

Peer Review File

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Reviewer A

Comments 1: First of all, adenocarcinoma in general (mixture of all histological subtypes of adenocarcinoma) is so rough and is not appropriate for correlation analysis. For adenocarcinomas, analysis should be performed in each subgroup stratified by histological subtypes.

Reply 1: We are appreciated for your essential advice; there is no doubt that the histological subtype subdivision of lung adenocarcinoma will greatly increase the richness and preciseness of the article and we are sorry for not further subdividing the pathology of lung adenocarcinoma. We have seriously explored the subtype classification of lung adenocarcinoma. We found that adenocarcinoma can be divided into minimally invasive adenocarcinoma type (mucinous type), minimally invasive adenocarcinoma type (non-mucinous type), invasive non mucinous adenocarcinoma type (adherent growth adenocarcinoma, acinar adenocarcinoma type, papillary adenocarcinoma type, micro-papillary adenocarcinoma type, solid adenocarcinoma type), invasive mucinous adenocarcinoma type, mucoid adenocarcinoma type, fetal adenocarcinoma type, adenocarcinoma type (intestinal type), adenocarcinoma non-specific type, definitely, correlation analysis based on each subtype of lung adenocarcinoma is a fabulous method, however, it requires a large sample size and a very accurate classification of LUAD samples. We used the public TCGA and GEO databases and these two databases can only be accurate to lung adenocarcinoma, lung squamous cell carcinoma or non-small cell lung cancer in terms of pathology. We cannot further classify them on the basis of the public database; meanwhile, our hospital is not equipped with database that can meet this requirement either. On the basis of the data we tried our best to acquire, we are very sorry that we cannot fully meet your requirements. May I have you understand in lacking of our ability and we are still grateful to your valuable suggestions for our articles.

Comments 2: The way of clinicopathological analysis is premature, i.e., survival analysis in silico was not complete to define pathological involvement of ADCY9.

a. First, disease-free analysis instead of OS is preferable. Second, analysis should also be performed among subgroups of the same age, the same p-stage, the status of driver gene (EGFR, ALK and KRAS, etc.) alteration and with/without the history of smoking and of adjuvant therapy, separately.

b. A major drawback in the in vivo analysis is the smaller scale of sample group (60 cases), which inevitably causes limitation to predict prognostic significance. It should be performed in larger number.

c. In addition, results of immunohistochemistry are not mentioned in the text.

d. The authors are advised to introduce the profile of samples (patients) according to the WHO classification of adenocarcinoma, TNM classification as well as age, gender,

smoking history, and molecular data in a table to show the data clearly.

Reply 2

a, d: Thanks for your valuable suggestion, I am firmly convinced that your advice will be of significant help in improving the logicality and rigorism of this article, we are truly sorry for our defects in immunohistochemistry and we will try our best to conduct subgroup analysis based on our existing clinical data such as gender and age. However, we are sincerely regretful that the pathological specimens provided by the pathology department of our hospital can only be accurate to the pathological classification of lung adenocarcinoma, lung squamous cell carcinoma or small cell lung cancer, please forgive us that we cannot continue to make pathological subdivision on the basis of lung adenocarcinoma. Due to the massive loss of follow-up data, the pathology department of our hospital can only provide clinical data like age, gender, survival time, while other clinical information such as TMN classification, smoking history and disease-free survival time really unable to be provided by the pathology department of our hospital, we are very sorry for not satisfying all your demand and may I have you understand in the lack of our ability, meanwhile, we still appreciate for your suggestions which makes our research better.

b, c: Thanks for your valuable suggestion, increasing the number of sample group has an undoubtedly positive effect on the accuracy of analysis results, Therefore, we tried our best to get a pathological tissue microarray (n=60) from the department of pathology. In addition to the previous one, a total of 115 samples were collected and immunohistochemistry staining was carried out. All pictures of the pathological tissue microarray samples and the ADCY9 gene expression level profile of each sample are in the supplementary materials.

