Peer Review File

Article information: https://dx.doi.org/10.21037/tcr-23-1272

Reviewer A

Ren et al use mainly databases on cancers which they use to find correlations between expression levels of the endosomal Cl/H exchanger ClC-3 and various data such as proliferation of cancers cells and patient survival. From this, they find that in some cases there is a positive, in others a negative correlation. For ovarian cancer cells, they actually perform one experiment: they knock down ClC-3 (at least shown on mRNA levels) by siRNA and measure cell amount (CCK8) and levels of PI3K and AKT.
In its present form, the manuscript is not very clear because there are changes in ClC-3 levels in different ways varying between different cancers, which is hardly discussed. The experiment is done with a single siRNA, no rescue experiment has been performed.
Here are some specific points to improve the manuscript:
Major:

1)On the only one experiment in the manuscript: a) how robust is the data when the knockdown was performed with only one siRNA and no rescue by re-expression has been done? Could it be unspecific off-target? b) Figure 9B: do the growth curves actually start at the same value (it is hard to see)? This is important because an exponential growth (which is not really observed here) will increase small differences in the beginning. c) The increase in PI3K and AKT (Fig.9C) should be quantified. d) An increase in PI3K and AKT alone does not mean a lot. Can the authors show that there is more active PI3K or AKT?

Reply 1: Thank you very much for your professional comments. a) We added another cell line and a brand new siRNA for double validation (see line 106-108，265-282，493-497 and figure 9) b) We try to pick initial values that are as close as possible for plotting (SKOV3: NC: 0.180,0.177,0.177; siRNA: 0.199, 0.202, 0.198. OVCAR433: NC: 0.359,0.334,0.346;siRNA:0.331,0.342,0.345.) c) We have added new plots in Figure 9 that quantify PI3K and AKT (see Figure 9C, D). d) We performed RT-PCR and WB duplicate validation of different siRNA knockdowns in two different cell lines and confirmed exactly that PI3K and AKT were also changed after CLCN3 alteration. Reference 27[1] in the article verified the PI3K total protein changes, which can prove that the total protein changes have a significant effect on the tumor, and as for the said more active proteins, they can be verified in future studies due to time and funding issues.

Changes in the text: line 106-108，265-282，493-497 and figure 9

---

2)Figure 1C: there are no signs of significance. Is there just no significant effect on ClC-3 protein levels? Isn't this what the manuscript is then based on?

Reply 2: Thank you very much for reminding us. We added the p-value in Figure 1C (see Figure 1C).
Changes in the text: Figure 1C

Medium:

3)The title is misleading. ClC-3 is not a channel and there are no data in the manuscript showing ClC-3 "mediates" the migration.

Reply 3:Thank you for pointing out our mistakes. We have changed the title to "CLCN3 mediated proliferation of human ovarian cancer cells" (see line 1).
Changes in the text: line 1

---

4)The introduction lacks important information about ClC-3, such as that ClC-3 is actually not a chloride channel but a Cl/H exchanger, and that it is not present on the plasma membrane but on endosomal compartments (Jentsch and Pusch 2018).

Reply 4: Thank you for pointing out our mistakes. We have modified our text as advised (see line 44-46)
Changes in the text: line 44-46

---

5)The text is also hard to read (basically at some point the reader just doesn't know what the authors are writing about) because there are so many abbreviations, some of which are spelled out nowhere in the manuscript: CESC? LAML? CPTAC? UCEC? LUAD? LUSC? ESCA? … and so on.

Reply 5: Thank you for your valuable advice. We revise the first occurrence of the noun in the article to the full name (see line 180-257).
Changes in the text: line 180-257

---

6)Lines 46-51: The literature deserves a bit more critical statements instead of just confirming what those

studies proposed. Rather “ClC-3 was reported to..” or “it was associated with…”

Reply 6: Thank you very much for your suggestion. We have modified our text as advised (see line 50-53)

Changes in the text: line 50-53

---

7)Line 230: do they actually interact (where and how was that shown?) or were they predicted to maybe (!) interact?

Reply 7: The p-values in Figure 6B-C demonstrate statistically significant interactions (see Figure 6B-C)

.

---

8)For Discussion: If ClC-3 has a reducing effect on cancer cell proliferation, what about the cancers with reduced ClC-3?

Reply 8: ClC-3 is indeed reduced in some cancers and there are some functions performed by low-expressed ClC-3. Cancer regulation is complex and there is heterogeneity in the regulatory mechanisms in different cancers. Thus, in some cancers, low expression of ClC-3 can perform similar or different functions.

---

9)Line 279: yes, link shown previously. But in references 30 and 31, the effect is opposite. This needs to be discussed.

Reply 9: Thank you for your comment. Different genes can have different effects in different cancers. For example, GPX, the iron death-related protein, which plays a promotional role in endometrial cancer[2], yet plays an inhibitory role in breast cancer[3], and there are many other genes that play different roles in different cancers, which is a reflection of genetic complexity.

