Author’s response to reviews

Title: Valproic Acid Sensitizes Metformin-resistant Human Renal Cell Carcinoma Cells by Upregulating H3 Acetylation and EMT Reversal

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Author’s response to reviews:

Dear Dr Chakrabarty:

Thank you very much for your letter and for giving us the opportunity to revise our manuscript entitled "Valproic acid sensitizes Metformin-resistant human renal cell carcinoma cells by upregulating H3 acetylation and EMT reversal", manuscript ID BCAN-D-17-01686. We also want to thank for the reviewers for their careful read and detailed comments on our previous draft. We have studied their comments carefully and have made correction which we hope could meet with their approval. We are sending back the revised manuscript according to the comments of the reviewers, and the revised portion are highlighted in red. We list the responses to comments point by point in this letter.
Thanks for considering our manuscript.

Editor Comments:

Reviewer reports:

Ekta Khattar (Reviewer 1):

1. The scientific writing and grammar needs tremendous improvement.

R1: We asked a professor and a foreign student who is fluent in English to perform a thoroughly revision on the manuscript and carefully corrected the mistake in English expression in our revision and rewrote some sentences to make sure our opinions were clearly stated.

2. In figure 8, vpa+metformin is not mentioned in the text.

R2: We add the descriptions of the cell morphology changes in VPA + Met group in the results part. Line 317-319

3. In Figure 2c and 3d, single time points are captured for cell cycle analysis. So, it is difficult to say whether it is arrest or changes in cell division rates. It would be better to show at least two-time points to understand the cell cycle status.

R3: Thank you for mention that it would be better to show at least two-time points to detecte the cell cycle. In the revision, we repeated the cell cycle test in 786-O and 786-M-R and settled two time points (24h and 48h). Besides, the machine we used for cell cycle analysis before was replaced by a now machine. You will find that the pictures put in the revision is not the same as before. We add the new results in Figure 2c, 3e, and the new describing in methods and results sections. Line 140-145, Line 224-229 and 246-248.
4. In Figure 7d the positive control for rapamycin treatment which is its target S6K phosphorylation should be checked to ensure that drug is working.

5. In figure 7d SC79 does not seem to increase Akt phosphorylation significantly. Please describe accordingly in the text section.

R4: R5: After thinking about the propose to use AKT activators and inhibitors, to detect the potential connections between AKT and aH3, we redesigned the groups of VPA and drugs. Like shown in figure 6e, we treated the 786-M-R cells with AKT activator (SC79), AKT inhibitor (AKT inhibitor V, LY294002) and rapamycin with or without VPA. The results showed that SC79 significantly increase the phosphorylation of AKT and S6K in the presence of VPA. Besides, according to reviewer’s suggestion, we use western blotting to detect the phosphorylation of S6K. The levels of pS6K were inhibited by rapamycin.

6. In discussion, line 294 states normal RCC cell lines. Please rewrite this as RCC cell lines. You cannot write normal.

R6: We are sorry for our mistake to wrote “normal” to describe cancer cell lines, which is clearly inappropriate. We rewrite this as RCC cell lines. Thank you for pointing out to us.

Anindita Chakrabarty, PhD (Reviewer 2): The goal of the reported study was to test Whether 1. Chronic metformin treatment induces resistance in RCC cells and 2. metformin resistance in RCC can be reversed by combining it with a histone deacetylase inhibitor valproic acid (VPA). While searching for the molecular mechanism that might contribute to metformin resistance, the authors identified up regulation of EMT markers, down regulation of pAKT, pAMPK and acetylated H3 (aH3). Combining VPA with metformin reversed this phenomenon. The authors implicated that metabolic reprogramming by pAMPK and pAKT determines the changes in histone acetylation levels in resistant cells which in turn affects EMT. Several points need to be addressed for consideration for publication in BMC Cancer. These are:
1. Authors should improve the English writing for clear representation of facts and findings.

R1: We asked a professor and a foreign student who is fluent in English to perform a thoroughly revision on the manuscript and carefully corrected the mistake in English expression in our revision and rewrote some sentences to make sure our opinions were clearly stated.

2. Authors should check cross resistance of metformin R line with similar class of drug (e.g. phenformin or related AMPK activators). This will help to deduce if the resistance is due primarily to modulation of AMPK activity following chronic metformin exposure.

R2: According to the suggestion of Dr. Chakrabarty, we performed CCK8 test to detected the IC50 to phenformin of 786-M-R. The result is in Figure 3b. The IC50 to phenformin in 786-O cells is 1.51mM, and in 786-M-R is 4.28mM. There exists a cross resistant to phenformin and metformin in 786-M-R cells, suggesting the resistance is largely due to modulation of AMPK activity.

3. EMT is often associated with gain of stem-like marker expression and functional properties of stem-like cells. Authors should check whether EMT induction in metformin R line is correlated with acquisition of stem-like phenotypes and whether this can be reversed by HDAC inhibitor like VPA.

R3: The stem-like marker expression was found related with EMT process. We use western blotting to check the expression of ZEB1, a well-known stem-like marker, in 786-O and 786-M-R. The results were appended into each figure. Like fibronectin and N-cadherin, ZEB1 can be inhibited by VPA and metformin and this inhibition can be enhanced by VPA and metformin combination.

