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20812 – Answering reviewers

Ze-Mao Gong,

Science Editor, Editorial Office

Dear Ze-Mao Gong,

We are glad to return to you our reviewed version of the manuscript entitled: **"*hsa-miR-29c* and *hsa-miR-135b* differential expression as potential biomarker of gastric carcinogenesis"**. All reviewer questions were answered and all editor suggestions were considered and incorporated to the final version. Here we provide the detailed answers to each question and suggestion.

Reviewer:

1. Materials and methods: Normal and cancer tissues were fresh frozen and the other two types were FFPE. How did the authors control the impact from the FFPE of gastritis and metaplasia tissues on the expression level of these two miRNAs?

Answer: We recognize that some studies have shown that the expression level in FFPE samples may be lower or disturbed in comparison to fresh samples of the same tissue due to a possible degradation. We tried to collect only fresh samples, but it was not possible to collect fresh frozen samples of gastritis and metaplasia. The hospitals that gave us all the samples have only gastritis and metaplasia in FFPE samples due the requirement of microdissection, *H. pylori* detection and immunohistochemistry of the histopathological service.

Besides, we believe that the FFPE did not have an impact in our results. We found a significant high *hsa-miR-135b* expression in gastritis and

intestinal metaplasia samples, indicating the absence of degradation in our FFPE samples.

Furthermore, our previous results of microRNA expression in gastric samples without tumor, gastric cancer samples and in tumor-adjacent samples [1,2,3,4,5] corroborate the results described in our manuscript.

1. **Ribeiro-dos-Santos Â**, Khayat A, Silva A, Alencar D, Lobato J, Luz L, Pinheiro D, Varuzza L, Assumpção M, Assumpção P, Santos S, Zanette D, Silva W, Burbano R, Darnet S. Ultra-Deep Sequencing Reveals the microRNA Expression Pattern of the Human Stomach. *PLoS ONE* 2010; **5**: 1-8 [PMID: 20949028 DOI: 10.1371/journal.pone.0013205]

2. **Moreira F**, Assumpção M, Hamoy I, Darnet S, Burbano R, Khayat A, Gonçalves A, Alencar D, Cruz A, Magalhães L, Jr. W, Silva A, Santos S, Demachki S, Assumpção P, Ribeiro-dos-Santos Â. MiRNA Expression Profile for the Human Gastric Antrum Region Using Ultra-Deep Sequencing. *PLoS ONE* 2014; **9**: 1-8 [PMID: 24647245 DOI: 10.1371/journal.pone.0092300]

3. **Gomes LL**, Moreira FC, Hamoy IG, Santos S, Assumpção P, Santana Á, Ribeiro-dos-Santos Â. Identification of miRNAs Expression Profile in Gastric Cancer Using Self-Organizing Maps (SOM). *Bioinformation* 2014; **10**: 246–250 [PMID: 24966529 DOI: 10.6026/97320630010246]

4. **Darnet S**, Moreira FC, Hamoy IG, Burbano R, Khayat A, Cruz A, Magalhães L, Silva A, Santos S, Demachki S, Assumpção M, Assumpção P, Ribeiro-dos-Santos Â. High-Throughput Sequencing of miRNAs Reveals a Tissue Signature in Gastric Cancer and Suggests Novel Potential Biomarkers. *Bioinformatics and Biology Insights* 2015; **9**: 1-8 [DOI: 10.4137/BBI.S23773]

5. **Assumpcao P**, Assumpção M, Moreira F, Hamoy I, Burbano R, Khayat A, Silva A, Santos S, Demachki S, Assumpcao P, Magalhães L, Vidal A, Pereira A, Ribeiro-dos-Santos Â. High-Throughput miRNA Sequencing Reveals a Field Effect in Gastric Cancer and Suggests an

Epigenetic Network Mechanism. *BBI* 2015; **9**: 111-117 [PMID: 26244015 DOI: 10.4137/BBI.S24066]

2. Both figures 1 and 2 show great variations of the 2 miRNAs in carcinoma samples. Is there anything that can stratify the samples, for example, grading or staging of the patients?

Answer: We previously tried to correlate the expression profiles to clinical data of carcinoma patients, but neither grading nor staging were able to stratify the samples due the high heterogeneity among the samples.

3. Figure 3: I assume the *H. pylori* detection was lumped together among all the samples (4 categories) for comparison. Anything interesting if the positivity was factored in figures 1 and 2?

Answer: If we subdivide the non-chronic gastritis group into *H. pylori* positive and negative, we will have 5 classes of samples. In this case, when we perform the ANOVA test and adjust by using Bonferroni's correction, the *P* value becomes too restrict and all the significances are lost to both *hsa-miR-29c* and *hsa-miR-135b*. Further, this subdivision is not necessary to be done since there is no difference between *H. pylori* positive and negative non-atrophic chronic gastritis *hsa-miR-29c* expression.

4. Figure 3: Where are the error bars?

Answer: The graphic with the error bars were submitted online as *20812-Fig. 3.pptx*.

5. Any justification of using Z30 but not the other small RNA controls or in combination with other controls? Have they been tested that the expression is rather constant?

Answer: Our laboratory has a long history on microRNAs expression. Along the years we tested some endogenous controls, such as RNU6B, RNU24 and Z30. In our experience, the most reliable in gastric samples was Z30. As requested, we measured the existing variance in the expression profiles in the samples of this manuscript by using the *Normfinder* algorithm. In this analysis, Z30 was suggested as the best gene to normalize the raw expression data.

Answers to the Editor's suggestions in the manuscript:

1. We provided an editorial certificate from American Journal Express (AJE), which certifies that our manuscript was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English editors.
2. The authorship with the full names was included in the manuscript.
3. The "Supportive foundations" was included in the manuscript.
4. Information about ethics approval, informed consent, conflicts-of-interest and data sharing was included in the manuscript.
5. The other files, such as audio core tip, institutional review board statement, language certificate, informed consent statement and copyright assignment were submitted online. We were not able to perform the CrossCheck analysis, because our institution does not have an account. We tried to request membership, but the process is too complex and will request further time and investment. We also tried to use some alternative softwares, such as DOC Cop and Viper, but these do not seem to be reliable.
6. All authors' abbreviation names and manuscript title were inserted in the manuscript.
7. All the original pictures were save as *pptx* individual files and removed from the manuscript. The legends of the figures 4, 5 and 6 were modified and the abbreviations were included.

8. The legend of table 1 was modified and the abbreviations were included.
9. We inserted the acknowledges, background, research frontiers, innovations and breakthrough, applications and terminology in the manuscript.
10. We inserted the PMID and/or DOI in all references.
11. We subject the final title to Google Scholar and found about 25 results, but none perfect match, as showed in the screenshots:

Yours sincerely,

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