

miR-200c regulates the proliferation, apoptosis and invasion of gastric carcinoma cells through the downregulation of EDNRA expression

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Abstract. This study aimed to investigate the regulatory effects of microRNA (miR)-200c on the proliferation, apoptosis and invasion of gastric carcinoma cells and to elucidate the underlying mechanisms involving the possible role of endothelin receptor A (EDNRA). The expression levels of miR-200c and EDNRA in the gastric carcinoma cell lines, BGC-823, SGC-7901 and HGC-27, and in GES-1 normal gastric cells were evaluated by RT-PCR and western blot analysis. The gastric carcinoma cells, particularly the BGC-823 cells, expressed significantly lower levels of miR-200c than the normal gastric cells ($P < 0.01$). Thus, the BGC-823 cells were employed as model cells. In comparison to the normal gastric cells, EDNRA was overexpressed in the gastric carcinoma cells ($P < 0.01$). Following the transfection of the gastric carcinoma cells with miR-200c mimics, or negative control vector (miR-200c NC), or with siRNA targeting EDNRA (siRNA EDNRA) or negative control siRNA (siRNA NC), the expression levels were assessed again by RT-PCR and western blot analysis. The successful transfection of miR-200c mimics was observed and this markedly elevated the expression of miR-200c ($P < 0.01$). The transfection of miR-200c mimics or siRNA EDNRA notably decreased the EDNRA mRNA and protein expression levels (both $P < 0.01$). In addition, dual-luciferase reporter gene analysis was performed to reveal the targeting relationship between miR-200c and EDNRA. EDNRA was found to be the downstream target gene of miR-200c. Moreover, methyl

thiazolyl tetrazolium (MTT) assay, Hoechst staining and Transwell assay were conducted to demonstrate the effects of miR-200c mimics or siRNA EDNRA on the proliferation, apoptosis and invasion of the gastric carcinoma cells, respectively. We found that transfection with miR-200c mimics and siRNA EDNRA were able to markedly suppress the proliferation and invasive capacity, and to promote the apoptosis of the gastric carcinoma cells (all $P < 0.01$). On the whole, our data indicate that miR-200c regulates the proliferation, apoptosis and invasion of gastric carcinoma cells through the downregulation of EDNRA expression.

Introduction

Gastric carcinoma is associated with the highest morbidity rates worldwide and is considered a lethal cancer; the mortality rate in China due to gastric carcinoma accounts for 40% of the mortality cases of gastric carcinoma worldwide (1). Traditional radical surgery and chemotherapy can extend the 5-year survival rate of gastric carcinoma patients to approximately 35%; however, this rate for patients with advanced-stage disease is only approximately 15% (2). Therefore, compared to traditional therapies, the targeted therapy of cancer may enhance the treatment efficacy of cancers, including gastric carcinoma to an extremely promising stage. Recently, the exploration of novel specific cancer biological targets and markers with high efficiency has become an international hotspot in the targeted therapy of cancer.

MicroRNAs (miRNAs or miRs) are endogenous non-coding micromolecular RNAs with approximately 21-25 nucleotides in length with highly conserved sequences in evolution. miRNAs can regulate gene transcription by binding to the non-coding domains of target mRNAs and then degrading the target genes or inhibiting target gene expression. It has been indicated that miRNAs have potent biological functions and are able to regulate approximately 30% of mRNAs (3,4). Recent studies have revealed that miRNAs play important roles in the genesis and development of gastric carcinoma, and they are also therapeutic targets in gastric carcinoma (3-5).

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As a type of miRNAs associated with cancer, the miR-200 family can block the development and metastasis of cancer by suppressing cancer neovascularization. The members of the miR-200 family include miR-200a, miR-200b, miR-200c, miR-141 and miR-429. However, the expression levels of the miR-200 family members in various tissues differ and their functions and regulatory mechanisms are diverse as well (6,7). Moreover, the miR-200 family members have been found to be low expressed at low levels in gastric carcinoma tissues and to be closely associated with a poor prognosis and unfavorable clinical parameters of patients, which renders the miR-200 family members effective biomarkers for gastric carcinoma diagnosis and prognosis (8). However, the specific functions and mechanisms of action of the miR-200 family members in gastric carcinoma remain unclear. Therefore, in this study, miR-200c, a member of the miR-200 family, was employed as the study object. We investigated the expression of miR-200c in gastric carcinoma cell lines and examined its regulatory effects and molecular mechanisms of action as regards the proliferation, apoptosis and invasion of gastric carcinoma cells.

