume were used for examination." (Methods see Page 9, line 185-186)

- bases of evaluation scale and cut off value of PD-L1

In response, we have revised the sentences in the methods section.

"The cut-off for classifying the positivity of PD-L1 was defined as greater than or equal to 1% of tumor cells of any intensity." (Methods see Page 10, line 219-221)

3. Authors may think of adding information about MSI status especially in colon cancer, please also check used references

- in the discussion part please correct/add:

We thank the Reviewer for this comment.

Response; We strongly agree with the reviewers' comments. However, we think that it would be better to focus on the original purpose of this study (to investigate the effect of *PIK3CA* mutations on the prognosis of CRC patients and the association between *PIK3CA* mutations and PD-L1) rather than expanding the parameters in this study. Some questions were raised in this study, which we plan to address in future studies. We think it would be very meaningful to add MSI studies with the resolution of these questions in future studies. Unfortunately, funding limitations also make it difficult to add MSI studies to the current studies.

4. References and more context of PD-L1 and *PIK3CA*

In response, we have revised the sentences and modified reference in the introduction section.

"Programmed death ligand-1 (PD-L1), a ligand for programmed death receptor-1 (PD-1), is expressed in tumor infiltrating immune cells and tumor cells. PD-1 is an inhibitory receptor expressed on activated T-cells. When PD-L1 expressed on tumor cells binds to its receptor, PD-1, it suppresses T cell function, downregulating the immune response. Targeting the PD-1/PD-L1 blockade has been proposed as a promising breakthrough in cancer treatment including CRC.

Previous studies showed that genetic alterations within tumors may influence immune system engagement; EGFR mutations or EML4-ALK fusions activate the

PD-1/PD-L1 pathway via PD-L1 upregulation. *PIK3CA* mutations are associated with PD-L1 overexpression in gastric cancer." (Introduction see Page 7, line 137-148)

5. If authors include information about MSI status it should be put in context to PD-L1 status, *PIK3CA* status and tumor location

We thank the Reviewer for this comment.

The response is the same as the response to the 3rd comment.

Response; We strongly agree with the reviewers' comments. However, we think that it would be better to focus on the original purpose of this study (to investigate the effect of *PIK3CA* mutations on the prognosis of CRC patients and the association between *PIK3CA* mutations and PD-L1) rather than expanding the parameters in this study. Some questions were raised in this study, which we plan to address in future studies. We think it would be very meaningful to add MSI studies with the resolution of these questions in future studies. Unfortunately, funding limitations also make it difficult to add MSI studies to the current studies.

6. Conclusions are pointing out interesting aspects but für immune checkpoint therapy there are already internationally accepted stratification factors not mentioned in this article. Please include.

In response, we have added the sentences in the discussion section.

"There are several Food and Drug Administration (FDA) approved immune checkpoint inhibitors (ICIs) targeting different pathways, including the programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1), and cytotoxic T lymphocyte antigen 4 (CTLA-4) pathway. PD-L1 expression is well known predictive biomarker for PD-1/PD-L1 inhibitors in advanced cancer patients." (Discussion see Page 18, line 390-396)

Response to Reviewer B

Authors aim to investigate the effect of the *PIK3CA* mutations on prognosis of CRC patients and the association between *PIK3CA* mutations and PD-L1 in a single institution from January 2018 to May 2019. Furthermore, *PIK3CA* mutations were analyzed by targeted next-generation sequencing using formalin-fixed paraffin-embedded specimens from 224 primary CRC patients. PD-L1 expression was evaluated by immunohistochemical staining. Their results disclosed that *PIK3CA* mutations and PD-L1 expression were detected in 21.4% and 10.3% of CRC patients, respectively. PIK3CA mutations were significantly correlated with right colon cancer colon (P = 0.011) and

were inversely correlated with lymph node metastasis (P = 0.026), distant metastasis (P = 0.047), and high TNM stage (P = 0.036). In univariate analysis, *PIK3CA* mutations were correlated with longer relapse-free survival in CRC patients. PD-L1 expression was correlated significantly with *PIK3CA* mutations (P < 0.001). In summary, the present study indicated that *PIK3CA* mutations were associated with favorable prognostic factors, longer relapse-free survival, and expression of PD-L1. The results seems informative and appealing; however, there are a lot of criticisms and have several issues that the authors need to address before the manuscript is suitable for publication.

