5 February, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 15912-manuscript).

Title: The downregulation of microRNA-382 is associated with poor outcome of esophageal squamous cell carcinoma

Author: Bo Qi, Jian-Guo Lu, Wen-Jian Yao ,Ting-Min Chang, Xiu-Guang Qin, Ying-Hua Ji, Tian-Yun Wang, Shang-Guo Liu, Han-Chen Li, Yu-Zhen Liu, Bao-Sheng Zhao

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 15912

We have revised the manuscript extensively. Therefore, no markings were used for changes in the revised manuscript except the title. Our detailed responses to the reviewers' comments are to be found below. Our reply to the reviewers is in italic.

The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewers

Reviewer 03035394:
I thank the authors for their submission and the opportunity to review the paper. Overall it is an interested topic aiming to advance management for this difficult disease. Through mainly "tightening" the paper in its scientific reporting, and partly by further expressing the clinical implications of their work, I believe the paper is worthy for publication. Below are my specific comments.

1) Abstract: methods - the term prognosis needs to be changed to "outcome" or "post-operative longevity" for example last para intro - "but different prognosis" - different wording should be used to make clear it was based on eventual patient outcome (prognosis is a term used before outcome is known)....I would suggest making this consistent throughout paper, including the figures, as outcome is a more accurate term and would make the paper clearer to read.....also state if the 2 patients previously studied was for published work or not.

Reply: *We have followed the reviewer's suggestion and extensively renamed the patients groups as "poor outcome and good outcome". Moreover, we carefully switched "prognosis" to "outcome" in terms of the*

context throughout the revised version of our manuscript. We have stated that the previous study from the 2 patients is published work by adding the reference number (Reference 15) when we mentioned the results from these 2 patients in the section "INSTRUCTION" in our revised version.

2) Methods: first paragraph - make clearer selections that applied. Consecutive patients? No note is made of post-operative treatments (esp. chemotherapy) which could confound results. This is also not reported in results section. It is implied but not stated that "mid-term" prognosis patients were excluded or not studied, given those surviving 1-5 years seem not to have been studied - this should be specified if this is the case. There is no description of the standardized follow-up? Did all receive the same? Did the clinicians know the miR-382 result when they were following the patients up and therefore could they have monitored those with higher levels more closely?

Reply: *We have addressed all above issues in the section "MATERIALS and METHODS" in the revised manuscript. 46 patients were grouped into "good outcome and poor outcome". The patients in the poor outcome group showed early metastasis but no recurrence and died within 1 year after surgery; the patients in the good outcome group had neither metastasis nor recurrence, and lived for 5 years or more after surgery. The patients who lived between 1-5 years were excluded here. All patients with good outcome did not receive any postoperative treatment; some of the patients with poor outcome had received postoperative chemotherapy treatment that was given after early metastasis occurring. Postoperative follow-up was a standardized process that included a routine CT scan and upper gastrointestinal endoscopy. Before we started this study, all patients' information and specimens had been collected. For this reason, we did not initially know the miR-382 result.*

3) Results:

- The lymph node metastases percentages do not add up (47.5% plus 67.5% does not equal 100!)....also the stage percentages do not add up despite being 46 patients??

Reply: *We deeply apologize for our mistakes. All numbers were recalculated and the errors are now extinguished in the revised manuscript.*

- Were there any stage 4 patients operated on and excluded?

Reply: *We have stated this question in the section "MATERIALS and METHODS" of our revised manuscript. When we started this study, we did not have stage IV patients who lived for 5 years or more, after surgery. Thus, stage IV patients were excluded in our study.*

-The sentence describing the level of miR-382 in each group does not specify if mean/STD or otherwise.

Reply: *The level of miR-382 was expressed as mean ± standard error of mean (SEM). We specified this in the section "MATERIALS and METHODS "of our revised version.*

-Figure 3 - no x-axis label.

Reply: *The x-axis has been labeled with "Survival time (months)" in the Figure 3.*

-No specification of what is "higher" level cut off and why it was chosen as the cut off - also not specified in the main text.

Reply: *This is particular constructive comment. We apologize for our inaccurate description and have carefully explained our results throughout the revised version of our manuscript. Additionally, we have mentioned that the total number of patients assessed in our study was relatively small, especially for the number of individual pathological stage patients. Thus, it is difficult to determine the cut-off level of miR-382 as the marker for clinical utility from our current results. When comparing miR-382 level between two groups, we found the miR-382 level (mean ± standard error of mean) in the poor outcome patients was lower than that in the good outcome patients. In another words, miR-382 level in the good outcome patients was higher than that in the poor outcome patients. Accordingly, either "higher" or "lower" level just means the relative quantification when comparing the results from two groups.*

4) Discussion:

- Second sentence is confusing in the way it is written. An assumption throughout is that the metastases relate to death. Was this the case for all patients or did some have local recurrence as their mode of death? Is the inference that miR-382 relates to metastases as opposed to local aggressiveness or is this not determined?