Materials and methods

Materials and cell culture. The BCA kit, Hoechst staining kit and HRP-labeled goat anti-rabbit IgG (H+L) (A0208) were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Luciferase assay reagent (LAR) and passive lysis buffer (PLB) were from Promega (Madison, WI, USA). Methyl thiazolyl tetrazolium (MTT) was purchased from Gibco (Grand Island, NY, USA). Rabbit anti-endothelin receptor A (EDNRA) polyclonal antibody (ab117529) was from Abcam (Cambridge, MA, USA). The transwell insert was from Corning, Inc. (Corning, NY, USA). Crystal violet was from Sigma (St. Louis, MO, USA). TRIzol and the one-step RT-PCR kit were from Takara (Dalian, China). The Lipofectamine™ 2000 kit, the wild-type (Wt) vector pGL3-EDNRA 3'UTR-Wt and the mutant (Mut) vector pGL3-EDNRA 3'UTR-Mut were obtained from Invitrogen (Carlsbad, CA, USA). siRNA targeting EDNRA (siRNA EDNRA) and siRNA negative control (NC) were prepared by Invitrogen and miR-200c mimics and miR-200c NC were synthesized by RiboBio (Guangdong, China) based on the primers listed in Table I.

The human gastric carcinoma cell lines, BGC-823, SGC-7901 and HGC-27, and the immortalized gastric mucosa epithelial cell line, GES-1, were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA).

Determination of miR-200c and EDNRA expression levels by RT-PCR and western blot analysis. For RT-PCR, total RNA from the BGC-823, SGC-7901, HGC-27 and GES-1 cells was extracted using TRIzol reagent according to the manufacturer's instructions and the RNA purity was evaluated by determining the absorbance at 260 and 280 nm with UV-vis spectrophotometry. RNA was then reverse transcribed into cDNA and amplified with the one-step RT-PCR kit. The primers as shown in Table II were added to the 25 µl of the PCR reaction system. The PCR parameters were set as follows: denaturation for 45 sec

Table I. Sequences of primers for siRNA EDNRA, siRNA NC, miR-200c mimics and miR-200c NC.

Gene	Primer sequences (5'→3')
siRNA EDNRA	F: UGGCAGUGUCUUAGCUGGUUG R: ACCAGCUAAGACACUGCCAUU
siRNA NC	F: UUCUCCGAACGUGUCACGUTT R: ACGUGACACGUUCGGAGAATT
miR-200c mimics	F: UACAGUACUGACCUGUCCACUCUGG R: CCGCCUUUGUGCUUCUGUUCUUCGU
miR-200c NC	F: UUCUCCGAACGUGUCACGUTT R: ACGUGACACGUUCGGAGAATT

EDNRA, endothelin receptor A; NC, negative control; F, forward; R, reverse.

Table II. Sequences of primers used for RT-PCR.

Gene	Primer sequences (5'→3')
miR-200c	F: GTAAATCGGTGTGTGTGTCTCGCGGGTCTG R: CAAACCCTCCGAAACCCCTCGACCG
EDNRA	F: GCTCTAGATAGGTAGCAACGTGGCTTT R: GCTCTAGAGCCCCAAAACCTTGTCAC
GAPDH	F: AGCCACATCGCTCAGACA R: TGGACTCCACGACGTACT

EDNRA, endothelin receptor A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

at 94°C, annealing for 45 sec at 59°C, elongation for 60 sec at 72°C and 35 cycles. The amplification products were loaded to conduct 2% (w/v) agarose gel electrophoresis. Electrophoresis strips were analyzed on a gel imaging system (ChemiDoc™ XRS; Bio-Rad, Hercules, CA, USA).

For western blot analysis, the BGC-823, SGC-7901, HGC-27 and GES-1 cells were seeded into 6-well plates. After 48 h, the cells were collected and mixed with moderate RIPA buffer. The mixture was centrifuged and the supernatant was carefully collected to obtain the total protein. The protein content was determined using the BCA kit. The protein was degenerated, quantified and loaded to conduct sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis for 1-2 h. The protein was then transferred to a membrane by a wet method for 30-40 min. The membrane was incubated with rabbit anti-EDNRA polyclonal antibody buffer (1:100 dilution) at 4°C overnight. After a rinse, the membrane was immersed in HRP-labeled goat anti-rabbit IgG (H+L) buffer (1:200 dilution) at room temperature for 1-2 h. Finally, after rinsing again, enhanced chemiluminescence (ECL) solution dripped onto the membrane and the membrane was exposed on a gel imaging system (ChemiDoc™ XRS; Bio-Rad). Quantity One software (Bio-Rad) was utilized to evaluate the gray values of proteins strips.

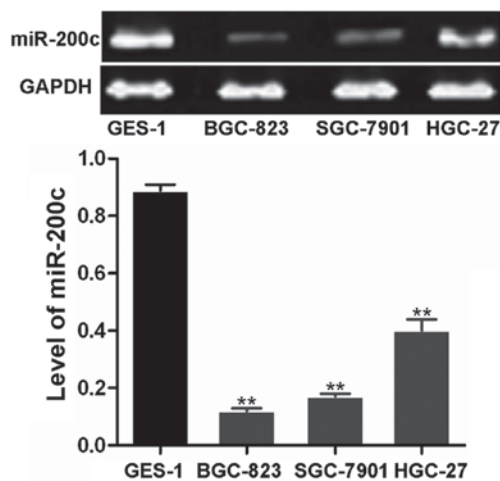


Figure 1. miR-200c expression levels in the gastric carcinoma cell lines, SGC-7901, BGC-823 and HGC-27, and in the normal gastric cell line, GES-1, evaluated by RT-PCR. GAPDH served as the loading control. ** $P < 0.01$ compared to GES-1 cells.

