Peer Review File

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Reviewer A

Breast cancer is the most common gynecological malignancy and the leading cause of cancer-related deaths in women. PIWI-interacting RNAs (piRNAs) are novel non-coding RNAs whose abnormal expressions have been closely associated with multiple cancers. In the manuscript "PIWI-interacting RNA-31106 promotes breast cancer by regulating METTL3-mediated m6A RNA methylation", authors explored the roles and possible mechanisms of piRNA-31106 in breast cancer.
Couple questions are required to be answered before it will be accepted.

Comment 1:The piRNA-31106 was the crucial topic in the study. Why to focus on piRNA-31106? Please state clearly in the introduction.
Reply 1: we have modified our text as advised
Changes in the text: see Page 3, line 82~33

Comment 2: It was advised to add related reference (DOI: 10.21037/tcr.2019.12.91) about the PIWI-interacting RNA in the introduction.
Reply 2:we have modified our text as advised
Changes in the text: see Page 3, line 82

Comment3: Please supplement the sequence of reverse primer of piRNA-31106.
Reply 3: we have modified our text as advised
Changes in the text: see Page 6, line 179-180

Comment4: The "**" was only showed in the figure 1. So, the illustration for "* and ***" should be deleted in the figure 1 legend. The "***" was only showed in the figure 2. The illustration for "* and **" should be deleted in the figure 2 legend. The same to figure 3, 4 and 5.
Reply 4: we have modified our text as advised
Changes in the text: see Page 18, line 586,Page 19, line 608,Page 18, line 586,Page 20, line 628, Page 20, line 641, Page 21, line 653

Comment5: The figure 7 was not clearly enough. Please replace it with a new.
Reply 5: we have modified our text as advised
Changes in the text: see Page 22, line 671-672

Comment6: It was better to further test the functions of piRNA-31106 by in vivo experiments.
Reply 6: We are currently experimenting with this at night
Changes in the text: NA

Comment7: The MDM2, CDK4 and cyclinD1 was the key topic in the study. What were the correlations between them and RNA methylation? Please state in the discussion.
Reply 7: we have modified our text as advised
Changes in the text: see Page 13, line 405-408

Comment8: It was better to add a schematic diagram of piRNA-31106/METTL3 pathway in breast cancer cells in discussion.
Reply 8: we supplemented this figure before publication
Changes in the text:

Comment9: The minor suggestion was that “room temperatire” should be changed to “room temperature”, and “C” should be changed to “C” in the methods.
Reply 9: we have modified our text as advised
Changes in the text: see “Method”

Reviewer B

Comment 1:First, I suggest the authors to indicate the research methodology in the title such as in vitro or in vivo.
Reply 1: we have modified our text as advised
Changes in the text: see Page 1, line 3~4

Comment 2: Second, the abstract is not adequate and needs further revisions. The background did not describe the research gaps and limitations of prior studies, as well as the potential clinical significance of the focus on piRNA. The methods need to describe the questions to be answered by these experimental procedures. In the results, please quantify the findings by using statistics and accurate P values, such as the expression levels. The conclusion needs some comments for the clinical implications of the findings.
Reply 2:thank you for the reviewer,s suggestions. However, we think that the contents in the abstract, background and results are more detailed, so we will not modify them. We changed the conclusion section
Changes in the text: see Page 14, line 446~447

Comment3: Third, in the introduction of the main text, the authors need to briefly review what has been known on the biomarkers associated with the etiology of BC and their physiological mechanisms, analyze their limitations and knowledge gaps, and explain the potential strengths and clinical significance of the focus on piRNA. Without these comments, I cannot see the necessity of the current research focus.
Reply 3: we have modified our text as advised
Changes in the text: see Page 3, line 82-87
Comment4: 4)Fourth, in the methodology of the main text, please have an overview of the experimental procedures and the questions to be answered by them. In statistics, please ensure $P<0.05$ is two-sided.
Reply 4: we confirm that $P<0.05$ is two-sided
Changes in the text: NA

Reviewer C

The present study explored the expression of piRNA-31106 in breast cancer tissues and cells, its regulation of malignant biological behavior in breast cancer, and its role in METTL3-mediated m6A methylation. The results demonstrated that piRNA-31106 is involved in the development of breast cancer through METTL3-mediated m6A RNA methylation, suggesting that piRNA-31106 may be a potential biomarker of breast cancer and/or a cancer therapeutic target. The study is well written, and provides relevant information on the action of piRNA-31106 in breast cancer.