Reply: *We thank the reviewer for the comments. We have rewritten the second sentence in the section "DISCUSSION" of our revised version. We apologize for confusing the reviewer. We have given more detail information about patients in the section "MATERIALS and METHODS" of our revised manuscript. All poor outcome patients in this study had metastasis but no local recurrence. All good out patients had neither metastasis nor recurrence. The inference therefore is that miR-382 relates to metastasis. Nonetheless, we cannot exclude miR-382 involving in ESCC local recurrence yet and further studies need to be done.*

5) Conclusion

- I recommend a further note being made about what specifically should be the next study point - ?determining the cut-off level of the marker for clinical utility? or prospectively comparing to TNM staging tool? or determining usefulness of the marker for post-op chemotherapy decisions, for example.

Reply: *We thank the reviewer for these valid comments. We follow the suggestions and discuss the future study points in the section "DISCUSSION" of our revised version.*

Reviewer 03026924:

It's an interesting well written study. Just 2 points for future studies or to add in the present study if possible:

1) It should be interesting to know if the analysis of the miR-382 is applicable also to forceps biopsies obtained during upper gi endoscopy and if it correlates to that expressed in the surgical specimen.

Reply: *The reviewer raises a good point. We have discussed this issue in the section "DISCUSSION" of our revised manuscript. We point out that miR-382 level in esophageal cancer tissue is dependent on the individual ESCC patient situation rather than specimen type used for examination. Hence, our results should be applicable to other specimen types such as fresh surgical specimen and forceps biopsies obtained*

during upper gastrointestinal endoscopy.

2) I would specify better how the stage was established before surgery (EUS, miniprobes, CT, Pet-ct) and how the patients were followed up(which imaging technique).

Reply: *We usually stage patients before surgery through CT, upper gastrointestinal endoscopy and gastro endoscopy. Nevertheless, all patients in our study were staged by classical TNM method after surgery. All patients were followed up through a standardized process which included routine CT scan and upper gastrointestinal endoscopy.*

3) Figure 3: please, specify the caption of the x-axis (weeks? Months?).

Reply: *The x-axis has been labeled with "Survival time (months)" in the Figure 3.*

Reviewer 03026970:

This study first provides the evidence of the divergent expression of microRNA-382 in tumor tissue from esophageal squamous cell carcinoma (ESCC) patients, which indicate that it is associated with prognosis and may develop a novel biomarker for both diagnosis and treatment. However, several questions regarding the manuscript should be addressed.

Major comments:

1) Many microRNAs that are related to ESCC have been reported. The authors found a new microRNA, microRNA-382, is also related to ESCC. It would be better if any introduction of microRNA-382 was added in the background.

Reply: *According to your comment, we have descripted more miR-382 in the "INTRUDUCTION".*

2) Materials and methods

-Did the authors detect the expression of microRNA-382 in normal population, or normal tissues adjacent to the tumor tissues, and what's the result?

Reply: *We addressed this issue in the section "MATERIALS and METHODS" of our revised manuscript. Para-cancerous esophageal mucosa (8 cm distant to the verge of the tumor tissue) from 4 ESCC patients was taken as controls. Table 1 shows the results.*

Table 1: qRT-PCR results of miR-382 in para-cancerous esophageal mucosa from 4 ESCC patients

Sample No.	*miR-382*		*Relative Quantitative Result*		
	Ct	*Mean Ct*	*U6 Mean Ct*	*-ΔΔCt*	*$2^{-\Delta\Delta Ct}$*
128	*18.70388*	*18.759363*	*9.168213667*	*-9.591149667*	*1.2965E-03*
	18.61417				
	18.96004				
123	*19.6067*	*19.480047*	*24.05109*	*4.571043333*	*2.3770E+01*
	19.54173				
	19.29171				

122	*18.53456*	*18.616283*	*9.399559*	*-9.216724333*	*1.6807E-03*
	18.50582				
	18.80847				
125	*18.3459*	*18.298857*	*9.771067667*	*-8.527789*	*2.7094E-03*
	18.38461				
	18.16606				
Average	***18.78864***	***18.78864***	***13.09748***	***-5.69115***	***5.943922***

-Did the patients receive other treatments after surgeries, such as chemotherapy and radiotherapy? Were there any differences between the two groups, because these treatments would influence the result?

Reply: *All patients with good outcome did not receive any postoperative treatment. Some patients with poor outcome received postoperative chemotherapy treatment that was given after early metastasis happening. Thus, we speculate our results were not influenced by the postoperative treatment.*

-As mentioned in this article, the high-throughput real-time quantitative PCR was used to evaluate 754 microRNAs levels from 2 ESCC patients previously. Were there any other microRNA downregulated in the ESCC patient with poor prognosis besides microRNA-382?

