Peer Review File

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Reviewer A

This article needs more information for publication. A research article should address an important question or knowledge gap in the field. The research should have practical implications and provide insights that can be applied in the real world.

Reply: Thank you for your precious comments on our article.

In this case, why choice these two miRNAs? What the parameters for the choice? It's not clear.

Reply: We thank the reviewer for the question about why miR-128-3p and miR-33a-5p was focused on in this study. These two kinds of miRNAs were chosen because previous researchers found that miR-128-3p and miR-33a-5p have tumor inhibitory effect in lung cancer, which can inhibit the proliferation and invasiveness of lung cancer cells. Moreover, the levels of these two miRNAs in lung cancer tissues were significantly lower than those in matched normal tissues, indicating that their contents were associated with lung cancer cells (1,2). However, limited to the collection of lung tissue, the clinical significance of these two miRNAs in the early diagnosis of lung cancer is not clear. However, compared with tissue samples, blood samples are easier to collect, which makes us wonder: can the changes in the content of these two kinds of miRNAs in blood represent the process of occurrence and development of lung cancer, and are expected to become new biomarkers for the diagnosis of lung cancer? In order to verify the clinical significance of these two miRNAs in the diagnosis of lung cancer, we carried out the experiment. Please see page 4,5 line 128-146 in manuscript.

Besides, the research should be based on a sufficient sample size and provide a thorough analysis of the data. The methodology should be sound, and the results should be presented clearly and accurately.

In this article, you recruited for only one month. Why? You used only thirty-six samples, and they analyzed with an amplification method, which can show confusion artefacts. What you did for anulate this? It's not clear, too.

So, please rewrite this paper clearly and concisely, explaining exactly your choices and the scientific impact of your discoveries.

Reply: We really appreciate your efforts in reviewing our manuscript. Firstly, our experiment was actually carried out for 4 months, from September 1, 2022 to December 30, 2022. However, due to writing mistakes, December was mistakenly written as September. Thank you for asking this question, and we corrected it in the article. Sorry for our mistake again. Due to the shortage of funds and the high cost of the study, only 36 patients were included, which is the main reason for the small sample size. If this study can continue to be supported, we will expand the sample size, further understand

the clinical value of miRNAs in the prediction of lung cancer, conduct self-verification and multicenter verification, and improve the experimental design. In the discussion part, we supplement the limitations of this study.

Secondly, qRT-PCR amplification method is a means to detect miRNA content in exosomes. In order to eliminate errors and interference from artificial substances, we conducted three repeated tests and took their average values for statistics.

Changes in the text: we added some contents in the text. (see Page 2, line 39; Page 6, line 175; Page 7, line 215; Page 11, line 354-359).

Reviewer B

Article has serious flaws, additional experiments needed, research not conducted correctly.

Replay: First of all, thank you very much for your careful reading of this manuscript. We are very sorry let you feel troubled. Due to various factors, including but not limited to funding, our research is not perfect, but in the follow-up research, we will make up for the shortcomings of the experiment in order to obtain more data for self-verification and external verification.

Reviewer C

The paper titled "Clinical significance of miR-128-3p and miR-33a-5p in the diagnosis of resectable lung cancer in humans" is interesting. Serum exosome miR-128-3p and miR-33a-5p showed good performance in lung cancer screening and may be used as new biomarkers for large-scale lung cancer screening. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) The abstract is not adequate and needs further revisions. The research background does not indicate the clinical needs of this research focus. The study results need to show the clinical characteristics of the two groups of patients.

Reply 1: We thank the reviewer for pointing this out and agree with the reviewer's advice concerning this issue. We have revised the abstract part of the manuscript as requested and red mark the revised part.

Changes in the text: we have modified our text as advised (see Page 1, line 32-34,48)

2) The content of this study is too simple. It is suggested to increase the function research of miR-128-3p and miR-33a-5p, which may be more meaningful.

Reply 2: Thank you for your careful consideration and valuable suggestion. In fact, it will be more convincing if we further examine the role of miR-126-3p and miR-33a-5p in lung cancer. In our study, we verified the potential of miR-126-3pandmiR-33a-5p as a biomarker of lung cancer, and further research is needed to detect the function of miR-126-3p and miR-33a-5p in lung cancer. On the other hand, many researchers have hypothesized and verified their function in lung cancer, trying to find possible ways to treat lung cancer, which is the focus and challenge of exosomes in cancer research. this is also the road that we can walk later. Thank you for pointing out the direction for our follow-up research. We give a supplementary description of the problem in the discussion part of the article.

