Peer Review File

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Reviewer A

In this study the authors evaluated the role of ATP synthase F1 subunit α (ATP5A1), in renal cell carcinoma through integrated bioinformatics analysis and functional experiments. This is a very well performed study with interesting results. I have some comments:

-RCC is essentially a metabolic disease characterized by a reprogramming of energetic metabolism (PMID: 36960789; PMID: 30983433, PMID: 36430837, PMID: 36310399). In particular the metabolic flux through glycolysis is partitioned (PMID: 29371925, PMID: 28933387, PMID: 25945836), and mitochondrial bioenergetics and OxPhox are impaired, as well as lipid metabolism (PMID: 30538212; PMID: 32861643, PMID: 29371925, PMID: 36430448, PMID: 38540735). This study showed thatvATP5A1 expression is downregulated in ccRCC in accordance with mitophagy described in RCC (PMID: 30538212). These findings should be referenced and discussed.

Reply: We extend our heartfelt appreciation for the invaluable feedback you provided on our article. We have carefully integrated the pertinent references into our manuscript.

Reviewer B

The study investigates the role of ATP5A1 in clear cell renal cell carcinoma (ccRCC). Via TCGA data, the investigators could show that ATP5A1 was significantly down-regulated in ccRCC tissues compared to normal kidney tissue. Furthermore, ATP5A1 correlated inversely with ccRCC aggressiveness, but directly with ccRCC prognosis. The suppressive influence of ATP5A1 on the malignant behavior of ccRCC cells was underlined be quite extensive in vitro data.

The manuscript is well written and of interest to the RCC research community. However, there are a few things that need to be improved before publication.

Abstract

“Compared to 293 cells and adjacent ccRCC tissues, renal cancer cells and tissues exhibited a significant reduction in ATP5A1 expression.” – The comparison is not against “adjacent ccRCC tissue” but rather adjacent normal kidney tissue, isn’t it? This should be amended.

Reply: We thank the reviewer for the comment. We have changed the adjacent ccRCC tissue to the adjacent normal kidney tissue.

Methods section

1) Please provide the manufacturer and the clone or catalog number as well as the dilutions of all antibodies used in this study.

Reply: We appreciate your valuable feedback. We have provided the catalog number

and dilution of the antibody in the manuscript (see Page 5, line 119-122).
2) Which secondary antibody was used for Western Blot? Which dilution? Please provide this information!
Reply: We appreciate your valuable feedback. We have provided the catalog number and dilution of the secondary antibody in the manuscript (see Page 5, line122).
3) "Cell transduction studies": Please amend this section to a logical order. Introduce the abbreviations for the plasmids and siRNAs including controls that are later used in the figures. How much siRNA was used for transfection?
Reply: We appreciate your valuable feedback. We have revised the section on Cell Transduction Studies (see Page 6, line137-146).
4) "Wound-healing assay": the start of the first sentence needs to be adjusted. "For transfection, …" should probably be "After transfection, …"?
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page 6, line148).

5) "Flow cytometric analysis of cell apoptosis": This paragraph is written like a manual instruction. Please adjust to the style of the other methods described! Were transfected cells used? In addition, it reads as if apoptosis was induced in all samples by adding a positive control. Was that so? Please clarify!
Reply: We appreciate your valuable feedback. We have revised the content of Flow Cytometric Analysis of Cell Apoptosis (see Page 7, line165-175).

6) Please clarify somewhere that 293 cells are normal cells and not ccRCC cells.
Reply: We appreciate your valuable feedback. We have added an explanation about 293 cells in the Cell Lines section of the manuscript (see Page4, line101).

Results section
1) "ATP5A1 expression is downregulated in ccRCC": In the last sentence cite Figure 3 and not only Figure 3 A C.
Reply: Thank you for your feedback. We apologize for our negligence and have revised the content in the manuscript (see Page8, line206).

2) "Association between ATP5A1 and the Clinicopathological Features of ccRCC": "T phase" and "M phase" in the first sentence need to be changed to "T stage" and "M stage".
Reply: Thank you for your feedback. We have revised this part of the content (see Page9, line209).

3) Figure 4H (ROC curve): This fit not in the prognostic data. The ROC curve should be moved to Figure 1. Which data were used to create the ROC curve? Data from Figure 1B or 1C? This needs to be indicated.
Reply: Thank you for your valuable feedback. The ROC curve was created from gene expression data of ATP5A1. We have moved the ROC curve to Figure 1.

