Cover Letter

Dear Editors and Reviewers,

Thank you very much for your efficient work and thank you for your letter and comments. Accordingly, we have revised the manuscript entitled ***"MicroRNA-596 acts as a tumor suppressor in gastric cancer and is upregulated by promotor demethylation" (Manuscript NO: 45055)***, and would like to resubmit it for your consideration. We have addressed the instructive comments raised by the reviewers, and the amendments are highlighted in red in the revised manuscript. Point by point responses to the reviewers' comments are listed for your consideration. We would like to express our sincere thanks to the editors and reviewers for the constructive and meaningful comments.

We are so grateful that you have offered us this opportunity to resubmit our manuscript. We hope that the revised version of the manuscript is now acceptable for publication in your journal.

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We shall look forward to hearing from you at your earliest convenience.

Yours sincerely,

Dong-Qiu Dai

Response to Reviewers

Reviewer # 1

Comment 1: PRDX1 as the target should be investigated in the remaining cell lines to substantiate the data indicating it is the principal target in gastric carcinogenesis.

Response 1: We are grateful for your meaningful comments. According to the Reviewer's comments, miR-NC or miR-596 mimics were also co-transfected into MKN-45 cells. qRT-PCR and western blot analysis approved that over-expression of miR-596 significantly inhibited PRDX1 expression at the mRNA and protein level (Figure 2d). Hopefully, this may address your concerns.

Comment 2:Transfection and 5-Aza-dC treatment analyses have been performed using only two cell lines and methylation analysis was performed using the MGC-803 cell line.

Response 2: We are so grateful for your meaningful comments. Data from our MSP analysis displayed that was a significant negative correlation between miR-596 promoter methylation and expression levels in GC cell lines. In order to make the results more meaningful, we selected the MGC-803 cell line with the highest degree of methylation for demethylation analysis, to confirm whether the expression of miR-596 can be restarted. Meanwhile, according to the Reviewer's comments, we also treated MKN-45 cells with demethylating agent 5-Aza-dC , to confirm whether miR-596 could be re-expressed. We observed that the methylation status of miR-596 promoter region was significantly decreased after treatment with 5-Aza-dC, the expression of miR-596 was obviously increased, and the highest expression occurred at a concentration of 5 μM (Figure 5b). Hopefully, this may address your concerns.

Comment 3: Different targets (BCL2L1, MEK1, Smurf1) have been suggested for miR-596 in different studies. What were the other possible targets resulting from bioinformatics database search ? Any concordance with the previous reports ?

Response 3: We are so grateful for your meaningful comments. Due to a non-strict hybridization of the seed match region, one miRNA can bind to multiple mRNA targets, allowing simultaneous down-regulation of multiple target mRNAs. Using three bioinformatic databases (TargetScan, miRWalk and miRanda) , PRDX1 is the common result of three databases and has the highest score (Figure 2A). Furthermore, the expression profile and potential role of PRDX1 in GC remains to be investigated. The results showed that overexpression of miR-596 decreased the expression of PRDX1 and luciferase reporter assays detected the direct binding of miR-596 to the 3'UTR of PRDX1 transcripts. Our results provided a theoretical basis for further study of the mechanism of miR-596 and PRDX1 in GC. Hopefully, this may address your concerns.

Comment 4:Only MGC-803 cells display promoter methylation (Fig. 5a) while others do not, although expression of miR-596 is lower than controls in all cancer cell lines. To suggest that miR-596 is regulated epigenetically in gastric cancer the mechanism should be valid for all cell lines.

Response 4: We are so grateful for your meaningful comments, and we fully agree with you. To further emphasize that the expression of miR-596 is regulated by epigenetic mechanism, we also treated MKN-45 cells with demethylating agent 5-Aza-dC , to confirm whether miR-596 could be re-expressed. We observed that the methylation status of miR-596 promoter region was significantly decreased after treatment with 5-Aza-dC, the expression of miR-596 was obviously increased (Figure 5b). Hopefully, this may address your concerns.

Comment 5:How do the authors explain upregulation of PRDX1 mRNA and downregulation of the protein following 5-Aza treatment ?

Response 5: Thank you for pointing this out. I think part of the reason may be that partial methylation may also be present in the promoter region of PRDX1, and demethylation can slightly upregulate the expression of PRDX1 mRNA. At the same time, demethylation can upregulate the expression of microRNA-596, inhibit the expression of PRDX1 protein by binding to the 3'UTR of PRDX1 transcripts. Hopefully, this may address your concerns.

Comment 6: The significances reported for differentiation and Borrmann type are only borderline

Response 6: We are very grateful for your meaningful comments, but we do not fully agree with you. Our data from western blot analysis showed that PRDX1 expression was significantly related to tumor differentiation grade（P=0.034）and Borrmann type (P=0.046). Their P values are all less than 0.05 and should be considered statistically significant. Of course, in order to make the results more statistically significant, it may be necessary to increase the sample size. Hopefully, this may address your concerns.

Reviewer # 2

Comment 1: Statistical analyses should be appropriate,i.e. non-parametric paired tests should be used for the analysis of results from initial 9 patients (a "repeated measures" t-test) ;

Response 1: We are grateful for your meaningful comments. According to the Reviewer's comments, through statistical analysis of experimental data, The expression of microRNA-596 was significantly downregulated in GC tissues compared with paired normal control tissues ($P<0.05$). Besides, In order to further study the relationship between the expression of microRNA-596 and clinicopathological factors, The expression of mir-596 and PRDX1 were analyzed in 55 paired GC tissues by qRT-PCR (Fig.1). We have re-written this part and re-analyzed the statistics. Hopefully, this may address your concerns.

Comment 2: Paired tests (either Student's or ANOVA) should also be used to compare tumor and non-tumor characteristics in the remaining 55 patients ?

Response 2: We are grateful for your meaningful comments. According to the Reviewer's comments, The expression of mir-596 and PRDX1 were analyzed in 55 paired GC tissues by qRT-PCR. The results demonstrated that miR-596 was significantly downregulated in GC tissues compared with paired normal control tissues (Fig.1a), PRDX1 expression was significantly upregulated in GC tissues compared to corresponding non-tumorous tissue(Fig.1c). Moreover, Pearson's correlation analysis revealed that the expression of miR-596 was inversely correlated with PRDX1 in GC tissues (Fig.1e). Then we further examined the relationship between miR-596 expression and clinicopathological factors in 55 paired GC tissues by Pearson's $\chi 2$ test. miR-596 expression was significantly related to tumor differentiation grade and TNM stage, but not with age, sex, tumor size, tumor site, Borrmann type or lymph node metastasis of the patient(Table 2). We have re-written this part and re-analyzed the statistics. Hopefully, this may address your concerns.

Comment 3: "Paracancerous tissue" (Figure 1) should be explained. Is that normal mucosal tissue ? How far away from the tumor were these specimens taken ?

Response 3: Thank you for your meaningful comments, and we fully agree with you and feel sorry for this mistake. We have made correction according to the Reviewer's comments. Yes, these specimens were taken more than 5 cm away from the tumor. Hopefully, this may address your concerns.

Comment 4: Did authors consider using healthy mucosa from patients without malignancy as a control group.

Response 4: We are so grateful for your meaningful comments, and we fully agree with you. However, healthy mucosa from patients without malignancy are not easily accessible in clinic. In addition, it has been reported that the expression of microRNAs may have individual differences, which may affect the statistical analysis of the results. Hopefully, this may address your concerns.