Changes in the text: we added some contents in the text.(see Page 12, line 372-378)

3) The number of patient samples in this study is too small, and a large sample study should be added for verification.

Reply 3: Thanks for your insightful comments. In fact, due to the shortage of funding and the high cost of the study, we only included 36 patients, which is the main reason for the small sample size. If this study can continue to be supported, we will expand the sample size, further understand the clinical value of miRNA in the prediction of lung cancer, conduct self-verification and multicenter verification, and improve the experimental design. In the discussion part, we supplement the limitations of this study.

Changes in the text: we added some contents in the text.(see Page 11, line 354-359)

4) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “Exosomal microRNAs in non-small cell lung cancer, Transl Cancer Res, PMID: 35116621”. It is recommended to quote this article.

Reply 4: Thank you for your careful consideration and valuable suggestion. We have read and studied the literature carefully and quoted it appropriately in the introduction.

Chang in the text: We quoted this article in the introduction（see Page 4, line 107,120）

5) What are the effects of miRNAs and immune microenvironment on the evolution of lung cancer? It is recommended to add relevant content.

Reply 5: Thanks for your insightful comments. We have added relevant content in accordance with your suggestion.

Chang in the text: We added some contents in the introduction（see Page 4, line 108-120）

6) What are the problems and challenges that need to be overcome in the clinical application of miRNA? It is recommended to add relevant content.

Reply 6: We thank the reviewer for pointing this out and agree with the reviewer’s advice concerning this issue. We have added relevant content in accordance with your suggestion.

Chang in the text: We added some contents in the discussion（see Page 10,11, line 322-330）

7) What are the new research advances of miRNA in lung cancer? It is recommended to add relevant contents.

Reply 5: Thank you for your careful consideration and valuable sugesstion. We have added relevant content in accordance with your suggestion.

Chang in the text: We added some contents in the discussion (see Page 10, line 317-322).

Reviewer D

First of all, my major concern for this study is the use of healthy volunteers as controls for this diagnostic test. Because of the focus of this study is the clinical significance, the research methodology is problematic. In clinical practice, the clinical question is to differentiate patients with lung cancer from those with benign lesions. From this, the clinical significance of this study is very limited.

Reply1: We thank the reviewer for pointing this out and agree with the reviewer's advice concerning this issue. We have deeply thought about this issue. The main purpose of our study is to test the expression differences of miR-128-3p and miR-33a-5p markers in lung cancer patients and healthy people, as well as their ability to assist in the diagnosis of lung cancer. How to distinguish patients with lung cancer and patients with benign lesions will be our further research based on this study. We are sorry that we didn't make it clear. We will revise relevant contents in the whole text.

Second, the title needs to directly indicate the research design of this study, i.e., a diagnostic test.

Reply 2: Thanks for your insightful comments. We have changed the original title to "A Test of miR-128-3p and miR-33a-5p in Serum Exosome as biomarkers for Auxiliary Diagnosis of Non-Small Cell Lung Cancer"

Changes in the text: we have modified the title as advised (see Page 1, line 3-4)

Third, the abstract needs some revisions. The background did not explain why the biomarkers are accurate for the diagnosis of lung cancer. The methods need to describe the inclusion criteria of the subjects, and how the levels of the two biomarkers were measured. The results need to quantify the findings by reporting the expression levels and accurate P values. The conclusion is misleading and even wrong because screening is for the general population and diagnosis is for the patient population with suspected lung tumor. Because of the expensive cost of the test of the biomarkers, such method is not feasible for population-based screening.

Reply 3: Thank you for your careful consideration and valuable suggestion. Firstly, for the abstract part, we modified it as required, but due to the word limitation, the inclusion criteria are described in detail in the text methodology. Secondly, the purpose of this study is to test the difference in the expression of miR-128-3pandmiR-33a-5p between

patients with lung cancer and healthy people, and its ability to assist in the diagnosis of lung cancer. Only on this basis can we further study its significance in the differentiation of benign and malignant tumors. Finally, the high cost of detection of the biomarker is also one of the limiting factors in our study, which we also described in the discussion. However, with the further understanding of human exocrine miRNA and the continuous progress of detection methods, this problem will be solved. Moreover, the ultimate direction of our follow-up research is to study the mechanism of miRNA in lung cancer and to find the key targets for regulating different biological processes such as lung cancer growth, progression, invasion, angiogenesis, metastasis and drug resistance.

Changes in the text: we have modified the abstract and discussion as advised (see Page 2, line 40,44,45,50; Page 10, line 325-330)

Fourth, in the introduction of the main text, please review known biomarkers for the diagnosis of lung cancer, have comments on their abilities for accurately diagnosing lung cancer at early stage and for the differential diagnosis between benign and malignant tumors, and have comments for the clinical implications of the findings.

