

Peer Review File

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Reviewer A:

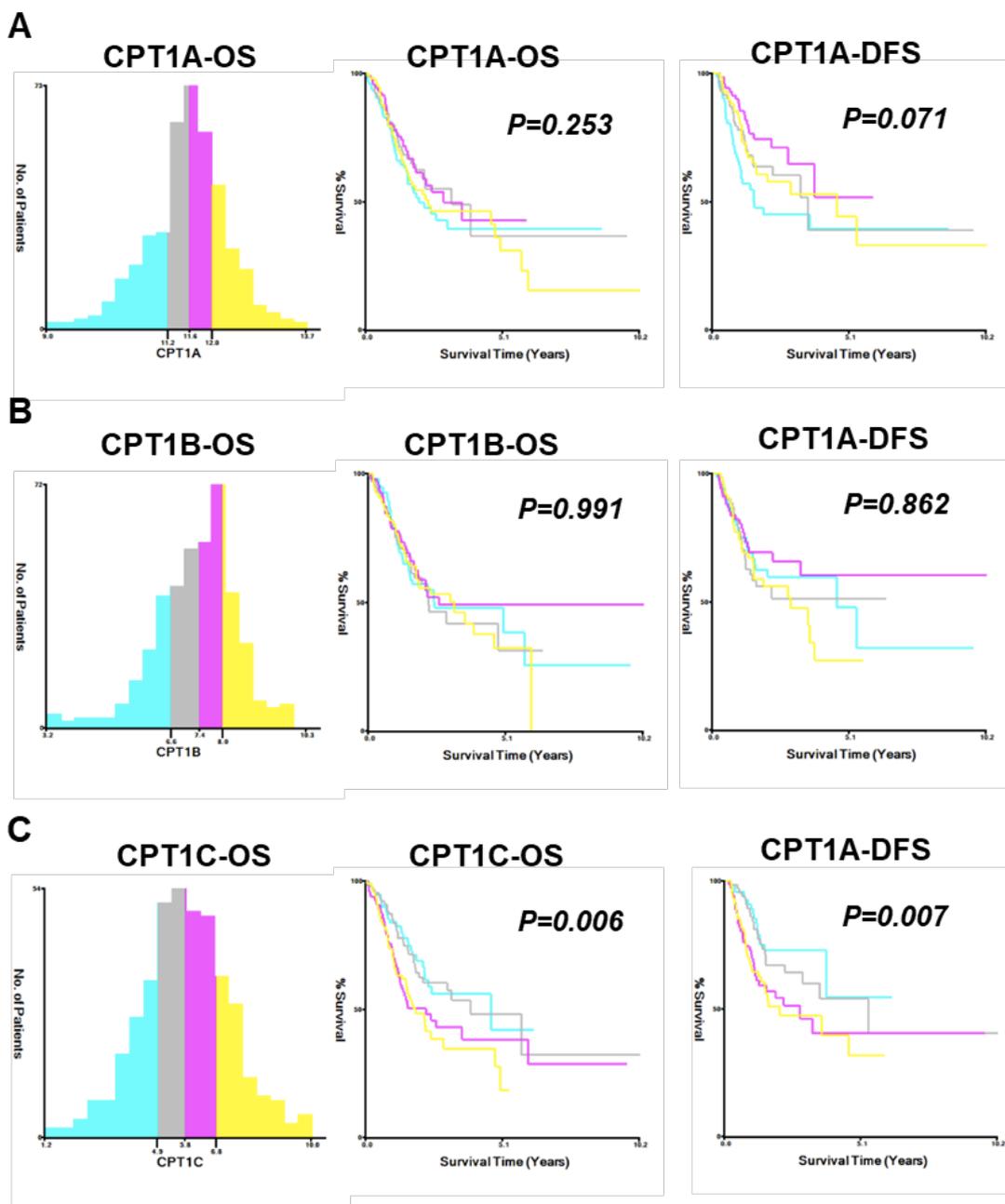
The report about CPT1C as critical enzyme impacting patients' poor prognosis and can promote the proliferation of gastric cancer cells. The topic is of interest. Nonetheless, a few major issues need to be addressed before proceeding with further versions.

