Peer Review File

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Reviewer A

The authors were trying to introduce novel biomarkers in CRC.

However, not involve any Inclusion/exclusion criteria for CRC patients!

In addition, the clinical significance of CRC patients is not clear.

The usefulness of the source could be included in the main text as" https://doi.org/10.1016/j.ctarc.2023.100787.

Thank you for your inquiry regarding CRC. The CRC samples were obtained from patients who had been clinically diagnosed and operated, while the control samples were collected from the serum of healthy individuals at the physical examination center. Detailed clinical data can be found in S Table 1. The study included 27 CRC patients (Tumor) and 27 healthy individuals (Normal) (refer to S Table 1). There were no significant differences observed in age and sex between the patients and healthy individuals.

S Table 1. Clinicopathological characteristics of colorectal cancer patients and normals.

Subgroup	**Patients(n=27)**	**Normal(n=27)**
Age (years) [mean ± SD]	60.6 ± 10.9	61.7 ± 10.3
Sex		
Male	18 (67%)	15 (56%)
Female	9 (33%)	12 (44%)
BMI (kg/m2) [mean ± SD]	23.0 ± 3.4	
Tumor size (cm)		
< 5	17 (63%)	
≥5	10 (37%)	
Tumor grade		
Low	2 (7%)	
Moderate	24 (89%)	
High	1 (4%)	
TNM stage		
I	2 (7%)	
II	12 (44%)	
III	7 (26%)	
IV	6 (22%)	

We have put this part of the results (S Table 1) at the end of the manuscript as supplementary materials.

Changes in the text: Lines 493-496.

Reviewer B

The paper titled “Prediction and validation of circulating G-quadruplexes as a novel biomarker in colorectal cancer” is interesting. The increased G4 is an important characteristic in patients

with CRC and has clinical application value as a novel biomarker. The relationship between G4 and TP53 regulation may be a potential target for future cancer studies, and as attention to this area of research increases, the underlying mechanisms may be better understood, potentially benefiting clinical cancer treatment. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) It is recommended to increase the evaluation of the correlation between G4 expression and prognosis and clinicopathological factors in patients with colorectal cancer.

We made a multi-omics analysis of this part of the prognosis evaluation, which will be presented in another article.

Changes in the text: None.

2) The abstract is not sufficient and needs further modification. The research background did not indicate the clinical needs of the research focus.

We have made some modifications to the abstract, and talked about the problems of clinical needs, which require a simpler diagnostic method.

Replace the first sentence of the abstract with the following:

There are some problems in the clinical diagnosis of CRC, such as the difficulty in saving samples, so it is the most popular research work to develop a diagnostic index and method that is easy to obtain, convenient to save and stable. G-quadruplex (G4) is a unique structure found in DNA and it plays a crucial biological role in tumor formation.G4 is derived from DNA with good stability, and the DNA of serum samples is easy to obtain. Therefore, G4 has the potential as an ideal marker for CRC diagnosis.

Changes in the text: Lines 38-44.

3) It is recommended to add in vivo and in vitro experiments to study the biological function of G4.

This is very good advice. We have detected the G4 content of serum DNA in clinical samples, and will detect the G4 content and changes in vivo in cells, so as to better clarify the biological function of G4.

Changes in the text: None.

4) How to judge the prognostic characteristics of colorectal cancer based on the results of this study? How to provide candidate targets for the treatment of colorectal cancer? It is recommended to include relevant descriptions in the discussion.

This is a very good suggestion. In the discussion part, we supplement the change of G4 in the process of CRC prognosis, which decreases with the increase of prognosis effect and increases with the decrease of prognosis effect, that is, the lower G4 is, the better the prognosis effect is, and the higher G4 is, the worse the prognosis effect is. Therefore, how to reduce the content of G4 by transplanting G4 formation pathway or protease may be a target or direction of treatment.

Changes in the text: Lines 289-297.

5) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “c-MYC and HIF1α promoter G-quadruplexes dependent metabolic regulation mechanism of berberine in colon cancer, Ann Surg Oncol, PMID: 22732839”. It is

recommended to quote this article.

Thank you very much for reminding me that we will cite these two articles in the discussion.

(39) Kim HR, Kim HC, Yun HR, Kim SH, Park CK, Cho YB, Yun SH, Lee WY, Chun HK. An alternative pathway in colorectal carcinogenesis based on the mismatch repair system and p53 expression in Korean patients with sporadic colorectal cancer. Ann Surg Oncol. 2013 Nov;20(12):4031-40. doi: 10.1245/s10434-012-2455-7. Epub 2012 Jun 26. PMID: 22732839.

(40) Wen L, Han Z, Li J, Du Y. c-MYC and HIF1α promoter G-quadruplexes dependent metabolic regulation mechanism of berberine in colon cancer. J Gastrointest Oncol. 2022 Jun;13(3):1152-1168. doi: 10.21037/jgo-22-389. PMID: 35837174; PMCID: PMC9274050.

Changes in the text: Lines 289-293.

6) What is the relationship between G4 and tumor-infiltrating immune cells? What role does G4 play in prognosis in tumor? It is recommended to add relevant content.

Thank you for your question. Currently, there is no clear association between G4 and tumor infiltrating immune cells, and there is a lack of research on this topic in the literature. While G4 may serve as a novel diagnostic marker, there is limited research on its prognostic value. However, we have discussed this in detail in the 39th reference of the manuscript.

Changes in the text: None.

Reviewer C

This is an interesting paper about a novel biomarker for colorectal cancer. However, I recommend several points addressed prior to publication.

-Overall I found the paper a bit hard to follow in current writing style.

Thank you for your opinion. We have made a slight adjustment to the writing style.

-The relationship between G4 and p53 was addressed but remains a bit unclear. Its stated that after tp53 ko the G4 activity was decreased. Yet many patients with colon cancer had increase in G4 activity. Many patients with colorectal cancer have tumor mutations in tp53 such as in the APC pathway of crc- I would discuss this.

Your question is highly valuable. In our experiment, we encountered this issue; the primary reason being that TP53 was knocked out, resulting in the loss of its function, whereas the mutation of TP53 altered its function without deletion, thereby leading to different reactions.

Changes in the text: None.

-Is this a colorectal cancer phenomenon or cancer in general to have elevated G4? Do specific mutations predispose to the increase?

G4 is associated with many cancers, see the following references (1, 2, 3, 4, 5).

Some mutations or deletions may also lead to the increase of G4 (References 6, 7 and 8).

1. Nakanishi C, Seimiya H. G-quadruplex in cancer biology and drug discovery. Biochem Biophys Res Commun. 2020 Oct 8;531(1):45-50. doi: 10.1016/j.bbrc.2020.03.178. Epub 2020 Apr 17. PMID: 32312519.