We thank the Reviewer for the insightful comments.

Major Compulsory Revisions:

1. The major flaw of the current study was that authors included stage I-IV colorectal cancer (CRC) patients for the detection of *PIK3CA* mutations and PD-L1 expression; however, the issue of *PIK3CA* mutations and PD-L1 expression is just associated with treatment of stage IV CRC patients. Therefore, the results were not compatible with clinical implications.

We agree with the reviewer's comments. This study was performed to investigate the effect of the *PIK3CA* mutations on prognosis of CRC patients and the association between *PIK3CA* mutations and PD-L1.

Based on the results of this study, it would be very meaningful to investigate the effects of immunotherapy-related *PIK3CA* mutation and PD-L1 expression in patients with advanced cancer in future studies.

2. The study objective was the correlation of *PIK3CA* mutation with PD-1 L1 expression and their clinicopathological significance in CRC, and *PIK3CA* mutations were correlated with longer relapse-free survival in CRC patients by univariate analysis, of which the adjuvant therapy and 1st-line treatment information of enrolled CRC patients was absent.

In response, we have added the sentences in the methods section.

"For clinical stage I-III patients, radical resection was the first choice of treatment, and for stage III patients, adjuvant chemotherapy (FOLFOX) was administered. Stage IV patients underwent palliative resection and received adjuvant chemotherapy." (Methods see Page 8, line 157-160)

3. In Introduction section: Previous studies have demonstrated that *PIK3CA* mutations are associated with PD-L1 expression (17). PD L1 expression is regulated by oncogenic activation of PI3K pathways (18). However, above studies showed that the observed

association of PTEN loss and/or activation of PI(3)K, with the increase in B7-H1 expression and the resulting effects on T-cell function can further contribute to the failure of adaptive immunotherapies in some breast and prostate cancer patients, and oncogenic activation of the AKT-mTOR pathway promotes immune escape by driving expression of PD-L1. The hypothesis of *PIK3CA* mutations is not equal to *PIK3CA* activation in CRC patients. Obviously, the study rationale should be refined and the preliminary results/references needs to be included.

In response, we have revised the sentences and modified reference in the introduction section.

"Programmed death ligand-1 (PD-L1), a ligand for programmed death receptor-1 (PD-1), is expressed in tumor infiltrating immune cells and tumor cells. PD-1 is an inhibitory receptor expressed on activated T-cells. When PD-L1 expressed on tumor cells binds to its receptor, PD-1, it suppresses T cell function, downregulating the immune response. Targeting the PD-1/PD-L1 blockade has been proposed as a promising breakthrough in cancer treatment including CRC.

Previous studies showed that genetic alterations within tumors may influence immune system engagement; EGFR mutations or EML4-ALK fusions activate the PD-1/PD-L1 pathway via PD-L1 upregulation. *PIK3CA* mutations are associated with PD-L1 overexpression in gastric cancer." (Introduction see Page 7, line 137-148)

4. In Methods section, no relevant information regarding the follow-up protocol and treatment modalities for their CRC patients.

In response, we have added the sentences in the methods section.

"Postoperative surveillance for CRC patients was performed at every 3 months. Laboratory tests including serum tumor marker CEA, CA19-9 were performed. Abdominal computer tomography (CT) was performed to detect recurrence and metastasis." (Methods see Page 8, line 173-176)

5. In Figure 2, Table 3 and 4, recurrence-free survival was not quite feasible in stage IV CRC patients.

There were 7 stage IV patients, and as a result of observation according to the follow-up protocol (response for 5th comment of Reviewer B), no patients had recurrence during follow up.