4) Table III: OS should be added to the table caption. Please also indicate how the multivariate Cox regression analysis was done. Were all parameters used in a step-wise fashion or were all significant parameters from the univariate Cox regression analysis or only selected parameters used? Furthermore, did you perform univariate and multivariate Cox regression analysis for DSS and PFI? What were the results?
Reply: Thank you for carefully reviewing the manuscript and providing valuable feedback. We have corrected the title of Table 3 and revised the content in the manuscript (see Page9, line218-221).

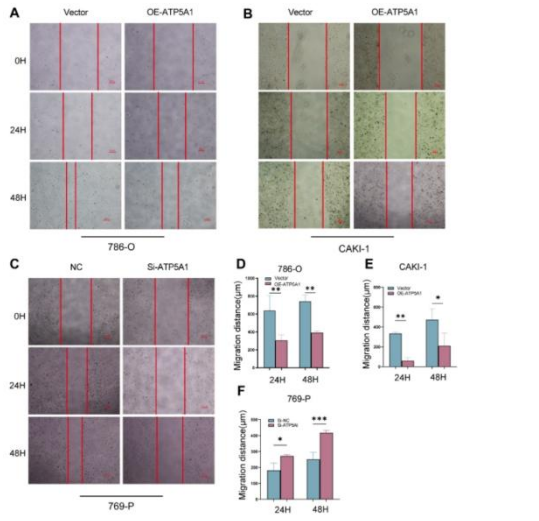
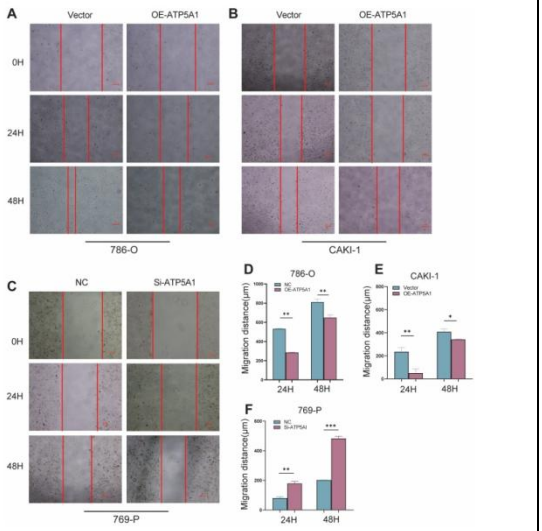
5) Table IV: Please define what the "q-value" is!
Reply: Thank you for your valuable feedback. q-value: Derived from Benjamini-Hochberg FDR correction procedure.The q-value is defined as the FDR analogue of the p-value. It represents the expected proportion of false positives among the discoveries (significant calls) made at that particular q-value threshold. Actually, deleting the q-value column from Table 4 does not affect the co-expression analysis results of the ATP5A1 gene, therefore we have made the necessary modification to Table 4.

6) "ATP5A1 Suppresses the Migration, Proliferation, and Invasion of ccRCC cells": Last sentence "… compared to ccRCC cells" should be "… compared to NC group".
Reply: Thank you for your valuable feedback. We have revised this part of the content (see Page10, line236).

7) Figure 4D legend: there is "and" missing between "expression" and "ccRCC".
Reply: Thank you for your feedback. We apologize for our negligence and have revised the content in the manuscript (see Page17, line454).

8) Figure 6B and 6D: The chosen examples for the microscopic images do not support the data in Figures 6E and 6F. For Figure 6C, for example, the migration distance for si-ATPA1 cells is less than in the NC group. The data should be checked carefully and adjusted appropriately.
Reply: Thank you for carefully reviewing the manuscript and providing valuable feedback. We have refined the data presented in Figure 6. (See the revised table below).

Before revision	After revision

Original Figure 6	Figure 6
	

9) Figure 8 (apoptosis data): Why is there such a huge difference in apoptosis rates for the respective NC groups (Figures 8A and 8C)? Sure, they are different controls, but 30% vs 4% apoptosis seems a bit much. What is the explanation?
Reply: Thank you for your feedback. Due to the use of different cell lines in the Si-NC group and the Vector group for the experiments, with the Si-NC group being transfected with si-RNA and the Vector group with plasmids, the apoptosis rates of the cells may exhibit differences after treatment with various reagents.

10) Figures 7, 8 and 10: There is a typo in the legend – "TP5A1" instead of "ATP5A1".
Reply: Thank you for your feedback. We have revised this part of the content (see Page17-18, line476, line482 and line492).