Changes in the text a, d: The changes can be seen in the figure 2. We hope our alterations can satisfy you standard.

b, c: The changes can be seen in the supplementary material. We hope our alterations can satisfy you standard.

Comments 3: a. The technical criticism is always raised in several experiments. Although authors introduced cell lines from cell banks, they presented the results of only one, SPCA1 cells. At least, 2 cell lines are mandatory for the experiments of overexpression, even if authors have only to confirm the results obtained from database.

b. Describe names of all cell lines used which are represented by “other cells” (p5, line 132).

Reply 3

a: We are grateful to your significant advice and we are sorry for applying one cell line. Actually, after submitting this article to journal of thoracic disease, we have realized that two cell lines are mandatory for the experiment; therefore, we have

already started the A549 cell overexpression since then. We adopted the A549 cell line and carry out a series of cell function assays like we performed on the SPCA1 cell line, the result of overexpression and cell function assays are displayed in the figures of this article.

b: Thanks for your mentioning our mistake in this article, other cells mean H1299 cell line, which were supposed to be applied in the WB and PCR experiment, however, passaging of cells were not very successful, therefore, we abandoned using this cell line in our article. We were supposed to delete this error description before submitting the article but we failed to find this mistake, here, we thanks again for pointing out our mistake.

Changes in the text a: The changes can be seen in the figure 9 of this article. We hope our alterations can satisfy you standard.

b: Changes in the text: Page 13, line 371-388

Comments 4: The unsatisfactory illustration of the immunoblot analysis makes difficult to evaluate the results;

a. All gels were cropped, but whole gel should be presented in Supplementary Figure.

b. No molecular weight marker protein, no positive control.

c. Authors used acrylamide gel. However, concentration is not described.

Reply 4:

a: We are appreciated for you opinion, we are sorry for not supplying the whole gel because we found that the effect of incubating antibody with cut membrane is better than that of a complete membrane, but we can put the cropped membrane together completely and upload a complete picture of WB membrane in the supplementary to you.

b: Thanks for showing the drawbacks of our WB picture, molecular weight marker protein is displayed together with the whole membrane picture that you mentioned in the previous comment and we are truly sorry for our negligence in this research.

c: Thank you for your valuable advice, we are sorry for not providing the concentration of acrylamide gel, it is essential to make the method of the WB clear, we have already put the concentration of acrylamide gel (10%) in the article and we are appreciated for pointing out our mistake again.

Changes in the text a: The changes can be seen in the WB figure in the supplementary material. We hope our alterations can satisfy you standard.

b: The changes can be seen in the WB figure in the supplementary material.

c: The changes can be seen in the text, Page 7, line 198.

Comments 5: Thorough discussion based on the notions reported in the past literature is also needed. In particular, describe and speculate the pathobiology of ADCY9 in lung AC more deeply in relation to microRNA and lncRNA, and ultimate transcriptional aberration.

Reply 5: We are glad to have your opinion concerning the discussion part; it is very important for an article to fully discuss the research molecules and its related RNA. I believe your suggestions will enrich the content of this article and make this article better. Therefore, we have made our changes in the text; we discuss the correlation between specific miRNA or lncRNA and ADCY9 gene in lung adenocarcinoma, meanwhile, articles mentioned by another two reviewers have also been cited in the text and I hope our alterations may satisfy your request.

Changes in the text The changes can be seen in the text, Page 14-15, line 416-437.

Reviewer B

Comments 1: The overall writing has some formatting issues, like wording and spacing. I suggest the authors check the grammar and avoid any typos. More importantly, the writing needs improvement.

Reply 1: Appreciation for your valuable comments. We apologize for the problems in language expression. We worked on the manuscript for a long time and delete the repeated addition and sentences or sections which obviously led to poor readability. We have used the medical writing service provided by to re-edit the language for us. We really hope that the language level has been substantially improved.

