Response to Editor and Reviewers

We thank Editor and Associate Editor's support to this work, and appreciate all the Reviewers for their constructive comments and the opportunity for clarification. We have revised our paper accordingly, with the responses to the individual comments given below. For the ease of reference, newly added results and revised text in the revision are highlighted in red front both in this letter and in the manuscript. According to the language requirements, the revised manuscript have been polished by professional native speakers of English.

Response to Editor

Response to Reviewers

Reviewer 1

Zhao et al. investigated SYPL2 expression in colorectal cancer. The following questions were raised.

Response: Thanks for your recognition of the study.

Comment 1: Authors wrote that SYPL2 expression was significantly lower than in normal tissue. However, in survival analyses they found that CRC patients with higher SYPL2 expression had worse survival. One would assume the opposite that the more similar results to normal would have better OS/DFS/etc. Therefore, authors must discuss this controversial observation.

Response: Following your comment, we have discussed this controversial observation.

In this study, SYPL2 expression in tumor tissues was significantly lower than that in normal tissue. However, higher SYPL2 expression was associated with worse survival in CRC. The paradox of the opposite effect of SYPL2 expression might be due to the following reasons. First, compared to normal tissue, tumor tissue can abnormally activate a series of signaling pathways and have special

tumor microenvironment. Some genes (including SYPL2) may play roles in promoting or suppressing cancer in specific signal pathways in the tumor microenvironment. Second, SYPL2 might work as a biomarker in CRC. Higher expression of SYPL2 could be associated with some specific and powerful activated oncogenic genes and enhanced malignant behavior of tumors. Finally, tumor-infiltrating immune cells are closely related to tumorigenesis, angiogenesis, and tumor cell growth and metastasis, which may in turn regulate the quantity and differentiation of immune cells. CD8+ T-cells are typically thought to be a homogenous group of cytotoxic cells that produce interferon-γ. In addition, CD8+ T lymphocytes are the major anti-tumor effector cells. In this study, CD8+ T-cell counts in the SYPL2 high-expression group were significantly lower than those in the SYPL2 low-expression group. Therefore, SYPL2 might contribute to the poor prognosis of CRC by affecting immune cell infiltration. However, the relevant molecular and pathway mechanisms still necessitate further experiments for verification.

Comment 2. In general, the Discussion is short compared to the many results authors described. Discussion should be more detailed.

Response: Thank you for your kind comment, we have added some necessary details about the discussion.

Comment 3. Further details about bevacizumab is necessary: how many patients received this treatment, when the treatment was received during the course of the disease, etc.

Response: Thank you for meaningful suggestion. We have added some details about bevacizumab in this manuscript.

We collected a total of 16 tumor samples entered into GSE60331 prior to undergoing treatment with bevacizumab; the responder group included seven samples and the non-responder group contained nine samples. Some detailed information can be retrieved from GSE60331.

We collected a total of 16 tumor samples prior to bevacizumab treatment from GSE60331 for validation because there is greater clinical practice value in predicting the treatment response by considering pre-treatment genes.

Comment 4. What is the relationship between SYPL2 and synchron vs metachron metastases.

Response: Thank you for your kind comment. It was a good and meaningful question. Tumor metastasis can be divided into synchronous and metachronous metastasis according to the time of metastasis. In this manuscript, we did not distinguish the two types of metastases specifically, because of lacking essential data. Due to time constraints, we felt very regret that we could not analyze the relationship between SYPL2 and two types of metastasis. And we will plan to study the question in next researches.

Comment 5. Why did not exclude authors those patients from the data analysis, who had several data missing (e.g.,TNM was missing for 45 patients, sex for 20). If all of these patients are excluded from the study, how do the results change?

Response: Thank you for meaningful comment. We have thought about this question carefully. We thought that those data still contained some useful information even though they had several data missing. Deletion of these data might lead to some errors. And this method was used in some articles too. (PMID:30496798)

And according to your advice, we analyzed the correlation between SYPL2 level and clinicopathological characteristics in CRC again. And the result was not changed obviously.

