Author's response to reviews

Title: The promoter of miR-663 is hypermethylated in Chinese pediatric acute myeloid leukemia (AML)

Authors:

Yan-Fang Tao (taoyanfang1982@163.com)
Jian Ni (ni_jian2008@163.com)
Na Wang (ontheway120@126.com)
Wen-Li Zhao (zhaowenli69@yahoo.com.cn)
Dong Wu (wudong.1987@163.com)
Li Pang (pangyeli2011@163.com)
Jun Lu (lujun_sz@yahoo.com.cn)
Jian Wang (wj196312@vip.163.com)
Xing Feng (xing_feng66@hotmail.com)
Jian Pan (panjian2008@163.com)

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Author's response to reviews:

Dear Reviewers,

Thank you for your review of our manuscript (MS: 1987043829792582: “MiR-663 is down-regulated with promoter hypermethylation in pediatric acute myeloid leukemia (AML)”).

The revised manuscript has a new Title “The promoter of miR-663 is hypermethylated in Chinese pediatric acute myeloid leukemia (AML)” We appreciate your concerns and suggestions and have revised our manuscript accordingly. Our point-by-point responses are detailed below and the key modifications to the text have been highlighted in yellow. In addition, we employed a professional language editing service that specializes in scientific manuscripts, to ensure the language and grammar meet the high standards required by this journal.

We look forward to your response.

Yours sincerely,

Pan Jian, PhD.

Associate professor of Department of Hematology and Oncology, Children’s Hospital of Soochow University, Suzhou, China.

Email addresses: panjian2008@163.com
Tel: (86)512-67788216
Fax: (86)512-67788216
Reviewer: hongwei wang

In this manuscript, the authors sought to evaluate whether miR-663 was inactivated epigenetically in pediatric acute myeloid leukemia, their results indicated that MiR-663 transcript was significantly lower in AML group compared to controls; meanwhile the miR-663 methylation was also investigated. This paper raises several concerns:

1) The meaning of the results need to be further addressed as there no any correlation between the clinical features and the miR-663 methylation. And also the transcript of miR-663 was almost same in patients with methylated and unmethylated miR-663, the author need to investigate whether promoter hypermethylation is a special event in pediatric AML which control the disease development. The author should be careful to give the major conclusion, and the written pattern should be changed accordingly.

In response to the reviewer’s suggestions, we have revised our manuscript carefully and added the following experiment to address the reviewer’s concerns: The relationship between miR-663 expression and promoter methylation status was determined by treating leukemia cell lines with 5-Aza-2#-deoxycytidine (5-Aza). This is an epigenetic modifier that inhibits DNA methyltransferase activity resulting in DNA demethylation (hypomethylation). Our results showed that expression of miR-663 in leukemia cell lines was significantly upregulated by demethylation. We then treated three primary pediatric AML cells with 5-Aza. Our results again showed upregulation of miR-663 expression. These results confirmed that promoter methylation directly affected the expression of miR-663.

2) The author claimed that a high frequency of miR-663 promoter hypermethylation was found in pediatric AML primary tumor cells but not in the control normal cells, but the result might not be enough to support this conclusion as there no any statistic analysis apart from some raw data in figure 2. And also the scale of the control samples is relative small in compared with the AML samples.

In response to the reviewer’s suggestions, we carried out statistical analysis of the results. Due to the difficulty in collecting sufficient numbers, the number of normal bone marrow control samples was smaller than that of pediatric AML samples. (Every year we can collect 120–150 pediatric acute leukemia samples but only 10–15 normal surgical bone marrow samples.)

3) The author should answer a critical question whether the promoter hypermethylation have any direct effects on the expression of MiR-663, which should be compared not only in the pediatric AML samples, but also in the leukemia cell lines and the control samples.

In response to the reviewer’s suggestions, we determined that the best way to demonstrate whether promoter hypermethylation directly affects expression of
miR-663 was by treatment with 5-Aza demethylation reagent. We treated three leukemia cell lines and three primary leukemia samples. Our results showed that miR-663 expression was upregulated by 5-Aza treatment, confirming that promoter methylation directly affected the expression of miR-663. These results are given in detail in the “Results” section of our manuscript.

4) Figure 2B is not necessary, as this figure do not add further information apart from showing the raw Q-PCR data.

In response to the reviewer’s suggestion, we agree that Figure 2B was not very important, and have moved it to Figure 4.

5) In the statistical analysis, the authors should show which method were used to compare the difference between the groups such as the data presented in figure B.

