MIIR-203 acts as a radiosensitizer of gastric cancer cells by directly targeting ZEB1

Objective: Gastric cancer (GC) is a common tumor malignancy with high incidence and poor prognosis. Radiotherapy is one of the main strategies for GC treatment, while development of radioresistance limits the effectiveness. microRNA-203 (miR-203) has been reported to participate in progression of GC, whereas its interaction with radiosensitivity of GC and the related mechanism remain largely unclear.

Methods: The expressions of miR-203 and zinc finger E-box binding homeobox 1 (ZEB1) were measured in GC tissues and cells by quantitative real-time polymerase chain reaction or western blot. Survival fraction, cell viability and apoptosis were measured in GC cells after treatment of radiation by colony formation, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay or flow cytometry, respectively. Tumor volume and weight were detected in murine xenograft model after radiation treatment. The interaction between miR-203 and ZEB1 was explored by bioinformatics analysis and luciferase activity assay.

Results: miR-203 expression was down-regulated and ZEB1 mRNA level was up-regulated in GC. The expression of miR-203 was associated with radiosensitivity of GC cells. Moreover, overexpression of miR-203 decreased survival fraction, cell viability and tumor growth but promoted cell apoptosis in radiation-treated GC cells. However, knockdown of miR-203 played an opposite effect. ZEB1 was validated as a target of miR-203, and it was involved in miR-203-mediated radiosensitivity of GC cells in vitro and in vivo.

Conclusion: miR-203 promoted radiosensitivity of GC cells by targeting ZEB1, indicating miR-203 as a promising radiosensitizer for GC treatment.

Keywords: gastric cancer, miR-203, ZEB1, radiosensitizer, radiosensitivity

Introduction
Gastric cancer (GC) is one of the most common tumor malignancies with high mortality and incidence worldwide. With the advance of understanding the pathogenesis, many strategies have been exploited in the diagnosis and management of GC, such as surgical resection, chemotherapy and radiotherapy. Radiotherapy is one of the main modalities of cancer treatment and plays important roles in treatment of gastrointestinal cancers, such as cancers of the stomach, pancreas, esophagus, liver and anus. Moreover, the progress of preoperative and postoperative radiotherapy has gained more attention in GC. However, development of radioresistance induces the failure of radiation treatment. Hence, it is urgent to explore novel radiosensitizers to improve the effectiveness of radiotherapy.

microRNAs (miRNAs) are a class of small noncoding RNAs, which have been indicated as potential targets for improving radiotherapy. Moreover, increasing works have demonstrated that miRNAs play essential roles in diagnosis, prognosis and...
radiotherapy in GC.\textsuperscript{6,7} For example, miR-196b enhances the radiosensitivity of GC cells by targeting radiation sensitive protein 23 homologue B (RAD23B).\textsuperscript{8} Former works suggest that miR-203 has a vital role in proliferation, metastasis and apoptosis in ovarian cancer, hepatocellular carcinoma and prostate cancer.\textsuperscript{9–11} In addition, miR-203 contributes to radiosensitivity by inhibiting DNA damage repair via regulating phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and Janus kinase (JAK)/signal transducer and activators of transcription 3 (STAT3) pathways in glioma cells.\textsuperscript{12} This reveals that miR-203 may serve as a radiosensitizer for cancer therapy. Increasing efforts have reported that miR-203 inhibits invasion, metastasis and growth by regulating multiple pathways in GC.\textsuperscript{13–15} However, little is known about the role and mechanism of miR-203 in radiosensitivity of GC cells.

Zinc finger E-box binding homeobox 1 (ZEB1) is a key element controlling epithelial-to-mesenchymal transition, which is implicated in tumorigenesis and development of human cancers.\textsuperscript{16} Furthermore, ZEB1 is suggested to have an impact on the outcome of therapy resistance, including radioresistance and drug resistance.\textsuperscript{17} Notably, ZEB1 is indicated as an oncogene to promote cell viability, migration and invasion in GC.\textsuperscript{18} Besides, available evidence indicates the regulatory network of miR-203/ZEB1 in progression of non-small cell lung cancer.\textsuperscript{19} Thus, we hypothesized that ZEB1 might be required for miR-203-mediated regulation of radiosensitivity in GC. In this study, we first analyzed the effect of miR-203 on radiosensitivity of GC in vitro and in vivo and explored the potential interaction between miR-203 and ZEB1.