Discussion
Are there any studies that investigated the effect of ATP5A1 on cellular function in other cancer cells? If so, this should be discussed.

Minor
1) Write the cell lines consistently with a dash throughout the manuscript (786-O, 769-P, CAKI-1)!
Reply: Thank you for your feedback. We have revised this part of the content (see Page6, line142-144, line148).

2) Abbreviation WB is introduced for Western Blot and should then be used throughout the manuscript.
Reply: Thank you for your feedback. We have revised this part of the content (see Page10, line257, Page17, line461 and Page18, line488).

Reviewer C
The study provides significant data on the association of the ATP5A1 gene with

unfavorable prognostic parameters in clear cell renal cell carcinoma. Despite being a promising marker, some of the findings of the in-silico analyses are not very innovative. What stands out in this study are the results of the in vitro tests.
One aspect that could be investigated, if the authors consider it relevant, is the expression profile and association of ATP5A1 in the papillary and chromophobe histological subtypes.
Major points
At the abstract:
1 - It describes that RNA sequencing data was evaluated in ccRCC samples. However, the results infer a broad analysis, considering different types of câncer. Since the goal was to evaluate ccRCC, these findings seem “deslocados”.
Reply: We began by conducting a comprehensive analysis of ATP5A1 expression across various human cancers to investigate its association with malignant tumors. Subsequently, we observed a significant decrease in its expression in clear cell renal cell carcinoma. As researchers specializing in urological diseases, we have chosen clear cell renal cell carcinoma as our research focus.

2 - The sentence “293 cells and adjacent ccRCC tissues” seems confusing. The 293 is the cell line used in the in vitro assays, which was not mentioned in the method. The first impression looks like 293 samples. I suggest the authors revise the abstract to align the study's proposal, methods, and results.
Reply: We appreciate your valuable feedback. We have added an explanation about 293 cells in the Cell Lines section of the manuscript (see Page4, line101).

3 - The sentence “a direct correlation with improved prognosis was identified” sounds subjective. Please consider rewriting it and explaining the exact findings such as positive correlation or association with overall survival.
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page2, line41).

4 - Please consider revising the second paragraph of the Introduction. Some repeated information about the enzyme would be avoided, which turns the text more objective and fluid. E.g., “fundamental enzyme responsible for energy production”, “plays a key role in ATP synthesis”, “essential role in intracellular energy production.”
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page3, line73-92).

5 - In the introduction, some sentences are generalist. Please, consider being more specific regarding the names of pathways, miRNA, and modifications such as protein phosphorylation. “correlates with the activation of oncogenic pathways”, “targeted miRNA-induced downregulation of ATP5A1”, “modifications in tumor-associated phosphorylation”.
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page3, line73-92).

6 - Please, refer to some of the studies that established a strong correlation between ATP5A1 and various tumors.
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page14-15, line352-355, line361-365, line371-391, line394-395 and line415-416).

7 - The relationship between the WNT pathway and ccRCC would be more evidenced. In the introduction, the authors refer to one review article from 2008, which basically describes that beta-catenin regulates the VHL gene expression. Additional scientific evidence should be included to justify the investigation of the WNT pathway in ccRCC and associated with ATP5A1.
Reply: We appreciate your valuable feedback.

In the methods section:
8 - The cell lines authentication through STR, as well as the cell source was described twice and from different cell banks. Which cell line was purchased from Wuhan Pricella Biotechnology and which one was from ATCC?
Reply: We appreciate your valuable feedback. We have made modifications in the Cell Lines section of the article (see Page5, line102-104).

9 -The sentence “For transfection, 786 O, CAKI 1, and 769 P cells were seeded at a density of 200,000 cells per well in 6-well plates.” is on the topic “Wound-Healing Assay”
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page6, line148).

10- For the Transwell Cell Invasion Assay, was the lower chamber filled with cell suspension solution or with medium supplemented with 20% FBS only (without cells)?
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page7, line160).

11- Please, revise the initial sentence of the topic Flow Cytometric Analysis of Cell Apoptosis. It sounds like a protocol instead of a methodologic description.
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page7, line165-166).

12 - The Statistical Analysis does not mention the tests applied for the in vitro assays.
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page8, line185-186).

The Results:
13 – Since there are many different tumors evaluated in Figure 1A. I recommend that

the acronyms for each tumor be described in the figure legend to make it easier to identify the types of tumors.
Reply: We appreciate your valuable feedback. The tumor names in the picture have been represented using acronyms (Figure 1A).