Figure 1 and 2: tumour cell expression and prognosis: Despite the functional role of CPT1 in human carcinogenesis, the relationship between CPT1 expression and the clinical prognosis of the diseases has not been clarified. Presently, Kaplan-Meier analysis showed the relationship between the expression of CPT1 and survival in GC patients with different clinicopathological factors (Table 1, 2) OS was significantly negatively correlated with CPT1 expression in GC patients, do the authors checked for post-progression survival (PPS), and first progression (FP) or event/progression-free survival? Moreover, please add the exact definition of CPT1high/low in "Statistical Analysis" section of "Methods" . Please include a figure visualizing absolute CPT1 expression across the entire cohort. Did the author select the median value as a class boundary? This explanation would rather confirm my concerns as the distribution of expression value might not favour a dichotomized separation. It would be crucial to see the respective OS and PFS data for the lowest and highest quartile while sparing the two intermediate, mostly levelled quartiles. Adding this comparison will be very helpful to provide robust conclusions on the presented data, or an alternative explanation should be provided instead.

Response: Thank you for your kind suggestions. In this study, OS and DFS are two outcomes that were used to evaluate the prognostic value of CPT1. Based on previous studies, death and relapse events are the two most important parameters for gastric cancer patients received surgical resection. Instead, PPS and FP were mainly used for metastatic cases and were established endpoints for therapeutic response assessment. We have to admit that analyzing the relationship between CPT1 and PPS or FP was also very important and interesting for evaluating the predictive value of treatment effect of CPT1 in GC patients. Though two large datasets TCGA and GSE62254 were used in this study, the two cohorts are in lack of these data. Future studies are warranted to be conducted to assess this interesting point.

In this study, the median value of CPT1 was used to divide patients into CPT1high/low cohorts and we have added the definition of CPT1high/low in the methods section. To further test the prognostic value of CPT1 in GC, we depicted the distribution of CPT1 in the TCGA database (Figure S1) and performed survival analysis based on quartile value of CPT1 (Figure below). All the isoforms were normally distributed in the entire cohort. Consistent with our previous result, survival analysis showed that CPT1A and CPT1B were not associated with OS and DFS. From the survival curves of CPT1C, the OS and DFS of patients with lowest quartile were notably poor than that of patients with highest quartile, confirming the relationship between CPT1C expression and prognosis. However, the curves of first/second quartile groups and third/fourth groups were overlapped with each other (Figure below). In other words, the

1 survival difference was not distinct between first and second quartile patients or third/fourth
 2 quartile patients. Thus, choosing cutoff value based on median value of CPT1C to depict the
 3 survival difference stratified by CPT1C may be more appropriate than quartile values.



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 6 Methods and patient' s data analysis: to my understanding, the authors interrogated data
 7 from a retrospective cohort. Therefore, it would be useful to better clarify the methodological
 8 statistical approach used in order to limit and even avoid statistical biases. I would strongly
 9 recommend taking into account the interesting networks that can better explain the reported
 10 findings.
 11 Firstly: a multivariate analysis can show the prognostic impact of several variables. Did the
 12 authors check for a statistical association between CPT1 and the other variables with
 13 significant impact within the uni- and multivariate analysis comprising additional factors that

1 can impact on patients' prognosis (i.e. TNM stages, therapy received, HER-2 status, etc.).
2 Those are fundamental details and information in this regard should be added to the results.
3 In the frame of this thinking, and regarding the methods declared, I would point out that the
4 biostatistical tests performed may be statistically significant but biologically less relevant if
5 placed into a more complex context, such as a statistically powered prospective study. To
6 compensate for these limits, the multivariate Cox' s proportional hazard regression models
7 are a worthy tool. Nevertheless, a mandatory assumption needs to be taken into account in
8 order to apply such a model: hazard proportionality. This assumption has to be made in order
9 to proceed with the Cox model. If the answer

10 is affirmative, this should be better highlighted in materials and methods. If it is not, it is
11 necessary to motivate and discuss the use of alternative models.

12 **Response:** According to your suggestions, a multivariate Cox' s regression analysis was
13 performed to test the independent prognostic value of CPT1s. All the variables in the
14 univariate analysis were incorporated into multivariate Cox model. Considering the potential
15 inner expression relationship among three isoforms, CPT1s were incorporated into
16 multivariate Cox model separately and it showed that CPT1C was independently associated
17 OS and DFS in both TCGA (Revised Table 3) and GSE62254 (Revised Table 6) datasets after
18 adjusting other significant confounding factors. In summary, CPT1C is a useful potential
19 biomarker for prognosis prediction.

