

Peer Review File

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Reviewer A

INTRODUCTION

Comment 1: Leading men cancer death cause is lung cancer (doi: 10.3322/caac.21708), please modify it;

Reply 1: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 3, line 64)".

Comment 2: Prostate cancer (PCa), in particular in the early phases, has no symptoms associated, please correct it;

Reply 2: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 3, line 67-68)".

Comment 3: In the diagnostic algorithm there is also MRI which is playing a crucial role (doi: 10.3390/currenol29100538), please improve your description of PCa diagnosis;

Reply 3: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 3, 4 line 76 and line77)".

Comment 4: line 71, correct PSE

Reply 4: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 3, line 75)".

Comment 5: Please describe miRNAs as small, single-stranded, non-coding RNA molecules containing 21 to 23 nucleotides and that their collection is possible also in the urine for a potential minimally invasive diagnostic test (doi: 10.3390/cancers14051112)

Reply 5: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 4, line 81-83)".

MATERIALS AND METHODS

Comment 6: line 94 please correct the mistaken word "partakes" with "partakers";

Reply 6: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 4, line 104)".

Comment 7: line 95 please primarily use the extensive form of the word and only after the short form. Please correct HCs.

Reply 7: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 4, line 105)".

Comment 8: Please specify included and excluded criteria.

Reply 8: Thank you very much for your advice. We have added the criteria for inclusion.

Changes in the text: We have modified our text as advised (see Page 5, line108-114)".

Comment 9: Please describe the 12 miRNAs included in your research and why them, from which panel did you choose these 12 miRNAs?

Reply 9: Thank you very much for your advice. We have added relevant literature related to these miRNAs, as shown in Supplementary Table 1.

Changes in the text: We have modified our text as advised (see Page 5, line116-line 122)".

Comment 10: line 113 why surgical resection for PCa? Do you mean radical prostatectomy?

Reply 10: Thank you very much for your advice. The sample we selected is patients diagnosed with prostate cancer based on pathological results after surgery. We did not pay attention to the specific surgical methods.

Comment 11: line 145 please write the extensive form for NCs before abbreviation.

Reply 11: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 6, line 165)".

RESULTS

Comment 12: Please reformulate the first paragraph of this section because it does not fit in that section of the manuscript, instead of it could be as inclusion and exclusion criteria.

Reply 12: Thank you very much for your advice. We moved the content to Material and Methods section.

Changes in the text: We have modified our text as advised (see Page 5, line 108-114)".

Comment 13: Please report instead the miRNAs selected for the analysis in the Material and Methods section.

Reply 13: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 5, line 120-line122)".

DISCUSSION

Comment 14: Please improve it with the latest novels in the field (doi:10.3390/cancers14133157; doi: 10.1016/j.urolonc.2020.03.007);

Reply 14: Thank you very much for your advice. Through studying these two articles, I have gained a deeper understanding of the role of miRNAs in the

occurrence and development of tumors, as well as the potential biological biomarkers for diagnosing prostate cancer in urine. And it has improved the description of the role of miRNA in the article.

Changes in the text: We have modified our text as advised (see Page 3, line219-line 224)".

Table 1

Comment 15: Please report the mean PSA value and its standard deviation.

Reply 15: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Table1)".

Reviewer B

Introduction:

Comment 1: It would be beneficial to provide a more detailed rationale for the selection of miRNAs included in the study and to discuss the importance of each miRNA in prostate cancer biology based on existing literature.

Reply 1: Thank you very much for your advice. We have added relevant literature related to these miRNAs, as shown in Supplementary Table 1.

Changes in the text: We have modified our text as advised (see Page 5, line116-line 120)".

Methods:

Comment 2: Participant Selection and Ethics: Provide more information about the criteria used to select participants, including age range and any exclusion criteria. Additionally, clarify whether the participants were from diverse ethnic backgrounds, as this could impact the generalizability of the findings.

Reply 2: Thank you very much for your advice. We have provided a more detailed description of the criteria for case selection. These patients are all Asians from hospitals in the region.

Changes in the text: We have modified our text as advised (see Page 4 ,5 line 108-112)".

Comment 3: RNA Extraction and RT-qPCR: Provide a brief explanation of why cel-miR-54-5p was used as an internal reference for RNA extraction normalization. This step might be crucial in ensuring the accuracy and reproducibility of the results.

Reply 3: Thank you very much for your advice. We added 2ul cel-miR-54-5p to plasma for normalization(1).

Comment 4: Statistical Analyses: While the statistical methods are mentioned, consider providing more details about the specific statistical tests used at various stages of the study. This would enhance the transparency of the analysis and its interpretation.