Reply 4: Thanks for your insightful comments. We have added relevant content in accordance with your suggestion.

Chang in the text: We have added some contents in the introduction (see Page 3, line 75-83).

Fifth, in the methodology of the main text, please describe the clinical research design, sample size estimation, data collection of clinical factors, and how the gold diagnosis of lung cancer was ascertained. Importantly, the authors emphasized biomarkers for early lung cancer, but the included cases are not at early stage of lung cancer. The other concern is the small sample size of this study, making the generalizability of the current findings unstable and difficult. In statistics, please describe how the two biomarkers were combined as the joint diagnostic test, report the threshold values of sensitivity and specificity for a good diagnostic test, and ensure $P<0.05$ is two-sided.

Reply 5: Thank you for your careful consideration and valuable suggestions.

Firstly, we add the relevant contents as required in the methodology of the text, please see Page 6, line 172,173,179-181).

Secondly, I'm very sorry for the mistakes in our work and writing, which make the main idea of the article ambiguous. The main purpose of our study is to test the expression differences of miR-128-3p and miR-33a-5p markers in lung cancer patients and healthy people, as well as their ability to assist in the diagnosis of lung cancer. Therefore, the patients included were not all in early lung cancer stage. We will modify the relevant contents of the full text. Moreover, in the follow-up study, we will test the expression performance of miR-128-3p and miR-33a-5p in serum exosomes of patients with different stages of lung cancer to further explore its clinical significance.

Thirdly, due to the shortage of funds and other problems, our research only included 36 samples, which made our research results limited. However, in the follow-up study, we will further expand the sample size for research and verification, and need to include more external data to further compare and verify by identifying and evaluating more predictive factors. In the discussion part, we describe the limitations of the experiment. Please see Page 11, line 354-359.

Finally, we added relevant contents in the statistics section as required, please see Page 7, line 227,228; Page 8, line 231,232.

Changes in the text: we have modified the manuscript as advised (Page 6, line 172,173,179-181; Page 7, line 227,228; Page 8, line 231,232; Page 11, line 354-359).

Reviewer E

1. **Abstract**:

1) Your abstract is too long. The abstract should be 200-350 words, but you have 381. Please revise.

2) Please check all abbreviations in the abstract, such as below abbreviations. All abbreviated terms should be full when they first appear.

28 **Background:** Lung cancer is the malignant tumor with the highest incidence in the
29 world today, which poses a serious threat to human health. Among them, NSCLC is the

47 control group. qRT-PCR method was used to detect the expression of two potential
48 markers miR-128-3p and miR-33a-5p in serum exocrine. The main indicators of

Reply: Thank you for your valuable advice. We have modified the words and contents of the abstract as required (See page 2; Line 32-62).

2. Please check if the citations of references need to be added in the below sentence since you mentioned the previous "studies".

105 there is still a lack of specific tumor markers for NSCLC screening. Although related
106 studies have confirmed that carcinoembryonic antigen, cancer antigen 125 and
107 cytokeratin fragment 21-1 are serum antigenic biomarkers associated with lung cancer.
108 And only by incorporating some clinical variables (nodule size, smoking history, age,
109 etc.) can these biomarkers improve the diagnostic accuracy of lung cancer and reduce
110 the invasive diagnosis and treatment of benign nodules, but also delay the treatment of
111 malignant nodules to a certain extent, and the clinical application is limited. Besides,
112 the value of circulating tumor DNA (ctDNA) as a biomarker of advanced tumor has
113 been well established. However, its role in lung cancer screening and auxiliary

Reply: We gratefully appreciate for your valuable suggestion. We cited the relevant literature and adjusted the citation sequence number of the full text (See page 3; Line 83).

3. Informed consent statement:
As this study included 18 healthy volunteers, did they sign informed consent form? If yes, please revise "patients" to "participants".

259 2013). The study was approved by Institutional Review Board of Changhai Hospital
260 (No.2020021) and informed consent was taken from all the patients.

Reply: Thank you for your reminding. In fact, all participants in the study signed an informed consent form. It was an oversight of us to use the wrong words, so we revised "patients" to "participants" (see Page 6, Line 184).

4. Please indicate the full name of "AUC" in Figure 3-4 legends.
Reply: Thank you for your reminding.The full name of "AUC" is area under the receiver operating characteristic curve.We have added it to legends section of Figure 3 - 4(see Page 19, Line 537; Page 20, Line 550).