20 Figure 3 A, E, figure 4 C and for all Western blot figures: densitometry readings/intensity ratio
21 of each band should be included; the whole blot showing all the bands with all molecular
22 weight markers on the Western should be included, at least in the Supplemental Materials.

23 **Response:** According to your suggestions, densitometry readings/intensity ratio of each band
24 have been added and the original uncropped western blots have been submitted as
25 Supplemental Materials (Figure S2)

26 RNA isolation, reverse transcription, and qRT-PCR: The authors show expression levels relative
27 to 1. For consistency reasons, absolute values should be added and RNA expression levels in
28 controls should be included.

29

30 **Response:**

31 All the RNA expression value depicted in this study were based on $2^{-\Delta\Delta Ct}$ which is the most
32 common used method in basic research. The $2^{-\Delta\Delta Ct}$ calculation is a convenient alternative
33 method to derive accurate quantitative information from real time PCR assays. It is indeed a
34 relative value. By using this method, we can illustrate the result of RNA expression more
35 concisely and conveniently.

36 General comments Do original clinical data exist about the translational relevance of the
37 mentioned result? If yes, these elements should be presented, at least in the form of
38 discussion and/or additional figure from a short literature meta-analysis, according to the
39 authors' preferences at least regarding CPT1C

40

41 **Response:** In this present, we for the first time revealed the prognostic value of CPT1C in
42 gastric cancer. To date, though several previous studies have uncovered that CPT1C
43 expression correlates inversely with mammalian target of rapamycin (mTOR) pathway
44 activation, contributes to rapamycin resistance in murine primary tumors, and is frequently

1 up-regulated in human lung tumors, limited studies focused on the prognostic value of
2 CPT1C. A previous study analyzing >9000 primary or metastatic tumor samples in TCGA
3 dataset established a gene signature containing CPT1C that is strongly associated with
4 epithelial-mesenchymal transition process, manifesting the potential translational relevance
5 of CPT1C and solid tumors. We have revised the discussion part according to your
6 suggestions.

7 General comments: An important link exists between hypoxic cell metabolism, tumour acidity
8 (lactic acid efflux), CPT1C function (Fatty Acid Oxidation Controls CD8+ Tissue-Resident
9 Memory T-cell Survival), and immune tolerance. This is because tumour acidity acts as a broad
10 immune escape mechanism by which cancer cells, inhibit anti-tumour immune effectors
11 (including T cells, NK cells and crucial antigen-presenting dendritic cells), while simultaneously
12 promoting the immunosuppressive and pro-tumour properties of regulatory T cells and
13 myeloid cells. This is of paramount importance in conditions deeply implicated in the immune
14 status equilibrium, as a major driver of GC initiation (PMID: 29393912). Moreover, this acidic
15 microenvironment may also specifically promote CPT1 orchestrated activity and thus further
16 impact cell survival and cancer progression. This author felt that this a good way of linking
17 these seemingly disparate topics of cancer metabolism, hypoxia, CPT1 phenotype and
18 immune function with immune surveillance and migration/invasion and the consequent
19 metastases that were highly relevant to the author study.

20 In the frame of this thinking, the emerging roles of lipid metabolism in cancer metastasis
21 (PMID: 28399876), especially taking into consideration connection between CPT1C and WNT
22 in immune-surveillance and nodal dissemination as demonstrated in several gastrointestinal
23 tumours (PMID: 31277479). This topic can offer fundamental combination strategies able to
24 increase the biological and translational landscape of the presented results.

25 Response: Thank you very much for your invaluable inspiration about the relationship
26 between lipid metabolism and the other critical signaling pathway. In our future work, we will
27 focus on exploring the potential mechanism of CPT1C promoting cancer progression and
28 uncovering novel function of CPT1C based on transcriptome profiling and mass spectrometry
29 method.

