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Regular paper

MiRNA-19b-3p downregulates the endothelin B receptor in gastric cancer cells to prevent angiogenesis and proliferation

Xiaodong Hu✉, Hongbing Liu and Chaoyu Li

Department of General Surgery, Medical Centre Hospital of Qionglai City, Qionglai, Sichuan, 611530, China

MiR19b-3p acts as a tumor suppressor gene in various cancers, but its function in gastric cancer is unknown. This study investigated the role of miR19b-3p in angiogenesis and the proliferation of human gastric cancer cells targeting ET_BR expression. Cell proliferation in SGC-7901 cells, cell transfection, luciferase reporter assay, detection of endothelin B receptor mRNAs by RT-qPCR, and a Western blot assay were carried out. RT-qPCR expression analysis showed a prominent ($p<0.01$) downregulation of miR19b-3p in SGC-7901 cells, which was inversely correlated with a substantial increase ($p<0.01$) in the endothelin B receptor (ET_BR). However, overexpression of miR19b-3p in SGC-7901 cells with its mimic ($p<0.01$) resulted in the loss of cell viability in the MTT assay. This effect was reversed ($p<0.01$) by the inhibitor. Western blot analysis revealed that ET_BR was significantly ($p<0.01$) decreased by miR19b-3p overexpression compared with that of the negative control or its inhibitor. Based on bioinformatics tools and luciferase reporter assays, we found that miR19b-3p interacts with the 3'untranslated region (3'UTR) of ET_BR. Restoring miR19b-3p overexpression with its mimic led to downregulation of ET_BR in gastric cancer cells (SGC-7901), which significantly ($p<0.01$) decreased the expression of vascular endothelial growth factor A (VEGF-A). These findings were considerably reversed by miR19b-3p inhibitors ($p<0.01$). The results indicated that miR19b-3p exerts its molecular action by targeting ET_BR at the post-transcriptional level by regulating angiogenesis and proliferation by overexpressing miR19b-3p as a potential treatment target for gastric cancer.

Keywords: miRNA, gastric cancer, proliferation, angiogenesis, endothelial B receptor

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✉e-mail: 3097317821@qq.com

Abbreviations: GC, Gastric cancer; RT-qPCR: Reverse transcriptase quantitative polymerase chain reaction; 3'-UTR, untranslated region; ETBR, Endothelin B receptor; VEGF-A, vascular endothelial growth factor A; FBS, fetal bovine serum; PVDF, polyvinylidene fluoride membrane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ET-1, endothelin-1; ECE-1 & -2, endothelin-converting enzymes-1 & -2; PBS, Phosphate buffer saline; RIN, RNA Integrity Numbers; NSCLC, non-small cell lung carcinoma cell line

INTRODUCTION

Cancer-related deaths from gastric cancer (GC) are among the fourth highest worldwide. The treatment strategies for GC depend on the stage of the disease. Early-stage GC can be treated with surgery, whereas advanced GC requires more aggressive treatments, such as chemotherapy and radiation therapy (Li *et al*., 2019). Combination treatment can be effective in treating non-metastatic stomach cancer. Gastric cancer treatment includes perioperative or postoperative chemotherapy with radiation treatment. Early-stage gastric cancers are best treated with endoscopic resection. Fluoropyrimidines, platinum, taxanes, and irinotecan are active cytotoxic agents in gastric cancer. However, combination regimens are more effective and offer improved survival rates. Immunotherapy is an emerging treatment strategy for stomach cancers. A monoclonal antibody (ramucirumab) against VEGFR-2, either alone or along with paclitaxel, decreases the risk of gastric cancer (Joshi & Badgwell, 2021). Novel therapies are required to treat gastric cancer, which can be achieved by fully understanding its pathogenesis and associated molecular changes. Many disease states, including cancer, are associated with miRNA dysregulation and serve as significant markers for early diagnosis, prognosis, and treatment. Gastric cancer may be caused by dysfunctional miRNAs that disrupt the target genes of these miRNAs. This results in the downregulation and overexpression of tumor suppressor genes and oncogenes (Choi *et al*., 2019).

miRNAs can regulate gene and miRNA expression in gastric cancer (Alessandrini *et al*., 2018). These miRNAs (miR-143, miR-200, and miR-145) regulate cell movement and metastasis in gastric cancer by inhibiting Myo6 expression (Lei *et al*., 2017a) and protein translation (N-cadherin) (Yuan *et al*., 2014). According to previous studies, gastric cancer cells exhibit abnormal miRNA expression compared to surrounding normal cells (Li *et al*., 2019). One of the earlier reports revealed that miRNAs have the potential to control or regulate several genes, and downregulation of miRNAs is correlated with the progression and development of gastric cancer (Zhu *et al*., 2020). A recent study suggested that miR-19b-3p/NRP1 may play a role in inhibiting the GC progression (Wei *et al*., 2020a). Another study reported that miR-19b-3p and miR-16-5p may be able to detect and predict the progression of gastric cancer (Zhang *et al*., 2015a).