2. Alessandrini I, Recagni M, Zaffaroni N, Folini M. On the Road to Fight Cancer: The Potential of G-quadruplex Ligands as Novel Therapeutic Agents. Int J Mol Sci. 2021 May 31;22(11):5947. doi: 10.3390/ijms22115947. PMID: 34073075; PMCID: PMC8198608.
3. Kosiol N, Juranek S, Brossart P, Heine A, Paeschke K. G-quadruplexes: a promising target for cancer therapy. Mol Cancer. 2021 Feb 25;20(1):40. doi: 10.1186/s12943-021-01328-4. PMID: 33632214; PMCID: PMC7905668.
4. Debbarma S, Acharya PC. Targeting G-Quadruplex DNA for Cancer Chemotherapy. Curr Drug Discov Technol. 2022;19(3):e140222201110. doi: 10.2174/1570163819666220214115408. PMID: 35156574.
5. Hänsel-Hertsch R, Simeone A, Shea A, Hui WWI, Zyner KG, Marsico G, Rueda OM, Bruna A, Martin A, Zhang X, Adhikari S, Tannahill D, Caldas C, Balasubramanian S. Landscape of G-quadruplex DNA structural regions in breast cancer. Nat Genet. 2020 Sep;52(9):878-883. doi: 10.1038/s41588-020-0672-8. Epub 2020 Aug 3. PMID: 32747825.
6. Brown RV, Wang T, Chappeta VR, Wu G, Onel B, Chawla R, Quijada H, Camp SM, Chiang ET, Lassiter QR, Lee C, Phanse S, Turnidge MA, Zhao P, Garcia JGN, Gokhale V, Yang D, Hurley LH. The Consequences of Overlapping G-Quadruplexes and i-Motifs in the Platelet-Derived Growth Factor Receptor β Core Promoter Nuclease Hypersensitive Element Can Explain the Unexpected Effects of Mutations and Provide Opportunities for Selective Targeting of Both Structures by Small Molecules To Downregulate Gene Expression. J Am Chem Soc. 2017 Jun 7;139(22):7456-7475. doi: 10.1021/jacs.6b10028. Epub 2017 May 19. PMID: 28471683; PMCID: PMC5977998.
7. Qin Y, Rezler EM, Gokhale V, Sun D, Hurley LH. Characterization of the G-quadruplexes in the duplex nuclease hypersensitive element of the PDGF-A promoter and modulation of PDGF-A promoter activity by TMPyP4. Nucleic Acids Res. 2007;35(22):7698-713. doi: 10.1093/nar/gkm538. Epub 2007 Nov 5. PMID: 17984069; PMCID: PMC2190695.
8. De Nicola B, Lech CJ, Heddi B, Regmi S, Frasson I, Perrone R, Richter SN, Phan AT. Structure and possible function of a G-quadruplex in the long terminal repeat of the proviral HIV-1 genome. Nucleic Acids Res. 2016 Jul 27;44(13):6442-51. doi: 10.1093/nar/gkw432. Epub 2016 Jun 13. PMID: 27298260; PMCID: PMC5291261.

Changes in the text: None.

