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RE: ESPS Manuscript NO: 5214

Dear Editor,

We are re-submitting a revision of our manuscript entitled: "A Novel Blood-based MicroRNA Biomarker Panel for Early Diagnosis of Pancreatic Cancer" for consideration of publication in WJGO.

Some of the major changes within this revision are:

- The patient population has been updated – we now included only pancreatic ductal adenocarcinoma patients and removed other histopathologic pancreatic cancer patients, which, as some reviewers expressed concern, might cloud the results. Consequently, all data analysis has been re-done and updated in this revision. The results and conclusions, however, remain the same.
- As one of the reviewers suggested, we have included a new set of data and ROC analysis, comparing CA19-9 to the three-miRNA panel.
- We have reorganized the structure of the Methods and Results sections in both the Abstract and Text, as one reviewer suggested, so that each section is better organized according to their contents.
- We have updated the Discussion section, including discussion about prior results in the published literature and each microRNA target.

What follows is our response to the reviewers' comments in a point-by-point format.

Thank you for considering our manuscript.

Sincerely,

Ganepola A. P. Ganepola, MD, FACS
David Chang, PhD

Reviewer 00505184 comment 1

Table 2 shows miRs that are upregulated pancreatic cancer patients and health controls. However, where is the comparable list of down-regulated miRs and possible inverse expression patterns in patients vs. controls? A selected subset of inversely expressed miRs (i.e. patients vs. controls) would also be an interesting subset to examine as potential prognostic factors.

Our Response:

Table 2 is divided into two columns, showing "up-regulated in patients" on the left-hand-side (with corresponding down-regulation of the same miRs in controls) and "up-regulated in controls" on the

right-hand-side (with corresponding down-regulation of the same miRs in patients). Therefore, we have shown both up-regulated and down-regulated miRs.

We agree and have done just what the reviewer suggested -- selected subsets representing both up- and down-regulated miRs in patients or controls. However, as stated in the Results section, "RT-qPCR Confirmed Three Potential miRNA Diagnostic Markers", of the 8 miRs tested, only 3 miRs, all up-regulated in cancer, gave consistent results and therefore were used as a diagnostic panel for validation study.

Reviewer 00505184 comment 2

The authors need to gear their discussion, in part, by comparing their results with other studies on miR expression in blood from pancreatic patients vs. controls.

Our Response:

We thank the reviewer's suggestion. The discussion with other studies have been included.

Reviewer 00505184 comment 3

The authors also need to discuss the known significance (functional/prognostic) of the three diagnostic miRs identified in this study. For example, serum miR-885-5p has previously been reported as a potential prognostic factor for detecting liver pathologies (Clinical Science 120, 183-193, 2011).

Our Response:

We thank the reviewer's suggestion. The discussion with known-significance of the 3 diagnostic miRs has been included.

Reviewer 02467561 comment 1

ABSTRACT The abstract gives a clear delineation of the research objectives, materials and methods, results and conclusions. Nevertheless, some contents should be moved from the Results to the Methods section, with reference to the different steps followed in carrying out the study and the statistical methods or laboratory tests applied (e.g. TaqMan RT-qPCR; ROC curve analysis…).

Our Response:

We thank the reviewer's suggestion. The abstract has been reorganized and updated to reflect the change.

Reviewer 02467561 comment 2

MANUSCRIPT The Results and Methods sections are not well organized. In particular, the Authors should insert in the Methods all those contents which are referred to the development and carrying out of the study (selection of patient groups, description of laboratory techniques/tests and statistical methods applied ...). The Results should contain only a lean description of the findings of the different steps of the study.

Our Response:
We thank the reviewer's suggestion. We have made some changes according to the recommendations. However, given the history of many publications relating to the similar experimental approaches in pancreatic cancer and other cancers, we feel strongly the need to provide at least some experimental details/methods in the Results section to minimize possible confusion and explain the critical experimental strategy.

Reviewer 02467561 comment 3
Regarding the discussion, some references should be indicated in different parts of the text (e.g. second paragraph: "We also combined potential miRNA targets identified from the results of our screening study with notable miRNAs published by other investigators…"; "Two miRNAs (miR-642b-3p and miR-885-5p) included in our final three-miRNA panel were shown to be significantly up-regulated in cancer by our screening process while the third miRNA (miR-22-3p) was shown to be up-regulated in the literature...".

Our Response:
The reference as stated has been provided, either next to the text, or in Table 2 next to each microRNA.

Reviewer 02467561 comment 4
Moreover, the full implications of the study findings are not sufficiently described and discussed.

Our Response:
The full implications of the study has been described and discussed in the Discussion section.

Reviewer 02467561 comment 5
REFERENCES Some references, although appropriate, should be updated.

Our Response:
The reference has been updated to the best of our knowledge.

Reviewer 00181289 comment
The only issue I have with the data is the inclusion of mucinous adenocarcinoma, ampullary carcinoma and neuroendocrine tumours as they have a different histopathogenesis and this could potentially cloud results

Our Response:
We thank the reviewer's suggestion. The questionable patients have been removed from the patient pool. All data has been re-calculated and re-analyzed accordingly. The results and conclusions, however, remain the same.