Reply: *The results from 2 ESCC patients were published (Reference 15). In addition to miR-382, some miRNAs, such as miR-31, miR-101, miR-196b and miR-652, were downregulated in the poor outcome patient compared to the good outcome patient. More detailed results can been found in Reference 15 (**Zhao BS**, Liu SG, Wang TY, Ji YH, Qi B, Tao YP, Li HC, Wu XN. Screening of microRNA in patients with esophageal cancer at same tumor node metastasis stage with different prognoses. Asian Pac J Cancer Prev 2013; 14: 139-143 PMID: 23534712).*

-Real-time RT-PCR is a benchmark technology for the detection of RNA levels for its simplicity, specificity and sensitivity, did you check the result?

Reply: *We did use real-time RT-PCR to examine the level of miR-382 in our study.*

-Definition of the down regulation is ambiguous. What are the control samples which are mentioned in the gene expression formula (materials and methods).

Reply: *We addressed this issue in our answer to reviewer 03035394 above. The total number of patients assessed in our study was relatively small, especially for the number of individual pathological stage patients. Thus, it is difficult to determine the cut-off level of miR-382 as the marker for clinical utility from our current results. When comparing average miR-382 levels between the good outcome patients and the poor outcome patients, we found the miR-382 level (mean ± standard error of mean) in the poor outcome patients was lower than that in the good outcome patients. Accordingly, either "higher" or "lower" level just means the relative quantification when comparing the results from two groups. Para-cancerous esophageal mucosas (8 cm distant to the verge of the tumor tissue) from 4 ESCC patients were taken as controls.*

3) Result

-In the study, 46 patients were divided into good and poor prognosis, but what are the specific criterions? A reference should be provided in this part. Did the authors exclude those patients survived more than 1year and less than 5years after surgery? This should be mentioned in the manuscript.

Reply: *The detail patients information is given in the section "MATERIALS and METHODS" of our revised manuscript. 46 patients were divided into 2 groups according to postoperative survival time: the poor outcome group, which showed early metastasis but no recurrence, and died within 1 year after surgery; the good outcome group, which did not have metastasis and recurrence, and lived 5 years or more after surgery. This study did not include the patients surviving between 1-5 years after surgery.*

-When the authors try to create a COX regression model, did they enroll other variables such as age, family history, alcohol consumption and smoking status?

Reply: *We only did the Cox single factor-related risk analysis. The results were shown in the section "RESULTS". We did not enroll other variables as mentioned in the comment.*

-It would be better if the authors could provide the RNA sequence of microRNA-382, as readers would be interest in this.

Reply: *We have provided human miR-382 sequence as well as gene number in the section "INTROUDUCTION".*

4) Discussion:

-The authors considered microRNA328 to be a potential biomarker for the prognosis of ESCC patients, it would be better if there was some exploration about the mechanism in the discussion part.

Reply: *We appreciate the reviewer's comment. We follow the suggestion and give a discussion on this point in the section "DISCUSSION".*

-The authors mention that microRNA-382 as a tumor suppressor could be a useful biomarker for prognosis and outcome prediction in ESCC. The research is not enough to prove microRNA-382 is a tumor suppressor. More research is needed for the conclusion, such as in vivo and in vitro study.

Reply: *You mention a very important point. We speculate miR-382 as a tumor suppressor in ESCC metastasis according to our current results, which indeed did not provide direct evidence for this speculation. We have discussed this issue in the section of "DISCUSSION ". We are now doing the relevant research and explore the effects and possible mechanisms by which miR-382 works as a tumor suppressor in ESCC.*

Minor comments:

1) Method:

-PCR methods description should report whether all samples' Ct value are in a reasonable range.

Reply: *We run PCR in triplicate for each sample. All samples' miR-382 Ct values are less 30, which is in a reasonable range. We describe this point in the section "MATERIALS and METHODS" of our revised manuscript.*

-Does the formula in correct form? Is it should be $F = 2^{-\Delta\Delta Ct}$?

Reply: *We have corrected the formula into* $F = 2^{-\Delta\Delta Ct}$ *in our revised manuscript.*

2) Results:

-There is a discrepancy between the total number of patients in table 1 and the text. Which is correct?

Reply: *We have recalculated all numbers and the errors are excluded in our revised manuscript.*

-The number of patients evaluated in this study was relatively small.

Reply: *We agree with you on this point. As we addressed this issue in our answer to reviewer 03035394 above, it is difficult to determine the cut-off level of miR-382 as the marker for clinical utility from our current results. A large size cohort study must therefore be an objective of future projects. We have collected 582 ESCC specimens since 2011 and all patients have been routinely followed up.*

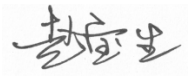
-Fig 3 would be better if the legend of X axis is added.

Reply: *We have added "Survival time (months)" as the x-axis label in the figure 3.*

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology.*

Sincerely yours,



Bao-Sheng Zhao, MD,
Department of Thoracic Surgery
The First Affiliated Hospital of Xinxiang Medical University
Weihui 453100, Henan Province
China
Telephone: +86-373-4404341
Fax: +86-373-4402573
E-mail: zhaobscn@126.com