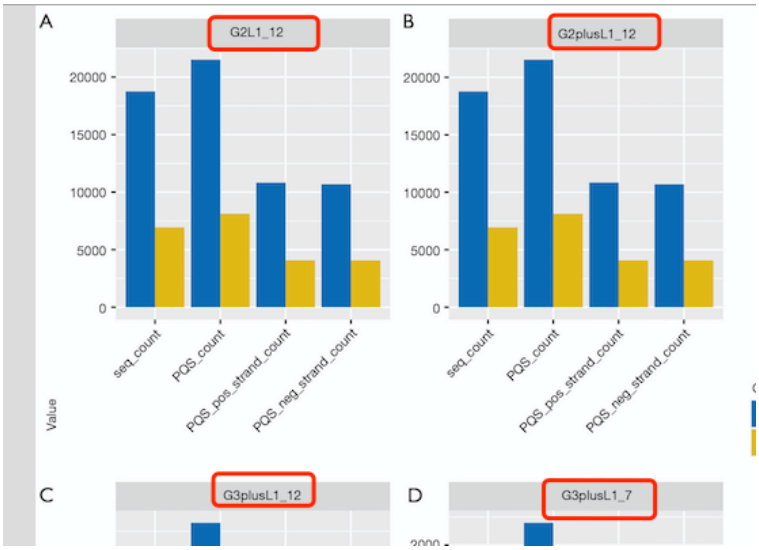
Reviewer D

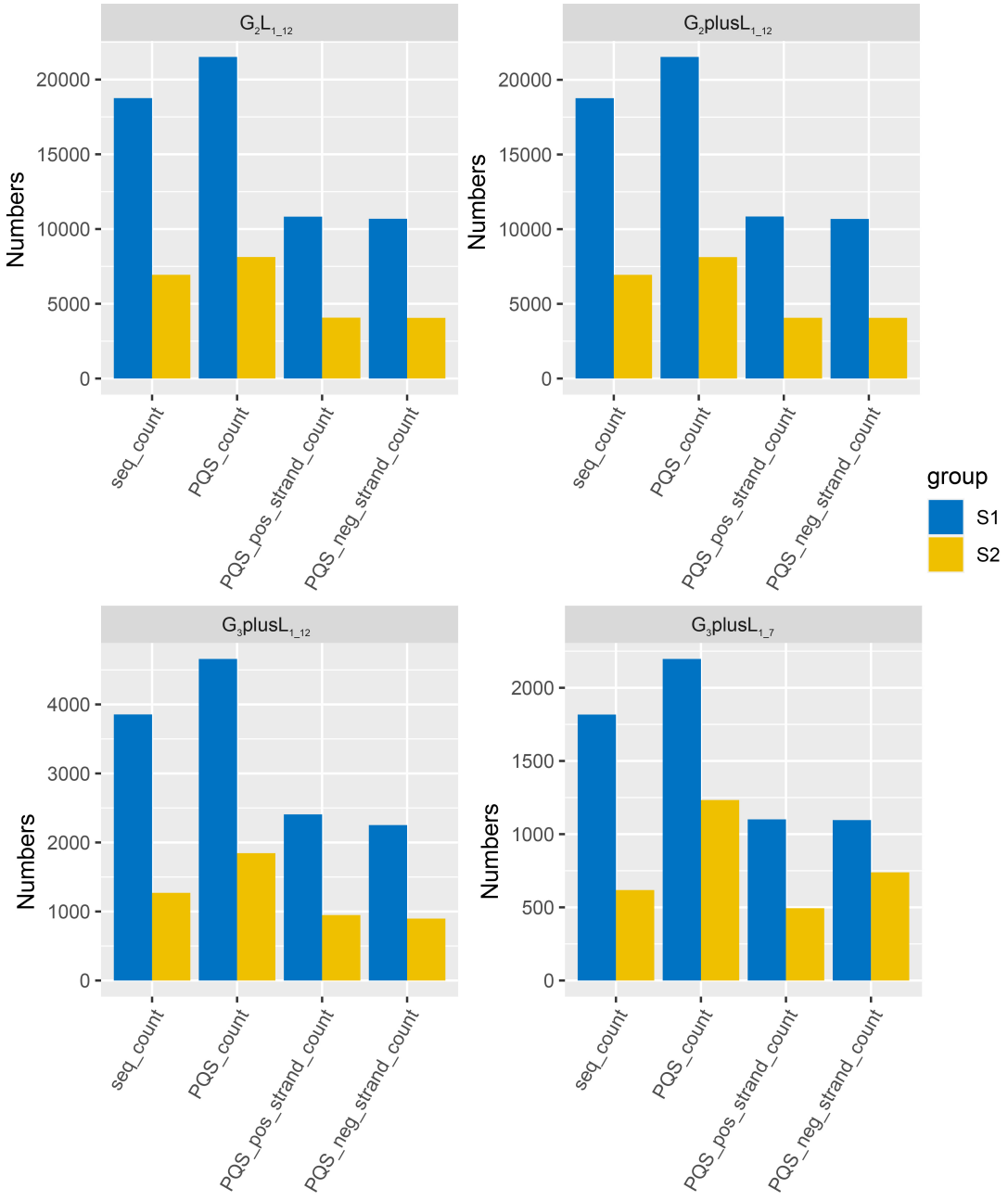
1. Figure 2

Please unify the style.

429 Figure 2. The number of PQSs and non-PQS sin the mutation types (A) $G_2L_{1\_12}$, (B) $G_{2+}L_{1\_12}$,

430 (C) $G_{3+}L_{1\_12}$, and (D) $G_{3+}L_{1\_7}$. The putative G-quadruplex sequences (PQSs) are the 4 PQS


A
G2L1_12
B
G2plusL1_12
20000
15000
10000
5000
0
seq_count
PQS_count
PQS_pos_strand_count
PQS_neg_strand_count
Value
C
G3plusL1_12
D
G3plusL1_7



Changes in the text: Lines 435-436.