There is growing evidence that miRNAs, such as miR-125a, miR-1, miR-125b, miR-199, and miR155 modulate ET-1 expression through the 3' untranslated region (3'UTR) (Jacobs *et al*., 2013). The small peptide endothelin-1 (ET-1) contains 21 amino acids and plays a variety of roles that affect various tissues, cells, and organs. However, the interaction between ET-1 and miRNAs in the gastric cancer is unfamiliar. Based on its own receptors, it has been established that it can exert a wide range of biological effects. Three endothelins (ET-1, ET-2, and ET-3), two G-protein-coupled receptors (ET_AR and ET_BR), and two endothelin-converting enzymes (ECE-1 and ECE-2) encompass the ET axis (Gu *et al*., 2019). Different types of cancers, such as lung, prostate, colorectal, liver, breast and ovarian cancers includ-

ing melanoma, have been found to express ET-1 more frequently. There is some evidence that this may contribute to the proliferation, metastasis, angiogenesis, and suppressed apoptosis of cancer cells (Kalles *et al*., 2019). The ET_AR receptor is primarily responsible for ET-1's ability to induce cell proliferation. A reduction in ET_BR expression has been implicated in the pathogenesis of various cancers, including those of the oesophagus, nasopharynx, prostate, and ovaries (Yin *et al*., 2020). High levels of ET-1 have been detected in the plasma of patients with gastric cancer. This suggests that ET-1 can be used as a reliable indicator for predicting disease recurrence (Aliabadi *et al*., 2022).

Currently, it is still unknown what biological function endothelin receptor B plays in gastric cancer and the mechanisms by which miR19b-3p regulates it. Our study was conducted to demonstrate that ET_BR plays a critical function in gastric cancer progression and to identify probable target sites for miR19b-3p and their cognate interactions within the 3'UTR of ET_BR employing a bioinformatics approach. We investigated the effects of miR19b-3p and its role in both the normal gastric epithelium and human gastric cancer cells targeting the endothelin B receptor (ET_BR) to prevent angiogenesis and proliferation. There is a possibility that this could lead to a new therapeutic approach and the identification of novel targets. Therefore, it is possible to use miRNAs as therapeutic targeting agents for various types of cancer.

MATERIALS AND METHODS

Cell culture

We obtained SGC-7901 (a human gastric cell line) and GES-1 (normal Gastric epithelial cell line) from the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 was used to grow cells (Corning Life Sciences, Wujiang, China) added with 10% foetal bovine serum (FBS) (Corning Life Sciences, Wujiang, China) and 1% antibiotics such as streptomycin. The cells were allowed to grow in an incubator at room temperature with CO_2 (5%). For each line, the lowest passage number was between 3–5. A total of 1×10^5 cells were seeded in six wells of a six-well plate. The cells were then exposed to saturated humidity for 24 h. It is generally recommended that cells pass through when they reach 80–90% confluence. Immediately after the medium was removed, the cells were rinsed twice with phosphate-buffered saline (PBS), incubated for 2 min with 0.3% trypsin, and then resuspended in RPMI 1640 medium (5 ml) with 10% FBS before passing a second time. PlasmoTest™ (InvivoGen, USA) was used for colorimetric detection of mycoplasma contamination in cell cultures. Positive results indicate contamination of the cell culture. We did not detect mycoplasma contamination in cell cultures.

Cell transfection

A density of 2×10^5 cells/cm^2 was seeded in six-well plates once the cells reached 80% confluence. As previously reported (Xin *et al*., 2019), miRNAs were transfected into the SGC-7901 cells. MiR19b-3p was evaluated by transfecting cells with miR19b-3p mimic (40 nM), miR19b-3p inhibitor (40 nM), mimic control (40 nM), or inhibitor control (40 nM) (Sangon, China) using HiPerFect transfection reagent from Qiagen (Hilden, Germany), according to the manufacturer's instructions. During the logarithmic growth phase, gastric cancer cells (SGC-7901) were transfected and divided into five groups: blank group (SGC-7901 cells without transfection), miR19b-3p-NC control group (SGC-7901 cells transfected with miR19b-3p mimic control), miR19b-3p mimic group (SGC-7901 cells transfected with miR19b-3p mimic), miR19b-3p inhibitor group (SGC-7901 cells transfected with miR19b-3p inhibitor), and miR19b-3p inhibitor control group (SGC-7901 cells transfected with miR19b-3p inhibitor control). Plasmid expression vectors encoding ET_BR and the accompanying empty vector (pGEM-T) controls were purchased from Origene (Rockville, MD, USA). For transfection of expression plasmids, 2.5 μg of empty vector or plasmid DNA was diluted with HiPerFect transfection reagent and added to cells grown in 6-well plates. Transfected cells were incubated for 24–72 h at room temperature, harvested for further assays with untreated cells, and used as blanks.