6. In Table 1: *PIK3CA* mutations were significantly correlated with right colon cancer colon ($P = 0.011$) and were inversely correlated with lymph node metastasis ($P = 0.026$), distant metastasis ($P = 0.047$), and high TNM stage ($P = 0.036$). However, patients with right side colon cancer were considered to be poor prognosis, especially in stage III and IV CRC. It seems to be contradictory among above parameters.

In response, we have added the sentences in the discussion section.

"Our results showed that right colon cancer had a higher rate of *PIK3CA* mutations, and these results are consistent with previous studies. The outcomes of patients with left-sided cancers were considered to be better than those of right-sided cancers in metastatic CRC. In relation to prognosis, the contradiction shown by tumor sidedness and *PIK3CA* mutations needs to be confirmed through additional studies." (Discussion see Page 17, line 377-382),

7. In Table 3: Patients with a *PIK3CA* mutation exhibited better RFS compared to the wild-type *PIK3CA* group, but no correlation between *PIK3CA* mutation and OS?

In response, we have added the sentences in the results section.

~~**"Patients with a *PIK3CA* mutation exhibited no correlation between *PIK3CA* mutation and OS."**~~ **"The PIK3CA mutation was not correlated with the patient's OS." (Results see Page 13, line 284-286)**

8. The low-T category CRC patients with *PIK3CA* mutations exhibited longer RFS compared to those with wild-type *PIK3CA* ($P = 0.028$) (Figure 2). The factors that had significant or borderline significant ($P < 0.1$) correlation with OS or RFS by univariate analysis were included in the multivariate analysis. Why did authors select low-T stage factor only here?

Response; Patients with *PIK3CA* mutations had better RFS compared to the wild-type *PIK3CA* group. RFS was considered to be a more important prognostic factor compared to other clinicopathologic parameters that showed significant correlation, so we tried to confirm that the RFS results were not biased.

9. Table 4. Multivariate Cox regression analysis for the overall survival and relapse-free survival of CRC patients. The results were quite confusing as no one variable was significantly correlated to both OS and RFS? And out study target of *PIK3CA* mutation and PD-L1 expression were lack?

Comment; The results were quite confusing as no one variable was significantly correlated to both OS and RFS?

Response; As described, no variables were significantly correlated with both OS and RFS.

Comment; And out study target of *PIK3CA* mutation and PD-L1 expression were lack?

Response; We searched thoroughly for published papers, but did not find similar studies, such as ours, regarding *PIK3CA* mutations and PD-L1 expression in CRC.

10. Appendix table 558 1. Summary of *PIK3CA* mutation in colorectal cancers. The different mutation exons were all meaningful for patients' outcomes? Is any further information available?

Response; All observed mutations are confirmed pathogenic in the public database (COSMIC) with the exception of one (c.277C>T). And in the patient with c.277C>T, two *PIK3CA* mutations were observed, and the other was confirmed as a pathogen. Thus, all patients with *PIK3CA* mutations carry the pathogenic mutation.

11. The TCGA data of *PIK3CA* mutations in survival outcome of CRC patients is suggested to be added on.

In response, we have added the sentences in the introduction section.

"*PIK3CA* mutations are observed in 24.7% (147 of 594) of colorectal cancers in The Cancer Genome Atlas (TCGA). Survival analysis from the TCGA database disclosed no prognostic value for *PIK3CA* mutations in colorectal cancer." (Introduction see Page 7, line 133-136)

Minor Essential Revisions:

1. Please correct the typo and grammatical error with an expert good at English-editing.

Response; We received expert proofreading when we first submitted the paper. This time, due to time constraints, we were unable to receive a separate correction, and we will do it again later.

2. Figure 1. Expression of PD-L1 in colorectal cancer. Authors have to show all three-point evaluation scale, not just only one positive PD-L1 IHC microphotography.

In response, we have modified figure 1. And we have revised figure legend, "Expression of PD-L1 in colorectal cancer. Only membranous staining of tumor cells was evaluated. (A) Tumor proportion score (TPS) 0 : <1% of tumor cells (B) TPS 1: ≥1% to 49% of tumor cells (C) TPS 2: ≥50% of tumor cells."