2. Figure 3 is a (A-P) combined picture, but 'A-Q' was indicated in the figure legend. Please revise.

the negative chain. A-Q are the four G4 numbers of G2L1_12, G2plusL1_12, G3plusL1_12, G3plusL1_7 in seq_count, PQS_cout, PQS_pos_strand_count, PQS_neg_strand_count, respectively. Ref, reference; Mut, mutant; mut _ anno, annotation of mutations.

Changes in the text: Lines 443.

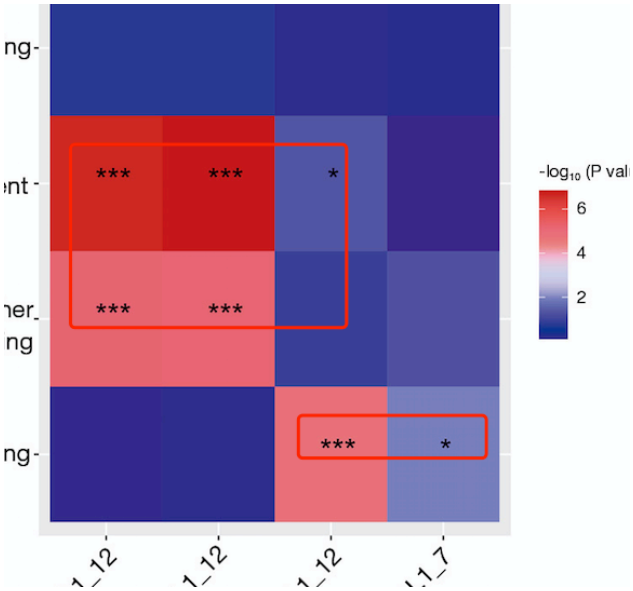
Changes in the text: Lines 449.

3. Figure 4

There is no symbol ** in figure 4, but you explained it in the figure legend. Please revise.

447 Figure 4. Fisher exact test of significance in damaging, other non-conserving, silent and oth

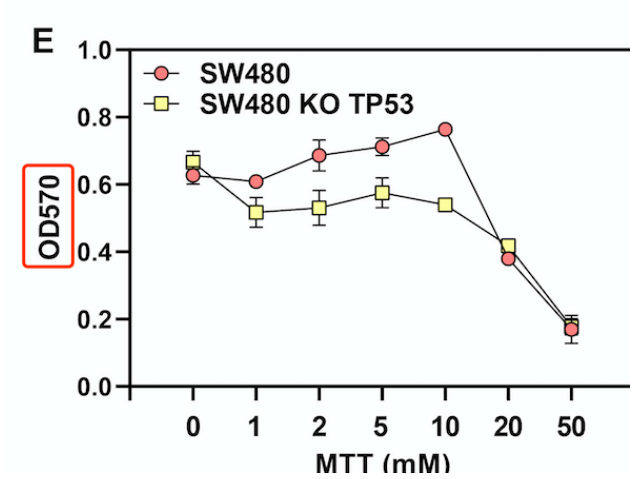
448 conserving. PQS, putative G-quadruplex sequence.***, P<0.001, **, P<0.01, *,P<0.05.



Changes in the text: Lines 457.

4. Figure 6

Please confirm if unit is needed for the Y-axis. e.g., OD (570nm).



Changes in the text: Figure6 E.

