

Reference: Jia-Ping Yan, Science Editor Office, Baishideng Publishing Group Inc.

Manuscript title: “Pivotal role of LncRNA-XIST in regulating immune checkpoint PD-L1 through a shared pathway between miR-194-5p and miR-155-5p in hepatocellular carcinoma”

Manuscript NO: 57925.

Dear Editor/ Prof. Jia-Ping Yan, Science Editor Office,

Please find enclosed the resubmitted revised manuscript for the paper titled; “**Pivotal role of LncRNA-XIST in regulating immune checkpoint PD-L1 through a shared pathway between miR-194-5p and miR-155-5p in hepatocellular carcinoma**” ID: 57925.

We have read thoroughly all the reviewer comments with the constructive criticism which I do respect for the perfection of the manuscript.

We have taken all comments into consideration and we answered the questions and revised the text accordingly (all changes are highlighted in bold).

I am herewith attaching the revised manuscript and the point to point replies to the reviewers.

I always do appreciate very much your support.

With my best regards,

Sincerely,

Hend El-Tayebi.

Reviewer #1:

1. In Introduction, the authors should refer to several papers by Dr. Tasuku Honjo's group and Dr. James P. Allison's group, in the part of cancer therapy using immune checkpoint. Especially regarding PD-1, the authors should need several more references, because Dr. Tasuku Honjo discovered it, another protein expressed on the surface of T-cells in 1992. Ishida, Y., Agata, Y., Shibahara, K., & Honjo, T. (1992). Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J., 11(11), 3887–3895.

Response: Several papers have been added and highlighted, as a reference for PD-1 discovery and immune checkpoint blockade for cancer therapy in the 3rd paragraph of **Introduction** section, lines 110-120.

Reference #9 Okazaki T, Honjo T. The PD-1-PD-L pathway in immunological tolerance. Trends Immunol. 2006;27(4):195-201.

Reference #11 Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J. 1992;11(11):3887-95.

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2. It is not needed the descriptions about the oleuropein from olive leaves and its effect for PD-L1 pathway, although it seems to have an effect on PD-L1 pathway. The authors need to fully explain the necessity of the description about the oleuropein.

Response: Based on a recent study, Oleuropein was reported to have a potential indirect impact on PD-L1 through modulating the expression of Hypoxia-inducible factor-1 in esophageal cancer. In addition, oleuropein immunomodulatory impact in HCC is not thoroughly studied. Thus, in our study we hypothesized that oleuropein might have a

potential immunomodulatory role in HCC through manipulation of PD-1/PD-L1 immune checkpoint.

(This part has been discussed and highlighted in the last paragraph of **Introduction**, lines 163-166)

3. In discussion, the authors should add the figure of the flowchart of pathway that the authors proposed in this study.

Response: A schematic representation of the proposed pathway was added as **Figure 9**.

4. It is not sufficient to explain the limitations of this study. The authors should describe the limitation.

Response: Limitations of the study are added in a separate paragraph under **Discussion** section, lines 462-469.

“Some limitations must be acknowledged for this study. First, the limited number of patients and subsequently, number of tissue biopsies. Further studies using a larger number of tissue biopsies should be implemented to validate the proposed pathway in a larger cohort of patients. Second, a further robust study design can be enforced through the analysis of this study key players in peripheral blood samples of advanced HCC patients as well as investigating the impact of mimicking of both miRNAs, miR-155-5p and miR-194-5p, on PD-L1 protein levels in HCC cell lines.”

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reflects the inflammation role. However, Huh-7 cells are not in the similar inflammatory microenvironment. And how to consider the effect of inflammation.

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Response: The significance of HCC bar is higher, despite showing a lower appearance. This is attributed to the low standard deviation (SD) between RQ values of PD-L1 readings in HCC biopsies on contrary to RQ values for PD-L1 readings in cirrhotic tissues that showed higher SD and hence, lower significance.

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Response: It is stated in the “materials and methods” section under “Patient and tissue samples” that tissue biopsies from 23 HCC patients are obtained as well as 10 healthy donor tissue biopsies, lines 181 and 185, respectively.

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Response: Limitations of the study are added in a separate paragraph under **Discussion** section, lines 462-469.

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4. The authors need to fill out the ARRIVE Guidelines with page numbers. No academic misconduct was found in the CrossCheck detection and Bing search.

Response: No animals were included in this study.

5. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

Response: Figures are compiled in a PowerPoint presentation and submitted along with the revised manuscript.

6. I found the authors did not write the “article highlight” section. Please write the “article highlights” section at the end of the main text.

Response: Article highlight section is added and highlighted.

7. The author should number the references in Arabic numerals according to the citation order in the text. The reference numbers will be superscripted in square brackets at the end of the sentence with the citation content or after the cited author’s name, with no spaces.

Response: Reference style is updated in the submitted revised manuscript.