Reply 4: Thank you very much for your advice. Backward stepwise logistic regression was used to examine the efficiency of diagnosis panel including the finally selected miRNAs. The software we used for statistical analyses was GraphPad 5.0 and SPSS.

Results

Comment 5: Graphs and Figures: Ensure that the graphs and figures are labeled clearly, and their relevance to the findings is explained in the accompanying text. This will help readers understand the data more easily.

Reply 5: Thank you very much for your advice. We have added some image labels to make it easier to understand.

Changes in the text: We have modified our text as advised (see Figure 2 and Figure 5)".

Comment 6: Discussion of Results: The results are described in a concise manner, but there is an opportunity to elaborate on the significance of the findings. Discuss how the identified miRNAs relate to existing knowledge about prostate cancer and how they may contribute to disease progression or prevention.

Reply 6: Thank you very much for your advice. We supplemented the importance of the research findings in the discussion section. We have already introduced the relationship between identified miRNAs and prostate cancer in the discussion section.

Changes in the text: We have modified our text as advised (see Page 10, line 279-line 280)".

Comment 7: Clinical Implications: Emphasize the potential clinical impact of the miRNA panel as a screening tool for prostate cancer. Discuss how the panel's high sensitivity and specificity could lead to improved patient outcomes, reduced overdiagnosis, and more targeted treatment strategies.

Reply 7: Thank you very much for your advice. We added the value of miRNA in clinical applications in the discussion section.

Changes in the text: We have modified our text as advised (see Page 8, 9, line 232-line 234)".

Comment 8: Summarize the main findings and their implications for prostate cancer screening and diagnosis. Reiterate the novelty and potential impact of the proposed four-miRNA panel.

Additionally, as you mentioned, consider incorporating the suggested additional studies (PMID: 37446024; PMID: 36294423) to strengthen the literature foundation and enhance the overall quality of the article.

Reply 8: Thank you very much for your advice. I have carefully read the two literature you recommended, which has given me a better understanding of miRNA and biological markers.

Changes in the text: We have modified our text as advised (see Page 8, line 219

and line 224)".

Reviewer C

Major comments:

Comment 1: In the "Material and methods" section, the "Study design" is not complete. How did the authors select those miRNAs? Why only those 12 miRNAs? The authors should clarify in this section and cite related works.

Reply 1: Thank you very much for your advice. We have added relevant literature related to these miRNAs, as shown in Supplementary Table 1.

Changes in the text: We have modified our text as advised (see Page 5, line116-line 120)".

Comment 2: In the "Results" section, the authors should also report differences in localised PC vs. not localised PC ($\leq T2c$ vs $\geq T3$. Be careful, $\leq T2$ vs $\geq T2$ reported in the "Table 1" is scientifically meaningless) by also reporting p-value in both "Table 1" and "Results" section. Serum levels or the presence of miRNAs could differ in these two populations.

Reply 2: We are sorry for our careless mistakes. We have changed "T2 \geq " to "T2 $>$ " in Table 1. Thank you for pointing out this error. Our study aims to compare miRNA levels in serum between normal individuals and prostate cancer patients, so we did not conduct a specific study in localized PC vs. not localized PC. This is the deficiency of our experiment and is worth further research.

Changes in the text: We have modified our text as advised (see Table 1)".

Comment 3: In "Table 1", the authors are invited to report grades of Gleason Score as numerical values. Is there any difference in the expression of miRNAs according to different GS scores?

Reply 3: Thank you very much for your advice. Our experiment did not study difference in the expression of miRNAs according to different GS scores. This is something worth exploring and we will continue to explore it in future experiments.

Minor comments:

Comment 4: In the "Introduction" section, I suggest citing data from guidelines as the first reference. Moreover, the second ref. is wrong. The article declares that PCa is the second most common cause of death in males, the first one is lung cancer.

Reply 4: Thank you very much for your advice. We have made modifications based on the latest guidelines.

Changes in the text: We have modified our text as advised (see Page 3, line64)".

Comment 5: In the "Introduction" section, the authors are invited to not write the full form of abbreviations after defining them (i.e., prostate cancer in line 69). In

the same line, BPH has not been defined before. The authors are invited to correct similar issues in the whole manuscript.

Reply 5: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 3, line 74)".

Comment 6: In the "Introduction" section, lines 70-71, how could screening cause overdiagnosis if it is made only by positive biopsy? The authors are invited to clarify this point. The same issue is present in the "Discussion" section.

Reply 6: Thank you very much for your advice. We have corrected the statement about PSA and tissue biopsy.

Changes in the text: We have modified our text as advised (see Page 3, Page4, line 73-79, Page8, line 218-219)".

Comment 7: An English form review is required, i.e, PSE in line 71 or "it plays" in line 75

Reply 7: We are sorry for our careless mistakes. We have modified "PSE" to "PSA".