Comments 2: The analysis methods are lack of details. More detailed descriptions are needed to explain the analysis methods.

Reply 2: I am very appreciated for your opinion as it does help a lot in reforming the structure of my paper so that the paper can be more readable and understandable. Therefore, we divided a complete paragraph “Bioinformatic analysis” into multiple paragraphs according to the type of biological information analysis. Each paragraph was matched with the title of the type of bioinformatic analysis, which can make biological analysis clearer than the previous edition that all the bioinformatics analysis completely mixed in a paragraph. Thanks again for your suggestions and I hope our efforts will make this article better

Changes in the text Page 4-5, line 111-157.

Comments 3: The labeled texts in some figures are very small to recognize, e.g. Figure 6. I would recommend the authors to polish the figures with high resolution.

Reply 3: we are grateful to have your suggestion which mentioned a very important problem that might be ignored by us. We are sorry for our negligence. High-resolution is of great essence in an article; On the one hand, we changed the order of images

according to the suggestion of another reviewer and added new pictures of new experimental results. We enlarged all the pictures on the premise that not affecting aesthetics of the overall figure and exported them in Adobe Illustrator in the highest resolution and TIF format. Appreciation again for your advice and I believe our efforts will make this article better.

Changes in the text The changes can be seen in the pictures 7 in the attachment. We hope our alterations can satisfy you standard.

Comments 4: In addition to the analyses and experimental results, I would suggest the authors discuss the possibility of using different approach (PMID: 26257336), or using single-cell multi-omics data (e.g. PMID: 35910046) as future perspectives, which helps expand the scope of the analysis results.

Reply 4: Thanks for your valuable opinion and we are sorry for poor theoretical knowledge in bioinformatics approaches. We are still lacking the code and technique in terms of single-cell multi-omics analysis, but single-cell multi-omics is indeed an advanced and promising bioinformatics analysis method; In the discussion part of this article, we have discussed the possibility of analyzing this gene with single cell multi-omics analysis, and cited relevant literature. I also expect that may be one day I can apply this method in future articles.

Changes in the text Page 15, line 439-446.

Reviewer C

Comments 1: Results: as previously indicated, it would be convenient to analyze other genes associated with ADCY9 that allow establishing functional routes that establish greater knowledge about the development and survival of tumor cells in lung cancer.

Reply 1: We are honored to receive your significant suggestions on this article; we firmly believe that your suggestions will significantly increase the readability and understandability of the article. Therefore, we have made a PPI network displaying the relationship between other genes and ADCY9 gene, Thanks for your advice again and I believe our efforts will make this article better.

Changes in the text The changes can be seen in the figures 5 of this article, we hope our changes may satisfy your request.

Comments 2: Introduction: a figure showing the functioning of the ADCY9 gene could be added in the introduction, allowing the reader to know more easily and

quickly the function of ADCY9.

Reply 2: We are appreciated for your valuable advice on the introduction segment and we are sorry for not making our description of the function of ADCY9 clear, however, we are sorry that we can only find part of the function of ADCY9 gene from the literature, but the specific pathway has not been reported in the document, therefore, we are sorry for not satisfying your request.

Comments 3: Discussion: since it is a topic already addressed in the literature, the results found should be discussed with the results of other studies already published. The discussion falls short given the topic being addressed.

Reply 3: We are sorry for not fully aware the current status and progress of molecule that we are looking into, we are sincerely appreciated for your pointing out our negligence timely and accurately. In our article, have expounded the specific research of correlation of ADCY9 and lung adenocarcinoma that you have mentioned in your comment (Jia S et al *Frontiers in Genetics*, 2021 12:666396 and Ma Y *Genes*, 2022 13:885) and cited these two articles. Furthermore, we have also elucidated the relationship of ADCY9 and lung tumor metastasis from colon cancer to make the discussion more sufficient. Appreciation again for your advice and I believe our efforts will make this article better.