After the siRNA EDNRA, siRNA NC, miR-200c mimics and miR-200c NC were respectively transfected into the BGC-823 cells as described below, the miR-200c expression level was assayed by RT-PCR, and EDNRA expression was evaluated by RT-PCR and western blot analysis once again.

Cell transfection. When the confluence of the BGC-823 cells reached 50-70%, siRNA EDNRA, siRNA NC, miR-200c mimics or miR-200c NC were respectively transfected into the cells using Lipofectamine™ 2000 kit according to the manufacturer's instructions. After 6 h, the culture medium was replaced with DMEM containing 10% (v/v) FBS and the cells were cultured at 37°C and 5% CO₂ for a further 48 h. EDNRA expression was then determined by western blot analysis and RT-PCR and miR-200c expression was measured by RT-PCR.

Determination of cell viability. The BGC-823 cells were seeded into 96-well plates. When the cell confluence reached 50%, siRNA EDNRA, siRNA NC, miR-200c mimics, or miR-200c NC were respectively transfected into the cells as described above. After 48 h, 20 μ l of MTT (5 mg/ml) were added to each well for 4 h. The culture medium was then discarded and 150 μ l of dimethyl sulfoxide were added to each well. Vibration was conducted to fully dissolve the crystal substances. Lastly, the plates were subjected to absorbance measurement at 560 nm on a microplate reader (Infinite F200/M200; Tecan, Männedorf, Switzerland). Relative cell viability was calculated based on the absorbance (relative cell viability = $(OD_{\text{treatment}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100\%$) and cells without any treatment served as the controls.

Determination of cell apoptosis. The BGC-823 cells were seeded into 6-well plates. When the cell confluence reached 50%, siRNA EDNRA, siRNA NC, miR-200c mimics, or miR-200c NC were respectively transfected into the cells as described above. After 48 h, the cells were stained and fixed using the Hoechst staining kit according to the manufacturer's instructions. The cells were then examined under a microscope (AF6000; Leica, Wetzlar, Germany).

Determination of cell invasive capacity. Matrigel was tiled uniformly onto the micromembrane (8 μ m) of the Transwell insert to prepare the gel. The BGC-823 cells were seeded into 6-well plates. When the cell confluence reached 50%, siRNA EDNRA, siRNA NC, miR-200c mimics, or miR-200c NC were respectively transfected into the cells as described above. After 48 h, the cells were digested and placed into the upper chamber of the Transwell insert. The lower chamber of the Transwell insert was filled with DMEM. After 48 h, the Transwell insert was removed, fixed with paraformaldehyde and stained with crystal violet. The cells in the lower chamber were then counted under a light microscope (AF6000; Leica) in 5 individual visual fields. The average cell number in each visual field represented the invasive capacity of the cells.

Luciferase reporter gene expression analysis. The miR-200c mimics and EDNRA recombinant vectors were co-transfected into the BGC-823 cells. The cells were divided into the following groups: the miR-200c mimics + Wt EDNRA, miR-200c NC + Wt EDNRA, miR-200c mimics + Mut EDNRA and the miR-200c NC + Mut EDNRA groups. The dual-luciferase reporting system (Promega) was employed to detect the activity of transfected luciferase according to the manufacturer's instructions. Briefly, the transfected cells were washed with phosphate-buffered solution (PBS) 3 times and mixed with PLB. Following full lysis, LAR was added to the mixture. The fluorescence intensity was then determined with a dual-reporting luciferase detector. Following the addition of stop buffer, the fluorescence intensity was determined again. The relative fluorescence intensity was calculated according to the following formula: relative fluorescence intensity = fluorescence intensity of firefly luciferase/fluorescence intensity of sea pansy luciferase.

Statistical analysis. All experiments were repeated at least 3 times and data are expressed as the means \pm standard deviation (SD). Statistical analysis was conducted using an ANOVA followed by Tukey's post hoc test with a SPSS 17.0 software. Significant differences were defined at $P < 0.05$.

Results

Original miR-200c expression levels in gastric carcinoma cells and normal gastric cells. The miR-200c expression levels in the SGC-7901, BGC-823 and HGC-27 cells were markedly lower than those in the GES-1 cells (Fig. 1). It was suggested that compared to the normal gastric cells, miR-200c expression was downregulated in the gastric carcinoma cells. The BGC-823 cells had the lowest expression of miR-200c, and thus they were employed for the cell model of gastric carcinoma in the following experiments.

miR-200c expression level in gastric carcinoma cells transfected with miR-200c mimics. At 6 h after the miR-200c mimics and miR-200c NC were transfected into the BGC-823 cells, RT-PCR demonstrated that the strip in the miR-200c mimics group was markedly lighter than that in the miR-200c NC group (Fig. 2). Moreover, as compared with the miR-200c NC-transfected cells (0.20 ± 0.02), miR-200c expression following transfection with miR-200c mimics (0.98 ± 0.10)