8. Please don’t include any *, #, †, §, ‡, ¥, @....in your manuscript; Please use superscript numbers for illustration; and for statistical significance, please use superscript letters. Statistical significance is expressed as aP < 0.05, bP < 0.01 (P > 0.05 usually does not need to be denoted). If there are other series of P values, cP < 0.05 and dP < 0.01 are used, and a third series of P values is expressed as eP < 0.05 and fP < 0.01.

Response: P values referencing is updated in the submitted revised manuscript.

Reference: Jia-Ping Yan, Science Editor Office, Baishideng Publishing Group Inc.

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I always do appreciate very much your support.

With my best regards,

Sincerely,

Hend El-Tayebi.

Reviewer #1:

This is a very interesting paper. It based on the analysis in silico further verified the epigenetic regulation in the anti-programmed death therapy in a solid cancer in vitro and in vivo. However, there are some questions should be answered. For example,

1. How many candidates' miRNAs totally were predicted to target PD-L1?

Response: According to microRNA.org and miRDB data base, a total of 146 miRNA were predicted to target PD-L1 mRNA.

This part has been added and highlighted in **Results** Section, under *In silico* analysis part, lines **331-332**.

2. How to rule out the others?

Response: Both miRNAs, miR-155-5p and miR-194-5p were thoroughly studied by our research group in HCC as well as breast cancer so it was interesting to build on the previous work and study into more depth the regulatory pathways orchestrated by both miRNAs in HCC. This part has been discussed in Discussion section, lines **419-422**

And here is of our research group publications including miRNAs miR-155-5p :

- El Tayebi HM, Waly AA, Assal RA, Hosny KA, Esmat G, Abdelaziz AI. Transcriptional activation of the IGF-II/IGF-1R axis and inhibition of IGFBP-3 by miR-155 in hepatocellular carcinoma. *Oncol Lett* 2015; 10: 3206-3212 [PMID: 26722313 DOI: 10.3892/ol.2015.3725].

And here is an abstract publication for miR-194-5p in breast cancer:

- Hamed MM, Eissa RAE, Kassem LAA, El Tayebi HM. XIST overrides the regulatory effect of miR-194 on its immunomodulatory target PD-L1 in breast cancer. *Annals of Oncology*. 2016;27:viii9 [DOI: 10.1093/annonc/mdw525.29].

3. Were they verified some other candidates in vitro or in vivo?

Response: Based on literature, several miRNAs were involved in an epigenetic immunomodulatory effect for immune checkpoint PD-1/PD-L1 as highlighted in references 19, 20 and 21 under **Introduction** section, lines **148-153**, as follows:

- 19 Zhao L, Yu H, Yi S, Peng X, Su P, Xiao Z, Liu R, Tang A, Li X, Liu F, Shen S. The tumor suppressor miR-138-5p targets PD-L1 in colorectal cancer. *Oncotarget* 2016; 7: 45370-45384 [PMID: 27248318 DOI: 10.18632/oncotarget.9659]
- 20 Cortez MA, Ivan C, Valdecanas D, Wang X, Peltier HJ, Ye Y, Araujo L, Carbone DP, Shilo K, Giri DK, Kelnar K, Martin D, Komaki R, Gomez DR, Krishnan S, Calin GA, Bader AG, Welsh JW. PDL1 Regulation by p53 via miR-34. *J Natl Cancer Inst* 2016; 108: [PMID: 26577528 DOI: 10.1093/jnci/djv303]
- 21 Yao K, Wang Q, Jia J, Zhao H. A competing endogenous RNA network identifies novel mRNA, miRNA and lncRNA markers for the prognosis of diabetic pancreatic cancer. *Tumour Biol* 2017; 39: 1010428317707882 [PMID: 28639886 DOI: 10.1177/1010428317707882]

4. The two candidates which called miR-155-5p and miR-194-5p, were examined only in huh-7 cell line, what about the situation in vivo?

Response: Based on literature, several studies have highlighted the impact of miR-155-5p and miR-194-5p knockout as well as overexpression in HCC as indicated in the following citations:

- 1- Sun JF, Zhang D, Gao CJ, Zhang YW, Dai QS. Exosome-Mediated MiR-155 Transfer Contributes to Hepatocellular Carcinoma Cell Proliferation by Targeting PTEN. *Med Sci Monit Basic Res.* 2019; 25: 218-228. [PMID: 31645540 DOI: 10.12659/MSMBR.918134].
- 2- Xie Q, Chen X, Lu F, Zhang T, Hao M, Wang Y, Zhao J, McCrae MA, Zhuang H. Aberrant expression of microRNA 155 may accelerate cell proliferation by targeting

sex-determining region Y box 6 in hepatocellular carcinoma. Cancer. 2012; 118(9):2431-42. [PMID: 21989846 DOI: 10.1002/cncr.26566]

3- Tang H, Zhao H, Yu ZY, Feng X, Fu BS, Qiu CH, Zhang JW. MicroRNA-194 inhibits cell invasion and migration in hepatocellular carcinoma through PRC1-mediated inhibition of Wnt/ β -catenin signaling pathway. Dig Liver Dis. 2019; 51(9):1314-1322.[PMID: 30948333 DOI: 10.1016/j.dld.2019.02.012].