Reviewer 01557573 comment 1

Circulating microRNAs have been proved to be detectable in peripheral blood with clinical significance. Due to its convenience and non-invasiveness, this method is drawing more and more attentions and has been tried in many different types of cancer, including PA. Two highly relevant and similar publications are found from PubMed[1, 2], this weakens the novelty of the reviewed manuscript.
1. Carlsen AL, Joergensen MT, Knudsen S, de Muckadell OB, Heegaard NH. Cell-Free Plasma MicroRNA in Pancreatic Ductal Adenocarcinoma and Disease Controls. Pancreas. 2013;42(7):1107-13. Epub 2013/09/21. 2. Liu R, Chen X, Du Y, Yao W, Shen L, Wang C, et al. Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. Clinical chemistry. 2012;58(3):610-8. Epub 2011/12/24.

Our Response:
We thank the reviewer's reference. We had overlooked one of the studies (ref#2) and did not anticipate the publication of ref#1, which was published after the manuscript was first submitted. Nevertheless, we have now updated our text and included discussion about these two studies in the Discussion section.

Reviewer 01557573 comment 2
The authours used totally health individuals as the control. Concomitant chronic pancreatitis (and some other diseases) is common in PA. From the clinical point of view, differential diagnosis of early stage PA from chronic pancreatitis is very challenging and thus of greater significance. Taken together, patients with chronic pancreatitis who have excluded PA would be a better choice of the control in this study. From the manuscript, I can not find the information about the concomitant pancreatitis or other situations in the PA patients group.

Our Response:
We disagree with this reviewer's assessment that "concomitant chronic pancreatitis (and some other diseases) is common in PA.

Dzeletovic et al. in "Pancreatitis Before Pancreatic Cancer: Clinical Features and Influence on Outcome." J Clin Gastroenterol. 2013 Oct 22. (PMID:24153158) found only 195 cases of pancreatitis among 2573 pancreatic cancer patients (8%). Yadav D and Lowenfels AB in "The epidemiology of pancreatitis and pancreatic cancer" *Gastroenterology*. 144(6):1252 (PMID:23622135), reviewed and demonstrated a weak link between the incidences of pancreatitis and pancreatic cancer. Therefore, there is no justification of using cases of pancreatitis as controls.

The purpose of this manuscript is to identify a panel of early detection markers for early stage pancreatic cancer, not a panel of "differential diagnostic markers for all pancreas-related diseases." None of the pancreatic cancer patients recruited for this study had pancreatitis or other pancreatic diseases and therefore were not mentioned in the manuscript.

Reviewer 01557573 comment 3
One pancreatic neuroendocrine carcinoma and one adenocarcinoma of ampulla were enrolled in the study. Later the former case showed different microRNA expression signature compared with other pancreatic adenocarcinoma, but the microRNA expression panel of the latter one

was not mentioned. So I assume that it was treated as a PA, but they are different diseases, so it should not be enrolled into the study.

Our Response:
We thank the reviewer's suggestion. The questionable patients have been removed from the patient pool. All data has been re-calculated and re-analyzed accordingly. The results and conclusions, however, remain the same.

Reviewer 01557573 comment 4
The rationale of using miR-3196 as the inner-control? U6 used to be widely used as the inner control, recently some researchers begin to question it. The authours argued this microRNA showed very stable expression in their study, but more explanation (about this microRNA or about this way to pick an inner control) would make it more acceptable.

Our Response:
We thank the reviewer's suggestion. We have included the rationale of using miR-3196 in the Methods section.

Reviewer 01557573 comment 5
Did the authours collected the resected samples of the PA? Since they were early-stage PA, I assume many of them underwent resection. It will be very interesting to compare the expression signature of peripheral and in situ microRNAs.

Our Response:
We thank the reviewer's suggestion. The current study focuses on identifying circulating miRNA markers for detection of early stage pancreatic cancer. The pancreatic tissue miRNA profiling is a completely different study. Although it would be of great interest to compare, mechanistically, between the expression signatures of peripheral circulating miRNA versus *in situ* pre- and mature microRNA expression in tissue. It is beyond the scope of the current study.

Reviewer 01557573 comment 6
The authours studied the diagnostic value of the panel of 3 microRNAs. I suggest they compare it vs. serum CA19-9 plus imaging (MR or CT), currently the latter combination is the widely used diagnostic tools of PA.

Our Response:
We thank the reviewer's suggestion. The CA19-9 result has been incorporated into this paper, and the diagnostic value of the three-miRNA panel has been compared with serum CA19-9 result.

It should be noted, however, that the pancreatic cancer patients recruited for this study are from a "confirmed cancer population", not a "suspected cancer population". They all had positive MRI and CT scans before undergoing surgery, and their staging was confirmed post-operatively by pathologists (as stated in the Methods). It would be of great interest to set up a future large scale clinical trial, based on this pilot study, to investigate the "suspected cancer population." However, that goal is clearly beyond the scope of the current pilot study.

Reviewer 01557573 comment 7

Sporadic spelling errors, like CA19-9 and CD19-9.

Our Response:

We thank the reviewer's suggestion. The errors have been corrected and checked to the best of our knowledge.