Materials and methods

Tissues samples
A total of 36 paired tumor and adjacent normal tissues (NT) were collected from patients with GC in Guizhou Provincial People’s Hospital and then immediately stored at \(-80^\circ\)C until RNA extraction. All patients enrolled in this study had no history of chemotherapy or radiotherapy prior to surgery and signed the informed consent. This study was approved by the Research Ethics Committee of Guizhou Provincial People’s Hospital and performed in accordance with the Declaration of Helsinki.

Cell culture and transfection
The human normal gastric epithelium cell line (GES-1) and GC cell lines (MKN-45, SGC-7901 and AGS) were purchased from American Tissue Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle Medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100 \(\mu\)g/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37\(^\circ\)C in a humidified atmosphere with 5% CO\(_2\).

miR-203 mimic (miR-203), miRNA negative control (NC) (miR-NC), miR-203 inhibitor (anti-miR-203), inhibitor NC (anti-miR-NC), ZEB1 overexpression vector (ZEB1), pcDNA empty vector (vector), short hairpin RNA (shRNA) against ZEB1 (shZEB1) and shRNA NC (scrambled) were synthesized by Genepharma (Shanghai, China). Cell transfection was performed in MKN-45 and AGS cells for 48 hrs by using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. The non-transfected cells were regarded as blank group.

Colony formation
MKN-45 and AGS cells were exposed with different doses of radiation (0, 2, 4, 6 and 8 Gy)\textsuperscript{8} using the X-ray apparatus (Rad Source Technologies, Alpharetta, GA, USA) with a dose rate of 200 cGy/min. Every sample was prepared in triplicate. After radiation for 24 hrs, cells were collected and seeded into 12-well plates at a density of 300 cells/well. After culture for 10 d, treated cells were fixed with methanol (Sigma, St. Louis, MO, USA) for 30 mins and then stained with 0.01% crystal violet (Sigma) for 15 mins. The colony formation was analyzed under a microscope (Olympus, Tokyo, Japan) and survival fraction was calculated as number of colonies/number of cells plated by normalizing to the control cells (non-irradiation).

Quantitative real-time polymerase chain reaction
Total RNA was isolated from tissues or cells by using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA was reversely transcribed using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) or M-MLV Reverse Transcription Kit (Thermo Fisher, Wilmington, DE, USA). The complementary DNA (cDNA) was used for quantitative real-time polymerase chain reaction using SYBR green (Applied Biosystems) following the manufacturer’s instructions. Every sample was prepared in triplicate, and the experiment was repeated three times. The expressions of miR-203 and ZEB1 were calculated using \(2^{-\Delta\Delta Ct}\) method with U6 small RNA or GAPDH as internal...
control, respectively. The primers were listed as follows: miR-203 (Forward, 5' - AACTCCAGCTGGGTGATGTTA-3'; Reverse, 5' - TGGTGTGCTGAGGTACG -3'), U6 (Forward, 5' - GCTTCGACACATACTACTAATAT -3'; Reverse, 5' - CGCTTCCAGAATTTCGCTCAT -3'), ZEB1 (Forward, 5' - AGCGAGGTAAGTTCTGCT -3'; Reverse, 5' - AGTTTCTGCGGCACTAAGC -3'), GAPDH (Forward, 5' - AACGATTGTTGGCTGTTTGGG -3'; Reverse, 5' - TCGCTCTGGAAGATGGTGATGAT -3').

**Cell viability**

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was conducted to measure cell viability. MKN-45 and AGS cells were exposed with different doses of radiation (0, 2, 4, 6 and 8 Gy). Treated cells were seeded into 96-well plates at a density of 1×10^4 per well and each group was prepared in triplicate. After the culture for 48 hrs, cells were interacted with 5 mg/mL MTT (10 μl) solution (Thermo Fisher) for another 4 hrs. Subsequently, 100 μL of dimethylsulfoxide (DMSO, Thermo Fisher) was added and incubated for 10 mins. The absorbance was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

**Cell apoptosis**

Cell apoptosis was detected using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Sigma) via flow cytometry. MKN-45 and AGS cells (2×10^5) were seeded into 6-well plates, treated with radiation of 6 Gy and cultured for 48 hrs. Each group was prepared in triplicate. Subsequently, cells were washed with PBS and resuspended in binding buffer, followed by staining with 10 μL Annexin V-FITC for 10 mins and 5 μL PI for 10 mins in the dark according to the manufacturer’s instructions. The apoptotic (FITC positive and PI positive or negative) cells were analyzed by using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