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32 **Reviewer B:**

33 The aim of this study was to analyze the relationship between fatty acid oxidation and gastric
34 cancer. The expression of carnitine palmitoyltransferase 1c (CPT1C) was analyzed according
35 to RNA sequencing and microarray data sets in TCGA and GEO cohorts. In both cohorts, high
36 expression of CPT1C was associated with shorter overall and disease-free survival rates.
37 Gastric cancer cell experiments demonstrated that silencing of CPT1C suppressed cell
38 proliferation and induced cell cycle arrest, while enhanced CPT1C expression had the opposite
39 effects. Moreover, the expression of CPT1C was up-regulated by HIF-1alpha. The results
40 demonstrate that CPT1C-dependent fatty acid oxidation stimulates proliferation of gastric
41 cancer cells. In contrast, the expression of CPT1A and CPT1B did not correlate with survival.
42 The topic and the results are of interest. However, there are also some concerns to be
43 addressed.

44 1) Introduction, page 4, paragraph 1, the phrase "5-year overall survival of GC remains very

1 short" is not correct. The 5-year survival rate can be poor but not short. If one talks about
2 short survival, the time period cannot be specified.

3 Response: According to your suggestions, we have revised our statements.

4 2) Page 4, paragraph 2, the phrase "which making CPT1 as a potential therapeutic target"
5 should be corrected to "which makes CPT1 as a potential therapeutic target" .

6 Response: Thank you very much for your correction. We have revised the grammar mistake.

7 3) Page 5, section entitled "Cell culture" : it should be specified which gastric cancer cell
8 line(s) was/were used.

9 Response: We have specified the choose of gastric cancer cell lines in Cell culture section.

10 4) More details about apoptosis and cell viability detection should be provided. In particular,
11 flow cytometry procedure should be described for FITC Annexin V Apoptosis Detection Kit
12 and method of absorbance measurement for CCK-8. Similarly, fatty acid oxidation assay
13 should be described in more detail.

14 Response: According to your suggestions, we have described the cell cycle and cell viability
15 detection and fatty acid oxidation assay more in detail.

16 5) Although CPT1C overexpression/silencing clearly affects fatty acid oxidation, there is no
17 direct evidence in this study that changes in fatty acid oxidation were involved in the effects
18 on cell viability/apoptosis. CPT1C could affect cell phenotype by changing fatty acid transport
19 to mitochondria irrespectively of the effects on FA oxidation. For example, FA oxidation may
20 not be stimulated in cancer cells despite high CPT expression due to hypoxia common in
21 many solid tumors.

22 Response: Fatty acid oxidation is an important metabolic process facilitating cancer cell
23 growth under metabolic stress. To further confirm the effect of FAO mediated by CPT1C in
24 GC cells, we used FAO inhibitor Etomoxir to treat cells with ectopic CPT1C expression. It
25 showed that Etomoxir treatment completely restrict the increase of FAO rate, cell viability and
26 the phase of DNA synthesis caused by enhanced CPT1C expression (Revised Figure 4).
27 Therefore, we believe that the increase of FAO rate mediated by CPT1C is essential for cell
28 growth of GC.

29 6) More details about Western blotting should be presented. The method of protein
30 extraction and SDS-PAGE should be described. It should be stated how much protein was
31 subjected to electrophoresis. Names and catalogue numbers of secondary antibodies should
32 be specified. The number of samples in each group should be listed.

33 Response: According to your suggestions, more details about Western blotting have been
34 presented. In addition, densitometry readings/intensity ratio of each band have been added.

35 7) Statistical analysis: how was normality of data distribution verified to choose between
36 Student and Wilcoxon tests?

37 Response: In this present study, because of limited sample size, we used Kolmogorov-
38 Smirnov (KS) method to test the goodness of fit. Once the p value of KS test was <0.05, it is
39 believed that the data is not normally distributed and Wilcoxon tests will be used, otherwise,
40 Student t test will be performed.

41 8) Why CPT1C silencing and overexpression were done in different gastric cancer cell lines?

42 Response: When choosing which cell line to silence or enhance CPT1C expression, we mainly
43 cared about the baseline expression level of CPT1C and the aggressive features of cell lines.
44 By detecting the baseline level of CPT1C, we silenced CPT1C by using small short hairpin RNA

1 technology in two cell lines, AGS and MGC803, which showed high baseline expression of
2 CPT1C and relatively strong aggressive feature. Instead, we enforced CPT1C expression in
3 MNK45 and SGC7901 cell lines, which showed low baseline expression of CPT1C and relatively
4 weak aggressive feature.