Reviewer D

1. Please define all abbreviations in Abstract.

Background: Breast cancer is the most common gynecological malignancy and the leading cause of cancer-related deaths in women. PIWI-interacting RNAs (piRNAs) are

Reply 1: All abbreviations correct

2. You should indicate in your manuscript (in both Methods section and Ethical statement in Footnote) that the study conformed to the provisions of the **Declaration of Helsinki (as revised in 2013)**, available at: https://www.wma.net/wp-content/uploads/2016/11/DoH-Oct2013-JAMA.pdf.

***- Suggested wording**: "The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013)."*

We helped add it, please confirm.

Reply 2: I have confirmed it

3. Ref.43 was not cited in your paper, please cite it in order in text.

Reply 3: I have modified our text as advised

4. Please check if any references are missing in this sentence.

401 *31106* and *METTL3*. Further studies confirmed that intervention of *METTL3* expression
402 could significantly inhibit the progression of breast cancer cells, and inhibit the
403 promotional effect of *piRNA-31106* on breast cancer.

Reply 4: I have modified our text as advised

5. You've mentioned "studies", while only one reference was cited in the below sentences. Please check. (You could either choose to revise them to "study" or to give **more than one reference** in those sentences. In the latter case, please keep the citations consecutively in text.)

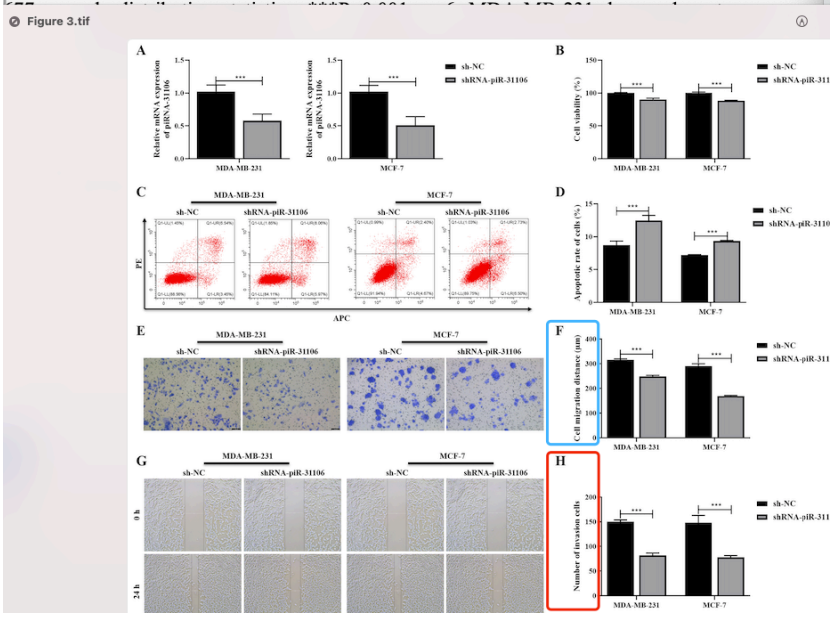
414 target for early diagnosis and prognosis of malignant tumors (27). Studies have shown
415 that *piR-5937* and *piR-28876* are upregulated in colon cancer and are promising
416 diagnostic biomarkers for colon cancer (28). The up-regulated expression of piR-1245

448 also believed to be closely related to cancers (39). Studies have shown that *METTL3* is
449 dysregulated and carcinogenic in a variety of malignant tumors (40). Knockdown of