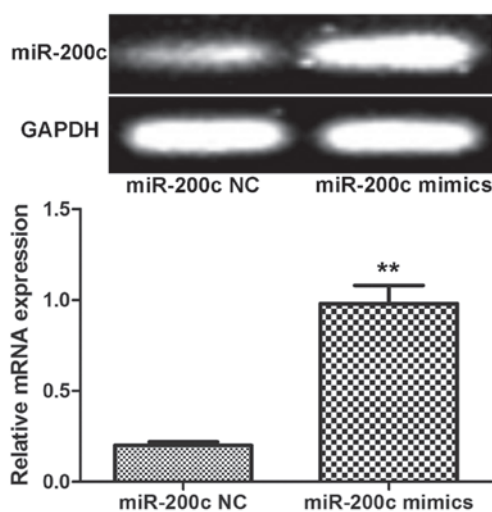


Figure 2. miR-200c expression levels in BGC-823 cells following transfection with miR-200c mimics and miR-200c NC evaluated by RT-PCR. GAPDH served as the loading control. ** $P < 0.01$ compared with miR-200c NC.

was significantly upregulated by almost 4-fold ($P < 0.01$). These results confirmed the successful transfection of the miR-200c mimics into the BGC-823 cells.

Effects of miR-200c mimics on the viability, apoptosis and invasive capacity of gastric carcinoma cells. We then examined the viability, apoptosis and invasive capacity of the BGC-823 cells following transfection with miR-200c mimics or miR-200c NC MTT assay, Hoechst staining and Transwell assay, respectively. We found that compared to the miR-200c NC-transfected cells, transfection with miR-200c mimics significantly reduced the viability of the BGC-823 cells ($P < 0.01$; Fig. 3A). As shown in Fig. 3B, the bright green-colored cells represented the apoptotic cells. It was observed that the bright green-colored cells in the miR-200c mimics group were notably greater in number than those in the miR-200c NC group. Quantitatively, the apoptotic rate following transfection with miR-200c mimics ($38.46 \pm 3.85\%$) increased by >10-fold as compared to transfection with miR-200c NC ($3.34 \pm 0.33\%$, $P < 0.01$). Furthermore, as shown in Fig. 3C, the number of invading cells in the miR-200c mimics group (43.50 ± 4.34) was markedly lower than that in the miR-200c NC group (149.52 ± 8.74 , $P < 0.01$). In summary, miR-200c mimics were capable of effectively suppressing the proliferation, promoting the apoptosis suppressing the invasive capacity of the gastric carcinoma cells.

Original EDNRA expression levels in gastric carcinoma cells and normal gastric cells. We examined the original EDNRA expression levels in the SGC-7901, BGC-823, HGC-27 and GES-1 cells by RT-PCR and western blot analysis. As shown in Fig. 4, the results revealed that the mRNA and protein levels of EDNRA in the SGC-7901, BGC-823 and HGC-27 cells were significantly higher than those in the GES-1 cells (all $P < 0.01$). It was thus indicated that EDNRA was overexpressed in diverse gastric carcinoma cells, particularly in the BGC-823 cells.

Luciferase reporter gene expression analysis. The miR-200c mimics, miR-200c NC, Wt vector pGL3-EDNRA 3'UTR-Wt, and Mut vector pGL3-EDNRA 3'UTR-Mut were transfected

into the BGC-823 cells. We found that the fluorescence intensities in the co-transfection groups transfected with miR-200c NC and either of the two vectors were similar (Fig. 5). There was no significant difference in the fluorescence intensity between the cells transfected with the miR-200c mimics or miR-200c NC and the pGL3-EDNRA 3'UTR-Mut vector ($P > 0.05$). However, following co-transfection with miR-200c mimics and the pGL3-EDNRA 3'UTR-Wt vector, the fluorescence intensity was significantly decreased as compared with the other 3 co-transfection groups ($P < 0.01$). These results suggested that EDNRA was the downstream target gene of miR-200c and that EDNRA may be regulated by miR-200c.

Effects of miR-200c mimics on the EDNRA expression levels in gastric carcinoma cells. RT-PCR and western blot analysis respectively revealed that compared to the miR-200c NC-transfected cells, transfection with miR-200c mimics markedly downregulated the mRNA and protein expression levels of EDNRA in the BGC-823 cells ($P < 0.01$; Fig. 6).

EDNRA expression levels in siRNA EDNRA-transfected gastric carcinoma cells. After the siRNA EDNRA or siRNA NC were transfected into the BGC-823 cells, the blots in the siRNA EDNRA group in both RT-PCR and western blot analysis were markedly fainter in comparison to those in the siRNA NC group (Fig. 7). As shown by quantitative western blot analysis, the cells in the siRNA EDNRA group (0.21 ± 0.02) produced significantly less EDNRA protein than the cells in the siRNA NC group (1.06 ± 0.10 , $P < 0.01$). This similar difference between siRNA EDNRA and siRNA NC was also found in the quantitative RT-PCR analysis for the EDNRA mRNA levels (0.27 ± 0.02 vs. 1.04 ± 0.10 , $P < 0.01$). It was thus suggested that siRNA EDNRA downregulated the expression of EDNRA in the gastric carcinoma cells, which was similar to the effects of miR-200c mimics on EDNRA expression.

