

Reviewer 1 (Reviewer's code: 00068458):

This manuscript entitled "PIWIL1 gene as a possible major player in gastric cancer" studied functions of PIWIL1 in gastric carcinogenesis. The authors found that PIWIL1 gene knockout in AGP01 gastric cancer cell line by using CRISPR-Cas9 system induced a significant decrease in gastric cancer cell migration and invasion. In addition, they identified a total 35 genes encoding proteins involved in cellular invasion and migration. Of them, 9 genes are possibly related to the progression of gastric cancer. They concluded that PIWIL1 be a therapeutic target of gastric cancer. The idea is interesting. However, the pathophysiological relevance of the postulated evidences remains unclear. The reviewer has the following suggestions:

1. Previously, it has been reported that downregulation of PIWIL1 (hiwi) inhibited the growth of gastric cancer cells (Int J Cancer, 2006;118:1922-9) and the epithelial-mesenchymal transition (EMT) process of cervical cancer cells by regulating cadherin, vimentin and snail (Oncology Letters, 2018;16:3874-80). The authors should provide evidences showing that PIWIL1 regulates expression of EMT-related proteins in gastric cancer cells.

Answer: We analyzed proteomic data and observed that *PIWIL1* knockout caused changes in the expression of several proteins involved in EMT. This information was added in discussion section.

2. Gene expression analysis identified a total of 35 genes encoding proteins involved in migration and invasion of cancer cells and 9 of them are possibly related to the mechanisms used by PIWIL1 to promote carcinogenic effects. 1) The authors should explain or provide evidences showing how PIWIL1 regulates expression of these genes.

Answer: There is no data available in literature correlating these genes with PIWIL1, but our results demonstrated that knockout of *PIWIL1* modified the expression of these genes, which was compatible with migration and invasion results.

2) They selected 9 of 35 genes showing differential expression in the cell line before and after PIWIL knockout. The reason why they chose 9 genes is unclear.

Answer: We chose these genes because they are possibly related to the mechanisms used by *PIWIL1* to promote carcinogenic effects related to migration and invasion, since their functions are consistent with the changes observed (being up- or down-regulated after knockout). This is stated at the end of the Results section.

3) A total of 251 mRNA were found to be differentially expressed after PIWIL1 knockout in the cell line. Data presented up to this stage of the manuscript does not justify stating that PIWIL1 be a therapeutic target of the treatment of gastric cancer.

Answer: This statement was included based on other research that supports/corroborates our findings, only to reinforce the importance of studying this so significant gene.

4) DNA sequences or genetic status of 9 genes in the cell line before and after PIWIL1 knockout should be shown.

Answer: The knockout experiment does not generate modifications in the DNA sequence of other targets, except for PIWIL1. Genetic status was provided by microarray assay that is presented in the results.

5) The authors should also point out that PIWIL1 expression is closely associated with expression levels of 9 genes in gastric cancer tissues.

Answer: The present research was developed using cell lines in order to demonstrate the hypothesis of PIWIL1 participation in gastric carcinogenesis, without using tumor tissue.

3. Mechanism for PIWIL1 overexpression in gastric cancer should be discussed.

Answer: The mechanism by which overexpression of this protein is observed in some types of cancer is still unclear. This was added to discussion section.

4. Improve the resolution of Fig. 5.

Answer: Fig. 5 resolution was improved.

Reviewer 2 (Reviewer's code: 03317069):

In this study, the authors evaluated only one gastric cancer cell line to knockout PIWIL1 gene. It seems to be difficult to evaluate that PIWIL1 plays a crucial role in the signaling pathway of gastric cancer, regulating several genes involved in migration and invasion processes.

The authors should evaluate the other gastric cancer cell lines except for AGP01 cell line.

Answer: Knockout experiment (including post-tests) using CRISPR-Cas9 system is extremely complex and expensive, making it impracticable to repeat it in other cell

line at this moment, especially because the hypothesis studied was clearly demonstrated. In addition, the AGP01 cell line is an experimental model of metastatic gastric cancer, increasing its importance.

Moreover, the authors should evaluate the expression of PIWIL1 after knock down by RT-PCR or Western blot.

Answer: The result of the sequencing is an unquestionable demonstration of the modification promoted by the using of CRISPR-Cas9 system, which is the change of the DNA sequence. Moreover, we analyzed the proteomic data, and protein expression did not appear in any of the cell lines (with and without knockout), perhaps because AGP01 cell line only has one copy of chromosome 12 (where *PIWIL1* gene is located) and its expression is low. However, the low expression is sufficient to trigger phenotypic modifications, because it should not be expressed in normal cells.

Many genes were thought as candidates for the mechanisms used by PIWIL1 to promote carcinogenic effects related to migration and invasion. It is difficult to confirm which genes really related to migration and invasion from the results of literature.

Answer: Our results demonstrated that knockout of *PIWIL1* modified the expression of these genes, which was compatible with migration and invasion results.

Reviewer 3 (Reviewer's code: 02687374):

This study established a permanent PIWIL1 gene knockout in AGP01 gastric cancer cell line using CRISPR-Cas9 system and analyzed phenotypic modifications, as well as gene expression alterations. The results showed that PIWIL1 plays a crucial role in the signaling pathway of gastric cancer, regulating several genes involved in migration and invasion processes; therefore, its use as a therapeutic target may generate promising results in the treatment of gastric cancer. The study is interesting. But it has some problems.

1. The English expression needs some modification.

Answer: We sent the text for correction and the certificate will be attached.

2. Cell line AGP01 used in this experiment is established by author's group in 2009. It is recommended to add more classic gastric cancer cell lines to verify tumor invasion and migration.

Answer: We have several articles published with this cell line, which in turn has the peculiarity of being obtained from metastatic gastric cancer, which favors the interpretation of the results.

3. This experiment lacks *in vivo* experiments to verify the role of the AGP01 in gastric cancer.

Answer: This paper, once published, will favor subsequent experiments including those using *in vivo* experimental models.

4. In Figure 4, it is recommended to compare the negative control group with the knockdown group.

Answer: This was done.

5. The conclusions are overstated. For example, this study did not test the gastric cancer tissue samples, but found that the gene is associated with poor prognosis.

Answer: Conclusion was rewritten.