**Tumor radiosensitivity assay in vivo**

BALB/c nude mice (male, 4-week-old) were obtained from Vital Laboratory Animal Technology (Beijing, China) and housed in specific pathogen-free conditions with a 12-hr light/dark cycle and free access to water and food for one week. Every experiment was made to minimize animals (n = 7 per group) under the approval of the Animal Research Committee of Guizhou Provincial People’s Hospital and conducted in conformity with the guidelines of Care and Use of Laboratory Animal. MKN-45 cells were transfected with the lentiviral vectors with miR-NC, miR-203, miR-203+Vector or miR-203+ ZEB1 constructed by GeneCopoeia (Rockville, MD, USA). AGS cells were transfected with the lentiviral vectors with anti-miR-NC, anti-miR-203, anti-miR-203+scrambled or anti-miR-203+ shZEB1. Stably transfected cells (5×10^6) were subcutaneously injected into the left flank of mice. After 1 week following the inoculation, mice were treated with radiation of 5 Gy and then tumor volumes were measured every 3 days with the formula: volume (mm^3) = width^2 x length/2. On 28 d after injection, the mice were sacrificed and tumor samples were weighted.

**Luciferase activity assay**

The potential targets of miR-203 were predicted by bioinformatics analysis using TargetScan Release 7.2 (http://www.targetscan.org/vert_72/). The 3' untranslated regions (3'-UTR) sequences of ZEB1 containing binding sites of miR-203 were amplified and then cloned into the firefly luciferase-expressing pGL3-Promoter vectors (Promega, Madison, WI, USA) to synthesize wild-type (Wt) luciferase reporter vectors (ZEB1-Wt). MKN-45 and AGS cells were co-transfected with 20 ng ZEB1-Wt or empty vector, 15 ng renilla luciferase vector (control vector) and 40 nM miR-203, miR-NC, anti-miR-203 or anti-miR-NC using Lipofectamine 2000 according to the manufacturer’s protocols. After the transfection for 48 hrs, luciferase activity assay was analyzed with luciferase assay kit (Promega) according to the manufacturer’s instructions.

**Western blot**

Proteins were isolated from cells by using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and quantified by BCA protein assay kit (Beyotime Biotechnology). After the denaturation, equal amounts of proteins were separated by SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 hr at room temperature, and then incubated with primary antibodies against ZEB1 (ab203829, 1:500 dilution, Abcam, Cambridge, UK) or GAPDH (ab181602, 1:10,000 dilution, Abcam) overnight at 4°C and horseradish peroxidase (HRP)-conjugated secondary antibody (ab205718, 1:10,000 dilution, Abcam) for 2 hrs at room temperature. GAPDH was regarded as loading control in this study. The protein blots were visualized
using enhanced chemiluminescence chromogenic substrate (Beyotime Biotechnology).

Statistical analysis
Three independent experiments were conducted for all analyses and the data were presented as the mean±standard deviation (SD). The relationship between the expression of miR-203 and ZEB1 was measured by Spearman rank correlation. Student’s t test or one-way analysis of variance (ANOVA) was performed to analyze statistical differences between groups by using GraphPad Prism 5 (GraphPad Inc., La Jolla, CA, USA). P<0.05 was regarded as statistically significant.

Results
miR-203 is lowly expressed and associated with radiosensitivity in GC
To investigate the role of miR-203 in regulating radiosensitivity of GC, its expression was first measured in GC. Compared with NT group, the expression of miR-203 was significantly decreased in GC tissues (Figure 1A). Similarly, the abundance of miR-203 was abnormally reduced in MKN-45, SGC-7901 and AGS cells compared with that in GES-1 cells (Figure 1B). Moreover, MKN-45 cells with lower expression of miR-203 were relatively more resistant to radiation, and AGS cells showed relative more radiosensitivity (Figure 1C). Hence, the MKN-45 and AGS cells were used for further experiments.