Characteristics	SYPL2 level		P-value
	Low (n=206)	High (n=206)	$\chi 2$

Age (years)	67.58±11.89	65.33±13.11	0.070
Gender			
Female	89	101	0.236
Male	117	105	
T			
T1+T2	50	31	**0.019***
T3+T4	156	175	
N			
N0	131	109	**0.028***
N1-2	75	97	
M			
M0	178	166	0.111
M1	28	40	

Reviewer 2

The authors investigated the association between SYPL2 expression and clinicopathological factors in colorectal cancer. I have following concerns.

Response: Thanks for your review of this manuscript.

Comment 1: The authors state that SYPL2 mRNA expression correlates with BRAF and NTRK1 mRNA expression. However, in General, BRAF and NTRK gene mutations, not mRNA expression, are used to select chemotherapy regimens for colorectal cancer. Please cite the articles that NTRK and BRAF mRNA expressions correlates with therapeutic effect and explain that SYPL2 affects therapeutic effect.

Response: Thank you for your kind comment and meaningful advice. We couldn't agree with you more. KRAS/BRAF gene mutation and NTRK gene fusions are used to select chemotherapy regimens for colorectal cancer. According to your advice, we added analysis and discussion about the

relationship between SYPL2 expression and KRAS, $BRAF^{V600E}$ and P53 mutation. And we deleted the paragraph about correlations analysis of NTRK genes expressions. In addition, we added related discussion about that KDR (also called VEGFR) and EGFR mRNA expressions correlates with therapeutic effect. Thanks for your great advice again!

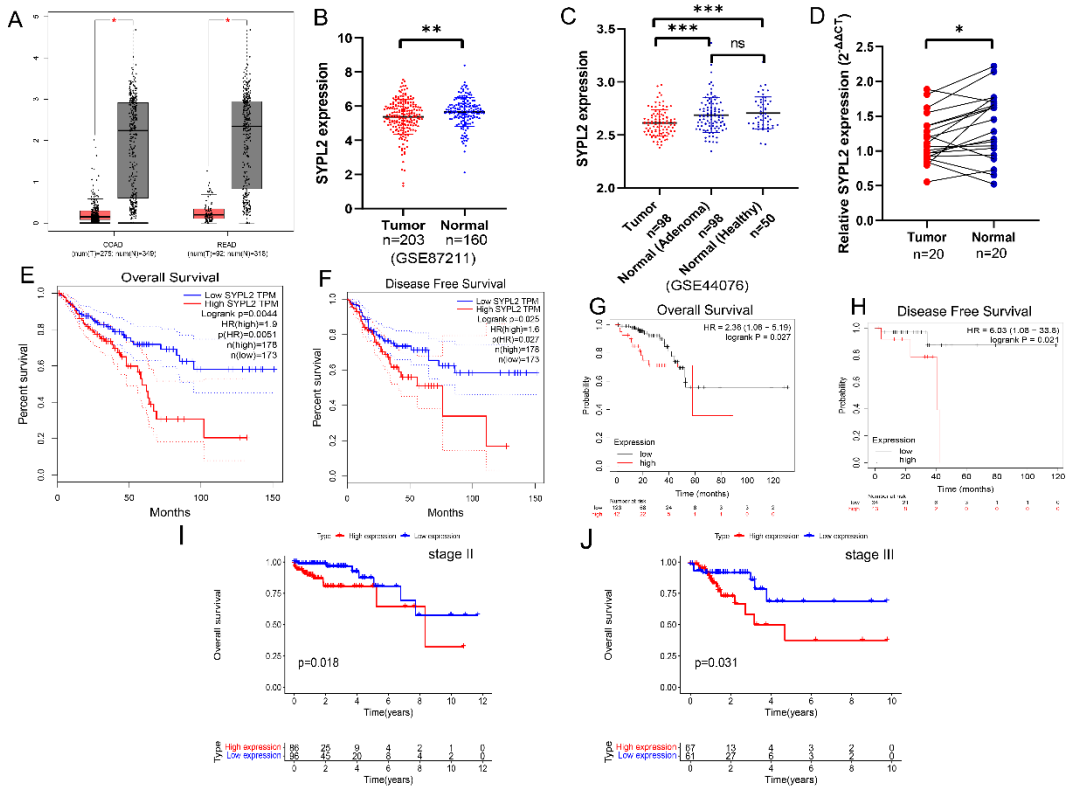
Correlation analyses showed that the SYPL2 gene-expression level was significantly associated with the expression of KDR (also called VEGFR) ($R > 0.4$) and EGFR ($R > 0.3$) and with $BRAF^{V600E}$ mutation ($P < 0.05$). Carvalho et al[24] reported that VEGFR expression is associated with the effect of bevacizumab therapy, and Szablewski et al[25] found that EGFR overexpression and mutations in KRAS and BRAF contribute to colorectal carcinogenesis. Moreover EGFR-directed molecular treatments could be investigated in a subset of patients affected by intestinal-type adenocarcinoma. These results of above studies were consistent with our research.