5. Figure 7

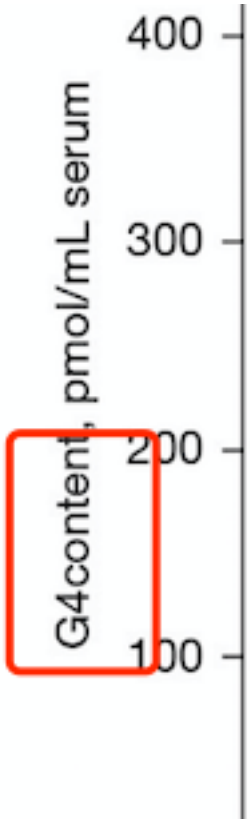
Please check and revise the figure legend (B).

600nm in five concentrations of G4 content. (B) Fluorescence value (RFU) at 490 nm forming a standard curve. RFU, relative fluorescence unit.

Changes in the text: Lines 472

6. Figure 9

Please revise ‘G4content’ to ‘G4 content’.



Changes in the text: Lines 478.

7. Please unify the style.

Fisher exact test requires the construction of contingency tables. With the damaging of $G2L_{1\text{-}12}$ used as an example, the constructed contingency tables are shown in Table 1. As shown in the above figure (Figure 4), the count numbers of reference and mutation vary significantly among different PQSs. This difference is reflected in the silent, other nonconserving sequences of G2L1_12 and G2plusL1_12. The damaging sequence showed the most significant difference in G3plusL1_12.

Mut_class was analyzed in the same way as above, and the result of the Fisher exact test is shown in Figure 5. This result indicated that there are significant differences between G2L1_12 and G2plusL1_12, including In_Frame_In, Nonstop_Mutation, De-novo-Start_OutFrame, Frame_Shift_Del, Splice_Site, Missense Mutation, Frame_Shift_Ins, Silent, In-Frame_Del, and Nonsense_Mutation,. However, in G3plusL1_12 and G3plusL1_7, Splice_Site and Frame_Shift_Ins were significantly different. This indicated that the different types of G4 varied in terms of mutations and were mainly focused in the G2L1_12 and G2plusL1_12 types, because the types have more differences.

475 Table1. The number of damaging and nondamaging types in reference and mutation sequences

476 for G_2L_{1-12}.

	Reference	Mutation
Damaging	52,481	46,008
Nondamaging	274,487+142,935+244	242,046+122,937+242

477

478 Table 2. Test results of PQS discrepancies between the tumor and nontumor gene reference–

479 mutation sequence pairs.

	Sequence Number	PQS number	-	+
G_2L_{1-12}	0.3738	0.0671	0.2343	0.1559
$G_{2+}L_{1-12}$	0.3440	0.0530	0.2556	0.1048
$G_{3+}L_{1-12}$	0.5161	0.0567	0.0544	0.4055
$G_{3+}L_{1-7}$	0.7332	0.0109	0.0489	0.0635

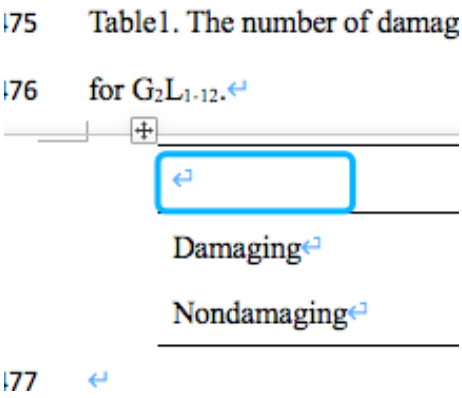
480 The table shows the P values for the Fisher exact test, which indicates that the classical PQS

481 $G_{3+}L_{1-7}$ had significant differences between neoplastic and nonneoplastic genes (P=0.0109).

All the types we mentioned in the article have been changed to subscripts.

8. Tables 1-2

Please add a table header in the first column.



Changes in the text: Added, see lines 485.

178 Table 2. Test results of

179 mutation sequence pair

$G_2L_{1\text{-}12}$	(
$G_{2+}L_{1\text{-}12}$	(
$G_{3+}L_{1\text{-}12}$	(
$G_{3+}L_{1\text{-}7}$	(

180 The table shows the P

Changes in the text: Added, see lines 488.