---

10)It would also be interesting to see what the authors speculate what a mechanism might be there for CLC-3 to affect proliferation, considering it is an endosomal protein (effect of vesicular Cl concentrations?). As plasma membrane Cl channel, VRAC has been shown to upregulate PI3/AKT (PMID: 28262948 (but not confirmed by PMID: 31151189)).

Reply 10: Thank you very much for your professional comments. In the future, we hope to conduct more in-depth mechanistic studies based on our current research.

Minor:

11)Line 54: “… greatly aided by gene.” The sentence does not seem to be complete.

Reply 11: Thank you very much for your suggestions. We have modified our text as advised (see line 54-55).

Changes in the text: line 54-55

---

12)Line 58: what do the authors mean be “mutation status”?

Reply 12: Mutation status are structural changes in the composition or arrangement of base pairs of genes. Inactivation of tumour suppressor genes and activation of oncogenes are associated with the development of many cancers[4]. Therefore, we analysed gene mutations in multiple cancers through the cBioPortal platform.

---

13)Spelling of some termini required. E.g., Cl with capital C, CLCN3 is the gene name and should be italic, name of protein is ClC-3, …

Reply 13: Thank you very much for reminding us. We have modified our full text as advised.

---

14)Lines 276-277: “exchange channel” makes no sense. It is an exchanger or antiporter, but no channel. Ref 29 does not fit here.

Reply 14: Thank you for pointing out our mistakes. We have modified our text as advised (see line 294-295,323-325)

Changes in the text: line 294-295,323-325

Reviewer B

The goal of identifying genes that may serve as markers or therapeutic targets for various cancers is an important one; toward that end the authors attempt to delineate the role of CLCN3 in ovarian (and others) cancer.

While this could be a very valuable contribution, there are a number of issues that need to be addressed.

1.There are issues with grammar; for example, see lines l 47, 38, 54, 60, 105, 175. Improved proofreading is necessary.

Reply 1: Thank you very much for advising us about the language of our manuscript. We will use your journal's language editor for grammatical touch-ups to publication standard.

---

2. line 329: The authors write: "The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request." this is not acceptable; the datasets should be deposited in a public database.
Reply 2: Thank you for pointing out our mistakes. We have modified our text as advised (see line 351-353).
Changes in the text: line 351-353

---

3. The authors should define all acronyms in the paper.
Reply 3: Thank you for your valuable advice. We revise the first occurrence of the noun in the article to the full name (see line 180-257).
Changes in the text: line 180-257

---

4. Way more detail is needed regarding the bioinformatics/gene expression analysis. For example, the authors state they used TIMER, but what parameters? What exact databases? Everything in the database? All of the experiments in databases? There is generally WAY too little data/explanation/detail in the methods used. All the sections are too brief but the promoter methylation is unacceptably brief. Software version is also paramount.
Reply 4: Thank you for your professional comment. We have cited references after each of the databases we have applied, which are used according to the instructions in the references.

---

5. Gels should be shown in full - not cut bands.
Reply 5: Thank you for your comment.The western blot gel is complete.

---

6. OD should not be used as the only indicator of cell proliferation.
Reply 6: Thank you very much for advising us about the methods of our manuscript. The OD value of CCK8 is very widely used[5], of course, other methods to prove proliferation such as EDU also exist, but due to the limitation of experimental funding, we will further verify it in subsequent studies.

---

7. The data on the PI3K/AKT signaling pathway is not completely new; as the authors state, this is no unexpected. The authors absolutely need to examine some other major pathways that are affected in their siRNA experiments; an RNASEQ experiment would be ideal here or at least a number of experiments (qRT) looking at other genes pathways.
Reply 7: Thank you very much for your professional comments. We performed RT-PCR experiments for changes in PI3K/AKT gene expression after knockdown of CLCN3 in SKOV3 and OVCAR433 cells (see line 142-147，279-282, Figure 9).

Changes in the text: line 142-147，279-282, Figure 9

---

8. A much more thorough discussion of the data is warranted. The data on this gene and its relationship to cancer is all over the place; in some cases decreased level are associated with cancer aggressiveness; in some cases upregulated levels are associated with tumor aggressiveness. The authors need to explain this incredible diversity of associations of this gene/protein in order to argue that it serves as a possible marker or therapeutic target.
Reply 8: The reviewer is right, the changes of genes/proteins are complex and the correlations with tumours are diverse, we have initially explored the important role of CLCN3 in tumours through raw letters and experimental validation, but the mechanism of action still needs to be further explored.

---

9. The authors should try additional cell lines to ensure their result could be generalized.
Reply 9: Thank you for your valuable advice. We added the OVCAR433 cell line and repeated the knockdown of siRNA and WB for validation.

Changes in the text: 106-108，265-282，493-497 and figure 9