Effects of siRNA EDNRA on the viability, apoptosis and invasive capacity of gastric carcinoma cells. We then examined the viability, apoptosis and invasive capacity of the BGC-823 cells following transfection with siRNA EDNRA or siRNA NC by MTT assay, Hoechst staining and Transwell assay, respectively. We found that compared to transfection with siRNA NC, transfection with siRNA EDNRA significantly decreased the viability of the BGC-823 cells ($P < 0.01$; Fig. 8A). As shown in Fig. 8B, the bright green-colored apoptotic cells in the siRNA EDNRA group were markedly greater in number than those in the siRNA NC group. Quantitatively, the apoptotic rate following transfection with siRNA EDNRA ($40.46 \pm 3.99\%$) was increased by >11-fold as compared to transfection with siRNA NC ($3.37 \pm 0.32\%$, $P < 0.01$). Moreover, as shown in Fig. 8C, the number of invading cells in the siRNA EDNRA group (40.51 ± 4.03) was significantly less than that in the siRNA NC group (138.53 ± 9.58 , $P < 0.01$). Thus, siRNA EDNRA were able to efficiently block the proliferation, enhance the apoptosis and inhibit the invasion of gastric carcinoma cells.

Discussion

As a member of the miR-200 family, miR-200c is located on human chromosome 12p13 and it is adjacent to and clustered with

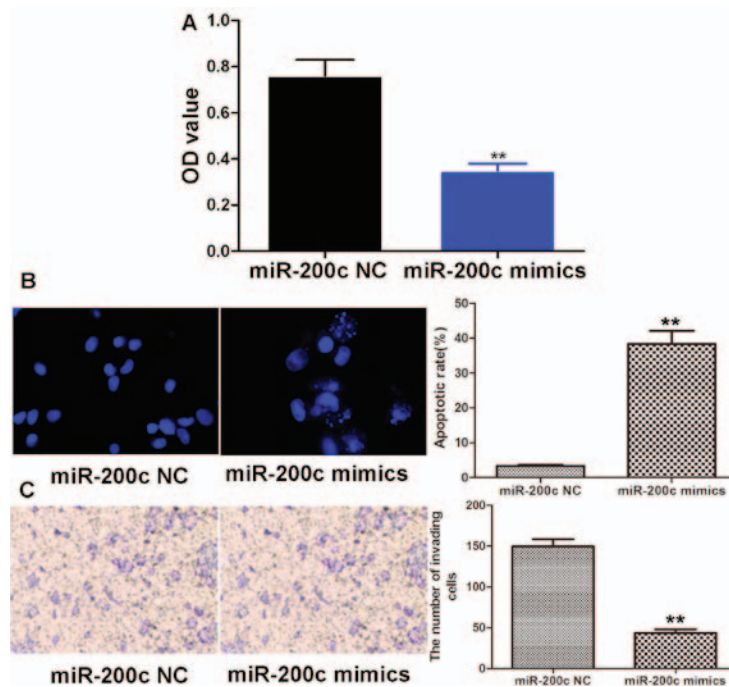


Figure 3. The (A) viability, (B) apoptosis and (C) invasion of BGC-823 cells following transfection with miR-200c mimics or miR-200c NC evaluated by MTT assay, Hoechst staining and Transwell assay, respectively. **P<0.01 compared to miR-200c NC. (B and C) Magnification, x200.

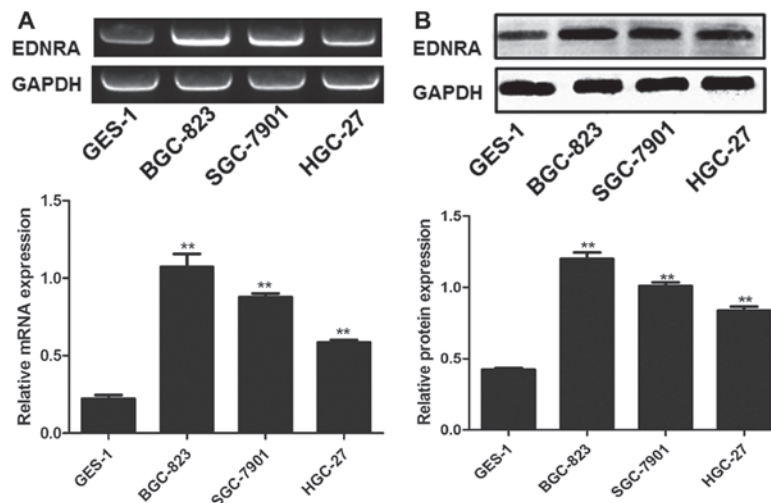


Figure 4. Endothelin receptor A (EDNRA) expression levels in the gastric carcinoma cell lines, SGC-7901, BGC-823 and HGC-27, and in the normal gastric cell line, GES-1, evaluated by (A) RT-PCR and (B) western blot analysis. GAPDH served as the loading control. **P<0.01 compared to the GES-1 cells.