Changes in the text: We have modified our text as advised (see Page 3, line 75)".

Comment 8: In the "Discussion" section, I suggest to better approach to miRNAs utility in all cancers and then in PC. Indeed, recent literature shows several data on miRNAs as diagnostic tools for PC in human fluids such as in PMID: 37446024 and PMID: 25496077.

Reply 8: Thank you very much for your advice. We added the role of miRNA in prostate cancer.

Changes in the text: We have modified our text as advised (see Page 8, line 224-226)".

Reviewer D

Comment 1: Authors very often used word "expression" for the miRNAs present in the blood serum, that is not correct since miRNAs could not be expressed in the extracellular space. It is better to use word "quantity" or similar.

Reply 1: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 4, line85-line 86)".

Reviewer E

Comment 1: The overall construction of the text can be improved to facilitate readability and to better transmit the message of the work as well as its objectives and results.

E.g: The title: The value of a group of serum miRNAs in screening prostate cancer'', although not misleading somehow lacks proper terminology, like spelling microRNA and using 'group' instead of panel or 'serum miRNA' instead

of circulating microRNAs.

Reply 1: Thank you very much for your advice. We changed the title to "The value of a panel of circulating microRNAs in screening prostate cancer".

Changes in the text: We have modified our title.

Comment 2: A widely more recognized and used acronym for prostate cancer is PCa, instead of PC, as used in the manuscript.

Reply 2: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 3, line64)".

Comment 3: Use miRNAs when referring to a plural - >1 miRNA.

Reply 3: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Page 4, line86)".

Comment 4: The introduction is very short and should also include a rationale about the choice of the 12 miRNAs analyzed in the study.

Reply 4: Thank you very much for your advice. We have added the selected 12 miRNAs in the introduction section.

Changes in the text: We have modified our text as advised (see Page 4, line93-95)".

Comment 5: Why the inclusion of 112 PCa + 112 control (healthy) samples exactly? So both groups can be symmetric?

Reply 5: The research hypothesis is that the area under the ROC curve of the diagnostic panel is greater than 0.8. The area under the ROC curve of index A was 0.9 in the previous pre-experiment (or checking the literature), with $\alpha=0.05$ (one-sided), $\beta=0.1$, and the ratio between groups was 1:1. PASS11 was used to estimate the sample size. It was found that at least 104 patients and 104 controls needed to be included. Considering a certain loss to follow-up rate, the study included 112 patients and 112 controls.

Comment 6: The authors should clarify what they mean by 'training phase'. This seems to be the preparatory step to validate their methodology before expanding the study.

Reply 6: During the training phase, we select more valuable miRNAs as candidate miRNAs through a small number of samples. In the validation phase, we further expanded the sample to validate these miRNAs.

Comment 7: In the section: "Selecting PC-related miRNAs during the screening phase" (line 157), I reiterate that more background must be given about the choice of target miRNA.

Reply 7: Thank you very much for your advice. We have added relevant literature related to these miRNAs, as shown in Supplementary Table 1.

Changes in the text: We have modified our text as advised (see Page 5, line116-line 120)".

Comment 8: In the section: "Building a four-miRNA panel for prostate cancer screening" (line 181). More detail is required to understand the choice of the 4-panel miRNA selected. This can be elaborated upon in the discussion.

Reply 8: Thank you very much for your advice. Backward stepwise logistic regression was wielded to examine the efficiency of diagnosis panel including the finally selected miRNAs. The software we used to statistical analyses was GraphPad Prism 8 and SPSS.

Comment 9: In Figure 1, panel B is missing the heading.

Reply 9: Thank you very much for your advice.

Changes in the text: We have modified our text as advised (see Figure 1)".

Comment 10: Figure 3 consists of a single panel. I suggest combining it with Figure 4 and elaborating on the figure caption about the selection of the 4-panel miRNA as well, since this is a key message of the study.

Reply 10: Thank you very much for your advice. But we recognize that Figure 3 and Figure 4 are two parts of content, and it would be better to separate them. We have already provided a detailed introduction in the results section on how to obtain this 4-panel miRNA.

Comment 11: In Figure 5, the captions on the graphs are not very legible.

Reply 11: Thank you very much for your advice. We have added a new title in Figure 5.

Changes in the text: We have modified our text as advised (see Figure 5)".

Comment 12: The manuscript has be checked to ensure that all figures are cited accordingly.

Reply 12: Thank you very much for your advice. We have rechecked all the figures in the manuscript.

Comment 13: An overall spell-check might be required before the manuscript can be published, to eliminate minor grammar errors.

Reply 13: Thank you very much for your advice. We checked the spelling of the words in the manuscript.