HiPerFect transfection solution (5 μL) was incubated at 37°C for 15 min in DMEM (300 μL) without serum. Five microliters of miR19b-3p mimic, miR19b-3p-NC, miR19b-3p inhibitor, or miR19b-3p-NC were placed in DMEM (250 μL) without serum. After incubation for 15 min, diluted miR19b-3p mimics, miR19b-3p mimic-NC, miR19b-3p inhibitor, and miR19b-3p inhibitor-NC were gently mixed with the diluted HiPerFect transfer reagent and incubated for another 20 min at 37°C. Phosphate-buffered saline (PBS) was used to wash the cells twice after removing the medium. Complexes (500 μL) were added to each well containing the cells and medium, and the plate was gently rocked to mix the solutions. To determine whether transgenes were expressed in cells, cells were incubated in a CO_2 incubator for 24 hours at 37°C.

Cell proliferation

The proliferation of transfected SGC-7901 cells with a miR19b-3p mimic, a miR19b-3p inhibitor, or their controls was studied using the MTT assay as previously described (Mu *et al*., 2019). After transfection for 48 h, 1×10^5 cells were grown in 96 well plates and the assays were carried out in triplicate. MTT reagent (Thermo Fisher Scientific, Waltham, USA) at a concentration of 5 μg/ml was added to each well of 96 well plates containing cells (Thermo Fisher Scientific, Waltham, USA). After incubation for four hours, 150 μL DMSO was added after removing the MTT reagent. The optical density of each sample was determined at 490 nm after shaking each sample at room temperature using a microplate reader (Molecular Devices, USA). SGC-7901 cells were seeded in 96-well plates and incubated at room temperature with 5% CO_2 in a humidified atmosphere for two weeks to examine their morphology. After 48 h of transfection, cell morphology was observed. To measure the amount of absorbance, cells were seeded (2×10^5 cells per well) in a 96-well plate, and the proliferative rate of the cells was assessed. The general morphology and proliferative rate of SGC-7901 cells was observed every day with the aid of an inverted microscope (Olympus, Japan). Proliferation rates were calculated by determining the percentage change in the number of viable cells in relation to the period over which they were measured.

Assay for Luciferase reporter activity

We used Target Scan Human (http://www.targetscan.org/vert_72/), an online prediction tool for miRNA targets, to determine miR19b-3p binding targets in humans. Based on the predicted results, miR19b-3p may play a role in the regulation of ET_BR expression. Human tar-

get scanning indicated a potential binding site. RT-qPCR was used to amplify the 3'-UTR of the human ET_BR. The amplified human ET_BR sequence was cloned into a pGL3 control vector containing the XbaI site. The Q5 site-specific mutagenesis kit (Q5® Site-Directed Mutagenesis Kit; catalogue no: E0554S, NE Biolabs Inc., China) was used to generate the mutated binding site of miR19b-3p in the 3'-UTR of ET_BR following the manufacturer's protocol. Prediction validation was performed using a dual-luciferase assay (Promega, USA) and a microplate luminometer (BioTek, USA). Briefly, the ET_BR 3'-UTR with wild-type (ET_BR-wt) miR binding site or mutant-type (ET_BR/mut) miR binding site was first cloned into the pmir-GLO luciferase reporter vector. According to a previous study (L. Zhang *et al.*, 2018) prior to transfection, SGC-7901 cells were maintained in nutrient-enriched medium for 24 h in a 24-well plate. Using the HiPerFect-Transfection reagent, 0.5 μg of 3'-UTR-ET_BR with reporter vectors (1 ng/μL, WT or MUT type) and 0.5 μg miR19b-3p-mimic or control were transfected into SGC-7901 cells. To quantify the luciferase activity of the cells, host cells were isolated, washed, trypsinized for 48 h after transfection, and assayed as directed by the manufacturer using a dual luciferase assay.

Detection of endothelin B receptor mRNAs by RT-q PCR

Agilent Bioanalyzers (Agilent Technologies Co. Ltd., Beijing, China) using microfluidics to classify RNA samples based on RNA Integrity Numbers (RIN) ranging from 1 to 10. According to a study (Padhi *et al.*, 2018), a RIN of more than 8.0 indicates intact and high-quality RNA samples, a RIN between 5.0 and 8.0 indicates moderately degraded samples, and a RIN of less than 5.0 indicates degraded samples. To ensure reliable measurement of gene expression by RT-qPCR, it is generally recommended that RNA samples with a RIN value above 5.0.