4. Authors should check whether another general HDAC inhibitor like TSA or vorinostat can be as effective as VPA against metformin R cells. This will confirm whether reversal of metformin resistance is truly associated with change in aH3 levels.

R4: We used TSA, another general HDACI, to combine with metformin and detected the function of TSA in 786-M-R. As the results showed in figure. 6d, TSA + Met significantly
inhibited the levels of pAKT, in 786-M-R, suggesting TSA can partly reverse the metformin resistance by upregulating the sensitivity of AKT. These results supported our conclusions that VPA can increase the sensitivity of AKT by upregulating aH3 in 786-M-R cells. Line 302-307.

5. Authors should include a pictorial representation of tentative roles of aH3, pAMPK and pAKT in conferring metformin resistance in RCC cells.

R5: We add fig. 8 to pictorial represent the potential roles of aH3, pAMPK and pAKT in RCC cells and in metformin resistance RCC cells. The long-term application induces resistance to metformin in RCC and this resistance mainly based on the low sensitivities of AMPK/ AKT and TGF- β/SMAD3 pathways. However, the addition of VPA can counteract the resistance through upregulating of aH3 therefore increasing the sensitivities of pAKT, and inhibiting pSMAD3/SMAD4 therefore reversing the EMT process

Common: major revision required.

Prathibha Ranganathan (Reviewer 3):

1. Metformin is a drug used to treat diabetes. It does have an advantage in treating renal cancers and other cancers as well. But it is not used as a stand alone treatment. The rationale for using metformin and valproic acid to demonstrate synergistic effect is not very clear in the manuscript. metformin alone has very little anti-cancer effect as the authors have mentioned.

R1: The comments of Dr. Ranganathan made us notice that our objectives of the present study had not been clearly stated in the manuscript. Thus, we rewrote a large part of the introduction section. Like we stated in line 61-79, the antitumor activities of metformin in experimental studies were always been detected, but there are many epidemiological and observationally analysis suggested that no significant reduction in risk of cancer under metformin treatment. Because the experiments performed in cells usually cared more about the short-term impacts of metformin, while the treat time of metformin in clinical trials was much longer. The time difference may be the predominant reason of the inconsistent results in studies, suggesting that the resistance of cancer cell caused by long-term metformin treatment may be the reason to explain use of metformin could not reduce the risk of cancer in clinical studies. Thus, we designed the present study to detect the possible metformin resistance in long-term metformin
treatment and the mechanism underlying the metformin resistance and to investigate whether this resistance can be broken by combination with VPA.

2. What is the basis of resistance caused by metformin?

R2: There are researchers who have studied the resistance caused by metformin. Scherbakov et al. suggested that acquired resistance was detected after long-term administration of metformin in breast cancer cells and partly based on the constitutive activation of AKT and E-cadherin signalling pathway maintaining the autonomous growth of the resistant cells[1]. In present study, our results showed that the resistance to metformin in 786-M-R is mainly based on the low activities and sensitivities of pAMPK/pAKT and TGF-β/SMAD3.

3. The concentrations of drugs used is in the milli molar range which seems rather high. The effect seen may be just due to toxicity and may not be a specific effect

R3: As the reviewer suggested, the concentrations of Met and VPA we use were higher than the therapeutic concentrations. Like we explained in results section, first we determined the IC50 of metformin (18.63-39.73 mM) and VPA (2.073-2.424mM), then we evaluated the combination index to choose the concentration group (Met 10mM + VPA 1mM) which has the least toxicity as well as the biggest synergetic effect. Besides, in previous studies about the antitumor activities of metformin in RCCs, the concentrations chose by researchers were 5-20 mM [2], 10 mM [3], 16-32 mM [4], the optimal proliferation inhibition concentration of Met was 10mM [5]. In previous studies about VPA and RCCs, the concentrations used by researchers were 1mM [6], and “a clinically safe dose1 mM of VPA” [7]. Like we discussed in Line 338-347, the inhibitory effects in most of laboratory studies were observed at concentrations which are at least 10-fold higher than the peak plasma concentration attained with typical dosing in diabetics, while there are emerging studies which show that even very low doses of metformin could have substantial anti-cancerous effects. Thus, we believe the concentrations we used in the present study are appropriate.
Minor points

1. The figure legends need to be more clear

R1: All the figure legends were carefully checked and corrected to make sure the abbreviations appeared in figures were well explained and the legends were adequate and clear to describe the figures.

2. Fig 1-- It would be better to represent the IC50s in a tabular form also

R2: As the reviewer suggested, we agree that it is not very clear that representing the IC50s in the curve. Thus, we added two bar graphs of IC50s in Fig 1 a, b.

3. Fig 2a-- the difference between Met alone and VPA + Met on ACHN cells is very marginal

R3: As the reviewer suggested, we repeated the CCK8 tests to detect the influences of Met, VPA and VPA+Met in ACHN cells. The new results are showed in Fig.2 a.

4. Fig 3d-- the differences in the cell cycle profiles not very significant

R4: In the revision, we repeated the cell cycle test in 786-O and 786-M-R. Besides, the machine we used for cell cycle analysis before was replaced by a now machine. You will find that the pictures put in the revision is not the same as before. We add the new results in Figure 2c, 3e, and the new descripting in methods and results sections. Line 140-145, Line 224-229 and 246-248.