Agents targeting VEGFR or EGFR and multiple tyrosine kinase inhibitors play an important role in CRC management[26]. The mutation of $BRAF^{V600E}$ residue occurs in approximately 10% of colorectal cancers, constituting a group with a particularly poor prognosis. And our result also found that SYPL2 was higher expression in the $BRAF^{V600E}$ mutation group, and associated with poor prognosis. The mutation of $BRAF^{V600E}$ is also extremely associated with targeted therapy of metastatic CRC[27]. Our result suggests that SYPL2 may be a biomarker for predicting targeted treatment of CRC patients.

Comment 2. The authors state that the expression levels of SYPL2 was associated with OS and DFS. However, SYPL2 expression was demonstrated prognostic factor by multivariate analysis, based on the data provide this article, patients with high SYPL2 expression were significantly likely to be grouped into late-stage colorectal cancer. To understand the significance of high SYPL2

expression for metastasis and recurrence, I think authors need to examine the association between SYPL2 expression and survival in colorectal cancer patients with stage II and III. The authors should show the cumulative survivals by stage.

Response: Thank you for meaningful comment. According to your advice, we added the survival analysis about SYPL2 expression in colorectal cancer patients with stage II and III. And the survival analysis was presented in Figure 1I and J.



24. **Carvalho B**, Lopes JM, Silva R, Peixoto J, Leitao D, Soares P, Fernandes AC, Linhares P, Vaz R, Lima J. The role of c-Met and VEGFR2 in glioblastoma resistance to bevacizumab. *Sci Rep*. 2021; **11**: 6067. [PMID: 33727583] [DOI: 10.1038/s41598-021-85385-1]

25. **Szablewski V**, Solassol J, Poizat F, et al. EGFR expression and KRAS and BRAF mutational status in intestinal-type sinonasal adenocarcinoma. *Int J Mol Sci*. 2013; **14**: 5170-5181 [DOI: 10.3390/ijms14035170]

26. **Khotskaya YB**, Holla VR, Farago AF, Mills Shaw KR, Meric-Bernstam F, Hong DS. Targeting TRK family proteins in cancer. *Pharmacol Ther*. 2017; **173**: 58-66. [PMID: 28174090] [DOI: 10.1016/j.pharmthera.2017.02.006]

27. **Grothey A**, Fakih M, Tabernero J. Management of BRAF-mutant metastatic colorectal cancer: a review of treatment options and evidence-based guidelines. *Ann Oncol*. 2021; **32**: 959-967. [PMID: 33836264] [DOI: 10.1016/j.annonc.2021.03.206]

Revision reiewer

Comment: The manuscript improved significantly during the revision. All of the questions riased were answered. In those questions, where no data were available, a "Limitations of the study" must be added.

Response: Thanks for your recognition of our work. In addition, we have added related “Research Limitations” in manuscript. Research Limitations In this research, we did not discuss the influence about SYPL2 on synchronous metastases and metachronous metastases. We plan to study the question in next research by collecting related clinical characteristics.