4- Krützfeldt J, Rösch N, Hausser J, Manoharan M, Zavolan M, Stoffel M. MicroRNA-194 is a target of transcription factor 1 (Tcf1, HNF1 α) in adult liver and controls expression of frizzled-6. Hepatology. 2012;55(1):98-107. [PMID: 21887698 DOI: 10.1002/hep.24658].

However our interest was to study the impact of the aforementioned miRNAs in In vitro model despite the interesting study that could be elaborated through highlighting our proposed regulatory pathway in in vivo model.

Reviewer #2:

The authors propose that the possibility of a novel shared upstream regulatory signaling pathway for PD-1/PD-L1 immune checkpoint between paradoxically acting miR-194-5p and miR-155-p through XIST expression modulation may exist in hepatocellular carcinoma, and they also suggest that such ceRNA circuits' key regulators could be employed as therapeutic targets for hepatocellular carcinoma. This is the well-written paper that presents interesting data and information. It will be of interest to readers of this journal, and is sufficient for publication.

Response: We would like to thank the reviewer for this comment and we do appreciate this kind support.

Reviewer #3:

1. In Introduction, the authors should refer to several papers by Dr. Tasuku Honjo's group and Dr. James P. Allison's group, in the part of cancer therapy using immune checkpoint. Especially regarding PD-1, the authors should need several more references, because Dr. Tasuku Honjo discovered it, another protein expressed on the surface of T-cells in 1992. Ishida, Y., Agata, Y., Shibahara, K., & Honjo, T. (1992). Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J., 11(11), 3887–3895.

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Response: Based on a recent study, Oleuropein was reported to have a potential indirect impact on PD-L1 through modulating the expression of Hypoxia-inducible factor-1 in esophageal cancer. In addition, oleuropein immunomodulatory impact in HCC is not thoroughly studied. Thus, in our study we hypothesized that oleuropein might have a potential immunomodulatory role in HCC through manipulation of PD-1/PD-L1 immune checkpoint.

(This part has been discussed and highlighted in the last paragraph of **Introduction**, lines **187-190**)

3. In discussion, the authors should add the figure of the flowchart of pathway that the authors proposed in this study.

Response: A schematic representation of the proposed pathway was added as **Figure 9**.

4. It is not sufficient to explain the limitations of this study. The authors should describe the limitation.

Response: Limitations of the study are added in a separate paragraph under **Discussion** section, lines **509-516**.

“Some limitations must be acknowledged for this study. First, the limited number of patients and subsequently number of tissue biopsies. Further studies using a larger number of tissue biopsies should be implemented to validate the proposed pathway in a larger cohort of patients. Second, a further robust study design can be enforced through the analysis of this study key players in peripheral blood samples of advanced HCC patients as well as investigating the impact of mimicking of both miRNAs, miR-155-5p and miR-194-5p, on PD-L1 protein levels in HCC cell lines.”

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Response: The main aim of the study was to investigate potential upstream epigenetic regulation of PD-L1 and hence, oleuropein was utilized then to study its impact on such immunomodulatory pathway. Mechanistically, we were not concerned with inflammatory effect of oleuropein, since it is thoroughly studied in previous literature, but rather our concern was how oleuropein can modulate the potential immune modulatory pathway in an attempt to boost tumor cytotoxicity.

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in HCC biopsies on contrary to RQ values for PD-L1 readings in cirrhotic tissues that showed higher SD and hence, lower significance.

3. The number of biopsy specimen is small and not clearly showed.

Response: It is stated in the “**materials and methods**” section under “**Patient and tissue samples**” that tissue biopsies from 23 HCC patients are obtained as well as 10 healthy donor tissue biopsies, lines **209 and 213**, respectively.

Several papers have used an approximate same number of tissue specimens:
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4. The authors need to fill out the ARRIVE Guidelines with page numbers. No academic misconduct was found in the CrossCheck detection and Bing search.

Response: No animals were included in this study.

5. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

Response: Figures are compiled in a PowerPoint presentation and submitted along with the revised manuscript.

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Response: Article highlight section is added and highlighted.

7. The author should number the references in Arabic numerals according to the citation order in the text. The reference numbers will be superscripted in square brackets at the end of the sentence with the citation content or after the cited author’s name, with no spaces.

Response: Reference style is updated in the submitted revised manuscript.

8. Please don’t include any *, #, †, §, ‡, ¥, @....in your manuscript; Please use superscript numbers for illustration; and for statistical significance, please use superscript letters. Statistical significance is expressed as $aP < 0.05$, $bP < 0.01$ ($P > 0.05$ usually does not need to be denoted). If there are other series of P values, $cP < 0.05$ and $dP < 0.01$ are used, and a third series of P values is expressed as $eP < 0.05$ and $fP < 0.01$.

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