miR-141, another member of the miR-200 family. miR-200c was screened from the miRNA of breast cancer cells, lung cancer cells, fibrosarcoma cells, melanoma cells and normal human bronchial epithelial cells by Hurteau *et al* (9) in 2006 and Zinc finger E-box-binding homeobox 1 (ZEB1) was confirmed to its target gene through bioinformatics (10,11). Subsequently, Ceppi *et al* revealed the differential expression of miR-200c between lung cancer and normal lung tissue (12). Thereafter, it has been successively demonstrated the abnormal expression of miR-200c in liver cancer, renal cancer, pancreatic cancer, bladder cancer and ovarian cancer tissues and cells (13 and refs therein). Moreover, miR-200c was found to regulate the growth, proliferation, apoptosis, metastasis and invasion of cancer cells by affecting the expression of target genes, such as ZEB1, 1-phosphatidylinositol-

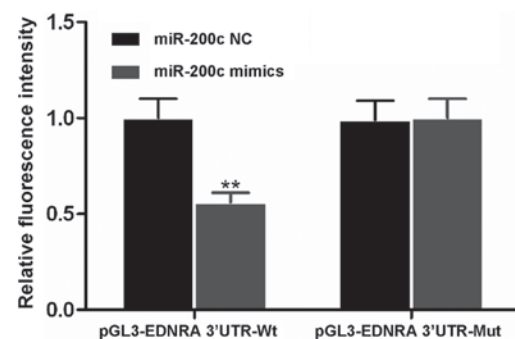


Figure 5. Fluorescence intensity of BGC-823 cells following co-transfection with miR-200c mimics, miR-200c NC, wild-type vector pGL3-EDNRA 3'UTR-Wt, and mutant vector pGL3-EDNRA 3'UTR-Mut which was probed by luciferase reporter gene expression analysis. **P<0.01 compared to miR-200c NC.

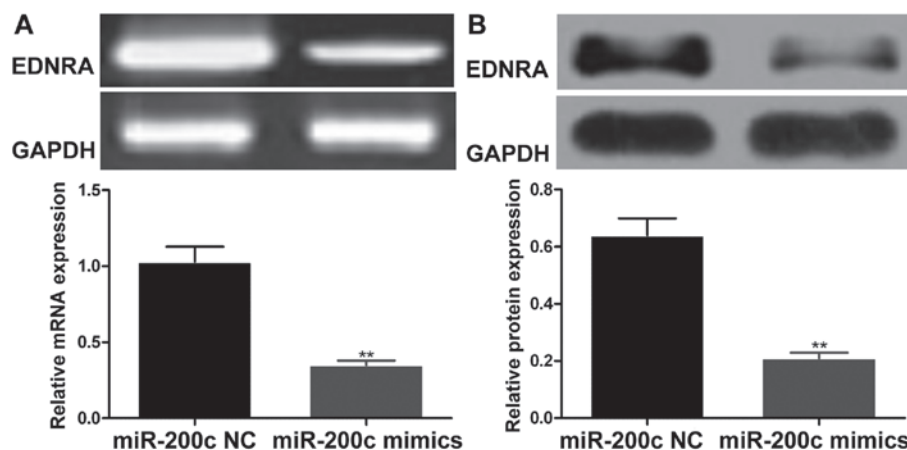


Figure 6. Effects of miR-200c NC and miR-200c mimics on the endothelin receptor A (EDNRA) mRNA and protein expression levels in BGC-823 cells evaluated by (A) RT-PCR and (B) western blot analysis, respectively. GAPDH served as the loading control. **P<0.01 compared to miR-200c NC.