Changes in the text Page 13-15, line 391-456.

Comments of minor changes:

1. Abstract: indicate the center where the study has been carried out and the date.
2. Introduction, line 68-69: the cancer with the highest incidence worldwide is breast cancer. Sung H, et al. *Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries*. *CA Cancer J Clin*. 2021;71(3):209-249.
3. Introduction, first paragraph: to make a correct introduction, it would be possible to talk about the most frequent genes in lung cancer such as TP53 or K-RAS due to their implications.
4. Introduction, line 79-80: survival rates in advanced or metastatic lung cancer would be more correct to give them at 5 years, being greater than 15% thanks to the introduction of immunotherapy with long responses to more than 3-5 years.
5. Introduction, line 99-101: this sentence belongs to the conclusions.
6. Results: The introductory titles of each section within the results should not indicate the findings that have been found but should indicate what is to be studied.
7. Results: please sort the figures in order of text.
8. Discussion, line 345: as I mentioned in the introduction, the data on survival in lung cancer should be changed.

Reply of minor changes:

Reply 1: Thanks for your suggestion for this article; we are sorry for not making our experiment center location and date clear, therefore, we attach acquisition time of IHC samples in the abstract part and according to the typesetting requirements of JTD, we have put the location of our experiment center in the part of correspondence authors' information, if it does not bother you, we are glad to tell you in this reply, this article was carried out from 2021/03-2021/12, in Nantong Key Laboratory of Translational Medicine in Cardiothoracic Diseases, Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu, China, this article was submitted to JTD in 2022/08.

Reply 2: We are grateful for your valuable advice, we are sorry for ignoring this basic common sense, We have made the changes in our article and cited "Based on Global Cancer Statistics 2020, lung cancer remains the second highest diagnosed incidence and still keep the highest cancer-related mortality rate all over the world", we express our gratitude again for updating our basic knowledge.

Reply 3: Appreciation for you advice, indeed, It is more reasonable to select common lung adenocarcinoma targets to discuss in Introduction part of this article. Revision has been made in this article that frequent lung cancer targets are given priority to be discussed such as TP53, K-RAS and EGFR and followed by the uncommon targets which I had discussed in this paper.

Reply 4: Thanks for your valuable advice; we are truly sorry for our mistake, what recorded in reference is "only 15.6% of all patients with lung cancer are alive 5 years or more after the diagnosis", which is due to our negligence, we wrote overall survival instead of five year survival rate; we have made changes in our article and change it into five year survival rate in our article.

Reply 5: Thanks for your valuable advice; as you mentioned, we are fully subscribe to the idea that it is inappropriate to put a conclusive description in the introduction section, therefore, we put this sentence in the conclusion section of the article and revised the conclusion part.

Reply 6: Thanks for your valuable advice. Your suggestion is of great significance to improve the strictness and readability of this article, revision has been made in this article according to your suggestion and changed the concluding statement into the research content.

Reply 7: we are grateful to have your suggestion, sorting the figures in order of text is of great essence for readers to comprehend the whole article, we are sorry for neglecting this important detail. Meanwhile, another reviewer suggests polish the figures with high resolution, therefore, we not only adjusted the order of pictures to make them consistent with the text order, but also improved the resolution of pictures and enlarged some pictures to increase the readability of the article. The changes can be seen in the pictures in the attachment. We hope our alterations can satisfy you standard

Reply 8: Thanks for your valuable suggestion; we have made the same changes (Reply 4) according to the comment 4.

Changes in the text 1. Page 2, line 51.

2. Page 3, line 67-69.

3. Page 3, line 73-82.

4. Page 3, line 83-85.

5. Page 4, 16, line 102-108, 459-465.

6. Page 10-12, line 295, 322, 355, 371.

7. The changes can be seen in the pictures in the attachment. We hope our alterations can satisfy you standard 8. Page 14, line 396-397.