14 - Which cell line is considered non-tumorous? It would be interesting to have this information described in the methods. Or explain why the other cell lines are compared with the 293 cells.
Reply: We appreciate your valuable feedback. We have added an explanation about 293 cells in the Cell Lines section of the manuscript (see Page4, line101).

15-Please consider revising the application of the word “association” instead of “correlation” in the results section and in the Figure 4 caption.
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page9, line213, and Page9, line452 and line455).

16 - Describe which statistical tests were used to analyze the data in Table 2.
Reply: We appreciate your valuable feedback. The chi-squared tests were used to analyze the associations between ATP5A1 expression and various clinical characteristics of ccRCC patients (see Page16, line431).

17 – Please, consider merging the results in the topics: “ATP5A1 Suppresses the Migration, Proliferation, and Invasion of ccRCC cells” and “ATP5A1 Promotes Apoptosis of ccRCC Cells”
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page9, line227).

18 - Why the 293 cell lines has not been used in the in vitro experiments?
Reply: In this study, the 293 cell lines was utilized to confirm the discrepancies in ATP5A1 expression levels between renal cell lines and renal cancer cell lines. Consequently, this cell line was exclusively employed in RT-qPCR and Western blotting experiments.

19 - The results describe 72 positively correlated and 33 negatively correlated genes. Were the GO and KEGG analyses conducted considering the total number of genes (72+33)? Was there an approach to performing the analyses with separate lists of positively and negatively correlated genes?
Reply: The analysis of positively correlated genes was prioritized in this study for GO and KEGG assessments due to their potential direct involvement in ATP5A1's functional regulation and the likelihood of providing a clearer indication of its mechanisms.

20 - The results described in the “ATP5A1 is Associated with the Wnt/B-Catenin Signaling Pathway” subsection can be merged in the topic before (ATP5A1

Enrichment Analysis of the Co-Expressed Genes) with an adjusted title. In addition, citations of the studies (13 and 14) in the results section can be avoided and addressed in the discussion section.
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page10, line239).

In the Discussion section
21 - The discussion on alterations in cell metabolism generally indicates an increase in aerobic glycolysis associated with tumor progression. Increased oxidative phosphorylation has been widely discussed concerning tumors resistant to different therapies. I suggest revising this approach in the discussion and citing studies that can consolidate the information described in the text.
Reply: We appreciate your valuable feedback. We have revised this part of the content (see Page10-11, line261-265).

22- Considering the deregulation of VHL - HIF1a in RCCcc, it would be interesting to discuss whether gene alterations ATP5A1 have a potential influence on the greater or lesser function of HIF1 in RCCcc cells. In Hela cells, It was also found that ATP5A1 could interfere with the alternative splicing of hundreds of genes associated with glucose homeostasis and HIF-1 signaling activation (https://doi.org/10.1177/153303382110391)
Reply: We appreciate your valuable feedback. We have noticed the relationship between ATP5A1 and cervical cancer and have cited this reference (25) in the original text.

23- The authors end their discussion by stating several limitations. However, it would be important to consider the term “several”, since only one limitation is described.
Reply: Thank you for your feedback.The limitations of this study are reflected in two aspects: the analysis results of the TCGA dataset require further validation with clinical data, and the precise molecular mechanisms by which ATP5A1 affects ccRCC need to be further investigated. Therefore, we believe the use of the word "several" is appropriate.

Minor points:
- Please, consider revising the nomenclature of human genes (uppercase and italic)
- To avoid ordinary terms such as “has been linked”
- Pathophysiological function of ATP5A1 instead of the physiological function of ATP5A1 (in the introduction section)
- To refer to the manufacturer’s name for the reagents and software: cDNA assay kit, BCA kit, ECL luminescent, software used to measure the fluorescence intensities, Apoptosis Positive Control Solution
- To describe: which protein-free solution was used for the WB membrane blockage; how many cells were seeded for the transfection assay with the

siRNA and plasmids;
- “Data analysis conducted using R software”. Does it mean gene expression data?
- Please, observe the citation of Figure 3. “Figure 3A C” (line 170)
- Regarding the Association between ATP5A1 and the Clinicopathological Features of ccRCC section, please, verify the correct use of “T phase (P<0.001), M phase (P<0.001)” or T stage, M stage.
- Please check whether the numbers in the tables are in Arabic or Roman numerals.

Reply: Thank you for reviewing our manuscript and providing insightful comments and suggestions that have helped us to significantly improve the quality of the manuscript. Based on your feedback and suggestions, we have made the necessary corrections to the manuscript, with the revised sections highlighted in red.