miR-203 enhances radiosensitivity of GC cells in vitro and in vivo
To explore the effect of miR-203 on radiosensitivity, MKN-45 cells were transfected with anti-miR-203 or anti-miR-NC and AGS cells were transfected with anti-miR-203 or anti-miR-NC. As a result, the abundance of miR-203 was effectively elevated in miR-203-transfected MKN-45 cells and obviously reduced in anti-miR-203-transfected AGS cells (Figure 2A and B). The results of colony formation revealed that overexpression of miR-203 significantly inhibited survival fraction in MKN-45 cells, while its knockdown increased the survival fraction in AGS cells after different doses of radiation treatment (Figure 2C and D). However, miR-203 overexpression showed little effect on radiosensitivity of GES-1 cells except at 8 Gy (Figure S1). Moreover, MTT assay demonstrated that addition of miR-203 led to great reduction of cell viability in MKN-45 cells after different doses of radiation treatment (Figure 2E). However, inhibition of miR-203 caused an opposite effect on cell viability in AGS (Figure 2F). Besides, as shown in Figure 2G and 2H, up-regulation of miR-203 promoted cell apoptosis in radiation-treated MKN-45 cells, but miR-203 down-regulation significantly impeded cell apoptosis in AGS cells after treatment of radiation. Additionally, the radiosensitive role of miR-203 was also analyzed in mice by xenograft and treatment of radiation. In MKN-45 cells-formed xenograft model, overexpression of miR-203 resulted in obvious loss of tumor volume and weight after treatment of radiation compared with miR-NC group (Figure 3A and B). However, after exposure of radiation, anti-miR-203 group showed higher tumor volume and weight than anti-miR-NC group in AGS-induced xenograft model (Figure 3C and D).

ZEB1 is a target of miR-203
To explore the mechanism allows miR-203 participating in GC, the potential target of miR-203 was probed by TargetScan Release 7.2. As shown in Figure 4A, bioinformatics analysis showed the putative binding sites of miR-203

Figure 1 miR-203 expression is down-regulated in GC. (A) The expression of miR-203 was measured in GC tissues and NT samples by quantitative real-time polymerase chain reaction. (B) The level of miR-203 was detected in GC cells by quantitative real-time polymerase chain reaction. (C) Survival fraction was analyzed in GC cells by colony formation assay. *P<0.05.
and ZEB1. This prediction was identified in MKN-45 and AGS cells by luciferase activity assay. Results showed that accumulation of miR-203 led to a great loss of luciferase activity in MKN-45 cells transfected with ZEB1-Wt, while it did not affect the activity in empty vector group (Figure 4B). However, abrogation of miR-203 significantly increased the luciferase activity in AGS cells transfected with ZEB1-Wt, whereas its efficacy was lost in response to empty vector group (Figure 4C). Moreover, overexpression of miR-203 notably suppressed ZEB1 expression at protein and mRNA levels in MKN-45 cells, but its knockdown evidently enhanced ZEB1 abundance in AGS cells (Figure 4D-4G). Subsequently, the expression of ZEB1 was measured in GC tissues and results showed that ZEB1 mRNA expression was abnormally elevated in GC tissues compared with that in NT samples (Figure 4H). Besides, the abundance of ZEB1 mRNA in GC tissues was negatively correlated with miR-203 level ($r=−0.462$, $P=0.005$) (Figure 4I).

Figure 2 miR-203 enhances radiosensitivity in GC cells. (A and B) The abundance of miR-203 was measured in MKN-45 cells transfected with miR-203 or miR-NC and AGS cells transfected with anti-miR-203 or anti-miR-NC by quantitative real-time polymerase chain reaction. Survival fraction (C and D), cell viability (E and F) and apoptosis (G and H) were measured in MKN-45 cells transfected with miR-203 or miR-NC and AGS cells transfected with anti-miR-203 or anti-miR-NC after treatment of radiation by colony formation, MTT or flow cytometry, respectively. *$P<0.05$. 

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ZEB1 is associated with miR-203-mediated radiosensitivity of GC cells

As a result, the abundance of ZEB1 protein was effectively enhanced in MKN-45 cells transfected with ZEB1 and was evidently reduced in AGS cells transfected with shZEB1 compared with that in their corresponding controls (Figure 5A and B). To investigate whether ZEB1 was required for miR-203-mediated regulation of radiosensitivity, MKN-45 cells were co-transfected with miR-203 and ZEB1 or vector and AGS cell were co-transfected with anti-miR-203 and shZEB1 or scrambled. As displayed in Figure 5C, 5E and 5G, restoration of ZEB1 significantly increased the survival fraction and cell viability but decreased apoptosis in MKN-45 cells with miR-203 overexpression after radiation treatment. However, the opposite occurred in response to ZEB1 interference in AGS cells with miR-203 deficiency after treatment of radiation (Figure 5D, 5F and 5H). Moreover, the role of ZEB1 was further evaluated in vivo. The mice were injected with MKN-45 cells stably transfected with miR-203 and ZEB1 or vector or AGS cells stably transfected with anti-miR-203 and shZEB1 or scrambled. Results showed that tumor volume and weight were significantly elevated in miR-203+ZEB1 group compared with those in ZEB1+vector group in MKN-45-induced xenograft model after exposure of radiation (Figure 6A and B). However, the tumor growth showed an opposite trend in anti-miR-203+shZEB1 group in radiation-treated AGS xenograft model (Figure 6C and D).