In response to the reviewer’s suggestions, we have included details of the method used to compare groups in the “Statistical” section.

6) The author needs to give a reason why different assay (Bisulfite genomic sequencing V.S methylation-specific PCR analysis) was used to detect the MiR-663 methylation in the leukemia cell lines and the pediatric AML samples. And especially the detected CpG islands by MSP assay need to be indicated, as the results of Bisulfite genomic sequencing assay might not be necessary.

MSP results can sometimes be false, due to the PCR primer or the conditions used for PCR amplification. Bisulfite genomic sequencing (BGS) provides additional information and can verify MSP results. Although it is preferable to analyze CpG islands using both techniques, BSG is an expensive method for analyzing large numbers of samples; therefore, we analyzed the cell lines and some AML samples using both methods, and the primary leukemia samples with MSP alone.

Reviewer: Bo Wen

1. The paper entitled “MiR-663 is down-regulated with promoter hypermethylation in pediatric acute myeloid leukemia (AML)” reported that DNA hypermethylation on the promoter of Mir-633 was observed in 41% of Chinese pediatric AML patients. However, their data showed that status of DNA methylation was not associated with expression level of Mir-633. Their data suggested that DNA methylation is not the epigenetic mechanism regulating mir-633 in AML. Thus, the title of this paper is very misleading, and the conclusion “epigenetic inactivation of miR-663 by promoter hypermethylation is frequent and tumor specific event in pediatric AML” was not well supported by the presented data. The authors should change the title and rewrite the paper based on data they have.

Inaccuracies in our language appear to have given a misleading Title and conclusion. Our intent was to present our study data to show that DNA methylation status was associated with the expression level of miR-633 in AML
samples, compared to NBM control samples. However, in pediatric AML samples, unmethylated miR-663 samples also showed low miR-663 expression. Due to the complications inherent in clinical samples, we presume there must be additional mechanisms that downregulate miR-663 expression in addition to promoter methylation. In order to address the reviewer's suggestions, and determine the relationship between miR-663 expression and promoter methylation status, we decided to carry out additional experiments. First, we treated leukemia cell lines with 5-Aza-2#-deoxycytidine (5-Aza). This is an epigenetic modifier that inhibits DNA methyltransferase activity resulting in DNA demethylation (hypomethylation). Our results showed that expression of miR-663 in leukemia cell lines was significantly upregulated by 5-Aza demethylation. We then treated three primary pediatric AML cells with 5-Aza. Our results again showed upregulation of miR-663 expression. These results confirmed that promoter methylation directly affected the expression of miR-663.

2. The function of DNA methylation relies on its location relative to genes. For example, promoter hypermethylation can be related to gene silencing but gene body methylation is usually associated with gene activation. The authors should provide a map to show the region they analyzed and its distance to mir-633 gene.

In response to the reviewer's suggestions, we have included a “map” of miR-663 to show the region of the promoter that was analyzed (Figure 1A).

3. Since DNA methylation and expression were not correlated, the authors should discuss other possible epigenetic mechanisms underlying mir-633 downregulation in AML.

In response to the reviewer's suggestions, we have revised our “Discussion” and “Background” sections by including other possible mechanisms that have been reported to downregulate miR-663.

Associate Editor's comments:

In the manuscript titled “MiR-663 is down-regulated with promoter hypermethylation in pediatric acute myeloid leukemia (AML)” Tao et al. reported that DNA hypermethylation on the promoter of Mir-633 was observed in 41% of Chinese pediatric AML patients but the data indicated that status of DNA methylation was not associated with expression level of Mir-633. This result largely limits its interest in cancer epigenetics area. The manuscript must be carefully revised following the comments raised by reviewers before being further consider to accept for publication.

In response to the Associate Editor’s suggestions, we have revised our manuscript carefully. In addition, we decided to perform some additional experiments. First, we treated leukemia cell lines with 5-Aza-2#-deoxycytidine (5-Aza). This is an epigenetic modifier that inhibits DNA methyltransferase activity resulting in DNA demethylation (hypomethylation). Our results showed that expression of miR-663 in leukemia cell lines was significantly upregulated by
demethylation. We then treated three primary pediatric AML cells with 5-Aza. Our results again showed upregulation of miR-663 expression. These results confirmed that promoter methylation directly affected the expression of miR-663.

2. Requesting copyediting
After reading through your manuscript, we feel that the quality of written English needs to be improved before the manuscript can be considered further.

We appreciate that this journal has high publication standards; therefore, we have had the language and grammar corrected by a professional copyediting service that specializes in scientific manuscripts.