Reply 5: I have modified our text as advised

6. Figure 3: Either the legends or the figures were misplaced, please check and revise.

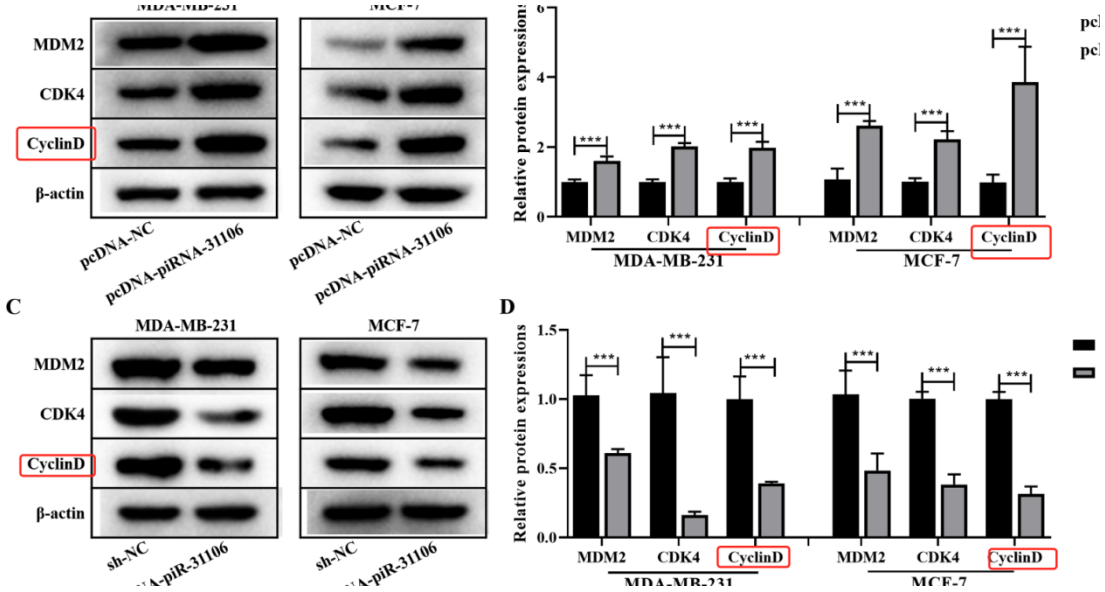
672 shRNA-piRNA-31106. Staining with crystal violet dye. Magnification ×100. **(F)**
673 Statistical analysis of the number of invasive cells. **(G)** The cell migration distance was
674 reduced after transfection with shRNA-piRNA-31106, as detected by the scratch assay.
675 Observation under light microscope. Magnification ×100. **(H)** Statistical analysis of the
676 cell migration distance. **(I)** The cell cycle was assessed by flow cytometry. **(J)** Cell



Reply 6: I have modified our text as advised

7. Figure 4: Is “cyclinD1” or “CyclinD”? Please check and unify them.

683 **Figure 4 The effects of *piRNA-31106* on oncogene expression in breast cancer cells.**
684 **(A)** Western blot was used to detect the protein expressions of MDM2, CDK4, and
685 cyclinD1 in the breast cancer cell lines MDA-MB-231 and MCF-7 transfected with
686 pcDNA-piRNA-31106 or pcDNA-NC. **(B)** Statistical histogram showing the protein
687 expressions. **(C)** Expression of MDM2, CDK4, and cyclinD1 after transfection with



Reply 7: I have modified our text as advised

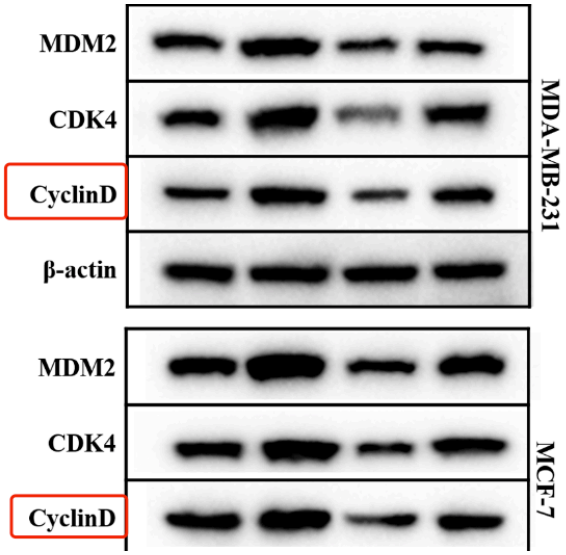
8. Figure 7: No "**" is included in the figure, please revise the legends.

728 Observation under light microscope. Magnification ×100. (H,I) Flow cytometry was
729 used to detect cell cycle. *P<0.05, **P<0.01, ***P<0.001; n=6. MDA-MB-231, human

Reply 8: I have modified our text as advised

9. Figure 8

a. Check if it should be "CyclinD1". You should unify.



b. Figure 7: No "**, *" were included in the figure, please revise the legends.

739 detected by Western blot. **(B)** Statistical histogram of protein expressions. *P<0.05,
740 **P<0.01, ***P<0.001; n=6. pcDNA-NC, pcDNA-negative control; si-NC, small

Reply 9: I have modified our text as advised