Discussion

In recent years, great advances have been gained in epidemiology, prevention and therapy of GC. However, aberrant molecular signatures related to resistance drive GC malignancy. The former findings have suggested that miRNAs play essential roles in diagnosis, prognosis and chemo- and radiotherapy in different cancers, including
Here we found that miR-203 expression was down-regulated in GC tissues and cells, which is also in agreement with the previous study. Moreover, we found that miR-203 expression was associated with radiosensitivity of GC cells. In this study, we first investigated the sensibilization role of miR-203 in radiotherapy of GC cells and revealed the regulatory network of miR-203/ZEB1.

The available evidence has indicated that miR-203 reduced radioresistance by regulating interleukin 8 (IL-8) and AKT signaling in nasopharyngeal carcinoma. Moreover, Shao et al revealed that miR-203 promoted cell radiosensitivity in vitro and in vivo by targeting B-cell-specific moloney leukemia virus insertion site 1 (Bmi-1) in hepatocellular carcinoma. These findings indicated that miR-203 might function as a radiosensitizer in human cancers. However, there is no work on the interaction between miR-203 and radiosensitivity of GC. In the present study, we disclosed that miR-203 promoted radiosensitivity of GC cells in vitro and in vivo, indicating the therapeutic value of miR-203 in radiotherapy of GC. Nevertheless, the potential mechanism allows miR-203 addressing radiosensitivity of GC remain poorly understood. Functional miRNA is known to regulate target expression by binding its 3′-UTR sequences. Previous studies have reported multiple targets of miR-203 in various cancers, such as slug (SNAI2), regulator of G protein signaling 17 (RGS17), ataxia telangiectasia mutated kinase (ATM) and progesterone-induced blocking factor 1 (PIBF1). In this study, we validated ZEB1 as a target of miR-203.
Figure 5 miR-203 regulates radiosensitivity in GC cells by targeting ZEB1. (A and B) The expression of ZEB1 protein was measured in MKN-45 cells transfected with ZEB1 or vector and AGS transfected with shZEB1 or scrambled by western blot. Survival fraction (C and D), cell viability (E and F) and apoptosis (G and H) were measured in MKN-45 cells transfected with miR-203+vector or miR-203+ZEB1 and AGS cells transfected with anti-miR-203+scrambled or anti-miR-203+shZEB1 after treatment of radiation by colony formation, MTT or flow cytometry, respectively. *P<0.05.
as a target of miR-203 in GC cells by bioinformatics analysis and luciferase activity assay, which is also reported in non-small cell lung cancer.  

This study showed high expression of ZEB1 mRNA in GC tissues, which is also in agreement with previous studies. Furthermore, available evidences have indicated that ZEB1 is involved in proliferation, migration, invasion and epithelial-to-mesenchymal transition in GC progression. These findings suggested that ZEB1 could serve as an oncogene in GC progression. Notably, emerging evidence suggested that miR-205 promoted tumor radiosensitivity by targeting ZEB1 in breast cancer, which indicated the inhibitory effect of ZEB1 on radiosensitivity. In addition, sever such reports uncovered that inhibition of ZEB1 might protect radiosensitivity in glioblastoma, rectal cancer and prostate cancer. According to these reports, we hypothesized ZEB1 might also be involved in radioresistance of GC cells. Intriguingly, this research confirmed that ZEB1 impaired radiosensitivity, revealed by it reversed miR-203-mediated promotion of radiosensitivity in GC, which suggested that miR-203 enhanced radiosensitivity of GC cells by targeting ZEB1 in vitro and in vivo. Previous effort has reported the importance of signaling pathway in radiosensitivity of cancers, such as PI3K/AKT/mTOR pathway and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway. Hence, the potential signaling pathway addressed by miR-203 is expected to be explored in further study.

**Conclusion**

In short, miR-203 expression was reduced and ZEB1 mRNA was enhanced in GC. Addition of miR-203 increased radiosensitivity of GC cells in vitro and in vivo, possibly by targeting ZEB1. This study...
indicates miR-203 as a promising radiosensitizer for GC treatment.

Disclosure
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Figure S1 The effect of miR-203 on survival fraction of GES-1 cells after radiation treatment. GES-1 cells were transfected with miR-203 mimic or miR-NC and then exposed to different doses (0, 2, 4, 6 and 8 Gy) of radiation. The survival fraction of cells was analyzed by colony formation assay. *P<0.05.