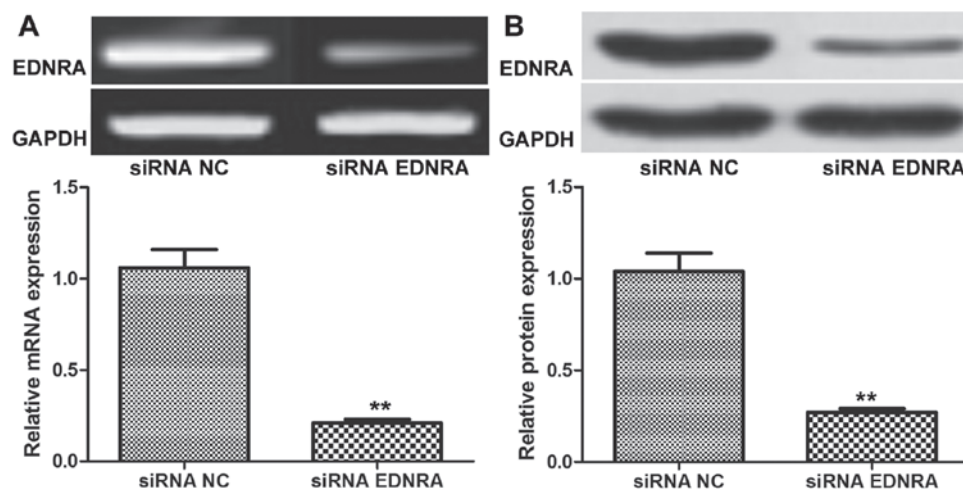


Figure 7. Endothelin receptor A (EDNRA) mRNA and protein expression levels in BGC-823 cells following transfection with siRNA EDNRA and siRNA NC evaluated by (A) RT-PCR and (B) western blot analysis, respectively. GAPDH served as the loading control. **P<0.01 compared with siRNA NC.

3-phosphate 5-kinase (FAB1), homeobox D10 (HOXD10) and BMI1, and to be closely associated with epithelial mesenchymal transition (EMT) in cancer (14,15). In recent years, miR-200c has also been found to be associated with the genesis and development of gastric carcinoma. Blanco-Calvo *et al* demonstrated that the expression of miR-200c in gastric carcinoma tissues was significantly inferior to that in normal tissues (16). Chang *et al* adopted the RT-PCR method to reveal that the expression levels of miR-200 family members, including miR-200c in 46 cases of gastric cancer were lower than those in the pericarcinomatous tissues, and that they were strongly linked to pathological patterns and angiogenesis (17). Consequently, it was suggested that miR-200c may be the judging indicator of gastric carcinoma invasion and may also be an effective indicator of patient prognosis. Although these studies have demonstrated the close association between miR-200c and the genesis and development of gastric carcinoma, the specific mechanisms involved remain unknown. Therefore, in this study, we investigated the molecular mechanisms involved in the influence of miR-200c on the biological behaviors of gastric carcinoma cells, such as proliferation, apoptosis and invasion. Firstly, we revealed the downregulated expression of miR-200c in 3 gastric carcinoma

cell lines in comparison to a normal gastric cell line. This result was in line with that of a previous study (18). After considering the lowest miR-200c expression level, histological grade and the toxicity of the transfection agents, the BGC-823 cells were employed as the model gastric carcinoma cells in the following experiments. We then successfully transfected the miR-200c mimics and miR-200c NC into gastric carcinoma cells using Lipofectamine 2000 which was verified by RT-PCR. MTT assay and Hoechst staining also demonstrated that as compared to transfection with miR-200c NC, transfection with miR-200c mimics markedly suppressed the proliferation and promoted the apoptosis of gastric carcinoma cells, which was consistent with the effects of miR-200c mimics on breast cancer cells, renal cancer cells and colon cancer cells in previous studies (19-21). Furthermore, Transwell assay revealed that transfection with miR-200c mimics notably suppressed the invasive ability of the gastric carcinoma cells, which was in accordance with the roles of miR-200c mimics in bladder cancer cells, colon cancer cells and head and neck squamous cell carcinoma (22-24). Thus, miR-200c mimics were able to strongly inhibit the proliferation and invasion, and promote the apoptosis of gastric carcinoma cells.

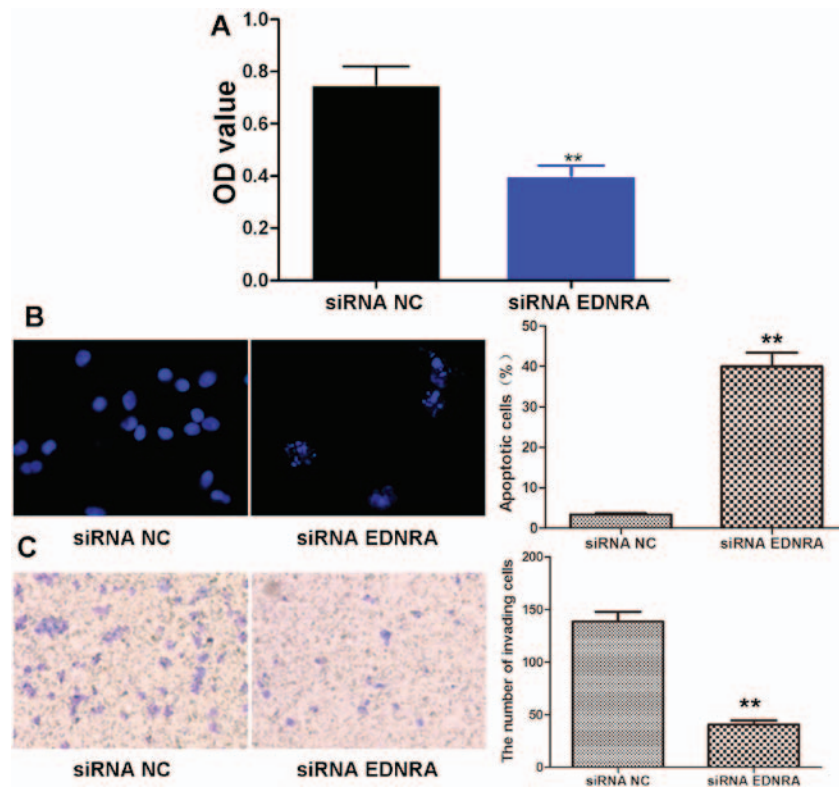


Figure 8. The (A) viability, (B) apoptosis and (C) invasion of BGC-823 cells following transfection with siRNA against endothelin receptor A (EDNRA; siRNA EDNRA) or siRNA NC examined by MTT assay, Hoechst staining and Transwell assay, respectively. ** $P < 0.01$ compared to siRNA NC. (B and C) Magnification, $\times 200$.

Similar to numerous miRNAs, miR-200c affected the biological behavior of cancer cells by regulating the target genes. Tang *et al* reported that exogenous miR-200c inhibited the growth and invasion of cancer cells by targeting DNMT3A, DNA methyltransferase 3 beta (DNMT3B) and SP1 (25). Saito *et al* demonstrated that miR-200c affected EMT in cancer cells by targeting ZEB and then regulating E-cadherin expression (26). Zhou *et al* revealed that the upregulation of miR-200c expression affected the metastasis and invasion of gastric carcinoma through the targeted downregulation of ZEB1/2 expression (27). Moreover, the alterations in EMT regulated by ZEB1 have been shown to be associated with the EMT-related gene, EDNRA (28), and the EDNRA signal can regulate EMT (29). As a G-protein coupled receptor, EDNRA contains 7 transmembrane domains and mediates signaling pathways regulated by G-protein coupled receptor kinases. Its affinities with different endothelin are distinctive with a strongest affinity with endothelin-1 (ET-1). Thus, it has a potent vasoconstrictive effect. The gene polymorphism of EDNRA is closely related to cardiovascular disease, immune disease and cancer (30,31). Furthermore, the overexpression of EDNRA in bladder cancer cells, epithelial ovarian cancer, and prostate cancer has been shown to be closely associated with the genesis and development of cancer, and ET-1/EDNRA regulates EMT and chemotherapeutic drug resistance in epithelial ovarian cancer cells (32-34). However, the specific mechanisms of action of EDNRA in gastric carcinoma have not yet been revealed. Furthermore, EDNRA may be the target gene of miR-200c (35). Therefore, the specific mechanisms of action of EDNRA in gastric carcinoma and the association between miR-200c and EDNRA were investigated in this study. Based on RT-PCR and western blot analysis, respectively, we first

demonstrated the mRNA and protein overexpression of EDNRA in gastric carcinoma cells in comparison with that of normal gastric cells, and the results were in agreement with the expression tendency of EDNRA in other cancer tissues (32,33).

We then investigated whether EDNRA is the target gene of miR-200c by utilizing luciferase reporter gene assay. The fluorescence intensity in the co-transfection group of miR-200c mimics and Wt vector pGL3-EDNRA 3'UTR-Wt was markedly inferior to that in the other 3 co-transfection groups, suggesting that EDNRA was the target gene of miR-200c. This interesting result was also confirmed by the results of RT-PCR and western blot analysis, showing that transfection with miR-200c mimics markedly downregulated the mRNA and protein expression of EDNRA in gastric carcinoma cells. In consequence, we speculated that miR-200c may affect the biological behavior of gastric carcinoma cells, such as proliferation, apoptosis and invasion by regulating the expression of the downstream target gene, EDNRA. However, as miR-200c may have multiple target genes, we transfected siRNA EDNRA into the gastric carcinoma cells to evaluate the effects of siRNA EDNRA on EDNRA expression and the biological behavior of gastric carcinoma cells. Following transfection with siRNA EDNRA, the results of RT-PCR and western blot analysis revealed that the mRNA and protein expression levels of EDNRA were downregulated, which was similar to the effects of miR-200c mimics on EDNRA expression. In addition, after the silencing of EDNRA by siRNA EDNRA, the proliferative and invasive capacity of the gastric carcinoma cells was markedly suppressed and apoptosis was promoted; these effects were similar to those observed with transfection with miR-200c mimics. Thus, this confirms our previous speculation that miR-200c mimics

disrupted the proliferation, apoptosis and invasion of gastric carcinoma cells by targeting EDNRA.

In conclusion, our findings demonstrated that miR-200c expression was downregulated in gastric carcinoma cells, while this downregulation was reversed by transfection with miR-200c mimics. EDNRA was overexpressed in gastric carcinoma cells, while its overexpression was downregulated by transfection with siRNA EDNRA or miR-200c. EDNRA was validated to be the downstream target gene of miR-200c. miR-200c regulated the biological behavior of gastric carcinoma cells by regulating their proliferation, apoptosis and invasion via the downregulation of the expression of EDNRA. These results may provide new insight into the diagnosis and treatment of gastric carcinoma by providing an effective biomarker (miR-200c and EDNRA).

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