As previously reported (Skovsted *et al.*, 2015), the total RNAs was extracted from normal and gastric cancer cells using TRIzol reagent. RNA extraction reagent was purchased from Dongsheng Biotech (Guangzhou, China). The extracted RNA was verified by agarose gel electrophoresis. Approximately five μg of RNA from both cell lines was reverse-transcribed using Moloney Murine leukemia virus reverse transcriptase (Sigma-Aldrich, Germany) and oligo (dT)18 primers in 20 μL reaction mixture. Then, the solution was made up to 50 μL with deionized water. For the PCR reaction, one μL of the solution was used as a substrate for PCR amplification. After 28 cycles, 25 μl of the solution was removed (91°C for 40 s, 57°C for 40 s, and 75°C for 50 s). Agarose gel electrophoresis was used to analyse approximately 10 μL of the solution containing the amplified products. PCR was carried out using pairs of primers specific for human GAPDH (5'-CATGTTCGTCATGGGGTGAACCA-3' and 5'-AGTGATGGCATGGACTGTGGTCAT-3') and ET_BR (5'-ACTGGCCATTTGGAGCTGAGAT-3' and 5'-CTGCATGCCACTTTCTTCTCAA-3'). GAPDH was used as an endogenous control for the normalization of target gene expression. Inverse logarithms of ΔΔCt values were used to calculate the relative levels of miR19b-3p. Table 1 lists the information of the primer sequence.

Western blot assay and antibodies

RIPA lysis buffer was used to collect total protein from cells. We measured the protein concentration using a BCA protein assay kit. Protein separation of proteins (30 μg) was carried out using SDS-PAGE (Bio-Rad Laboratories Co., Ltd. Shanghai, China). A polyvinylidene fluoride membrane (PVDF) was then used to transfer the proteins. A 5–6% non-fat dry milk solution was used to occlude the membranes for one hour to prevent nonspecific binding. After blocking, antibodies such as anti-ET_BR (1:200) or anti-VEGF-A (1:3000) were transferred to the membranes and allowed for incubation for 10 hours at 4°C. Subsequently, HRP-conjugated secondary antibodies (1:2500) were applied to the membranes at 37°C for 1 hour. The protein bands on the membrane bound to antibodies were detected by a chemiluminescence system (GE LAS4000 Image, Quant, CA, USA). Protein expression was analysed using ImageQuant software. The bands were detected and photographed using a chemiluminescence analyser (Biotech Co., Ltd., Beijing). Densitometry was used to quantify protein bands using Quantity One software. The difference in loading between groups was corrected by dividing the volume per individual group by the volume of normalised β-actin for each group. The differences between each group and the control group were then determined by a normalisation procedure. The results were presented as percentages based on the control data (Sun *et al.*, 2022).

Statistical analyses

A minimum of three independent analyses of each experiment were performed to achieve the most accurate results. Data were analysed using GraphPad Prism (Version 9.0) using a student's *t*-test. In this study, we used the t-test to determine whether there was a significant difference between two groups. The values of the experimental and control groups were compared using one-way ANOVA. Tukey's multiple comparison test was used to compare the groups. We define statistical significance as a p-value <0.05.

RESULTS

miR19b-3p inhibits proliferation in SGC-7901 cells

Quantitative real-time PCR findings revealed that miR19b-3p expression in SGC-7901 cells was substantially ($p<0.01$) reduced compared to GES-1 cells (Fig. 1A). To examine the role of miR19b-3p in gastric cancer, the role of miR19b-3p in SGC-7901 cells was investigated by

Table 1. List of primers used in the study

No	Primers	Sequences
1	miR19b-3p-mimic	forward: 5′-UGUGCAAAUCCAUGCAAAACUGA-3′ and reverse, 5′-AGUUUUGCAUG GAUUUGCA-CAUU-3′
2	miR19b-3p-mimic control	forward: 5′-UUCUCCGAACGUGUCACGUTT-3′, and reverse, 5′-ACGUGACACGUUCGGAGAATT-3′
3	miR19b-3p-inhibitor	forward: 5′-UCAGUUUUGCAUGGAUUUGCACA-3′ and reverse, 5′-ACGAGAAGGAUGGATTUGGAU-3′
4	miR19b-3p-inhibitor control	forward: 5′-CAGUACUUUUGUGUAGUACAA-3′ and reverse, 5′-ACGAUTTUAATGCCGUUAAUUA-3′

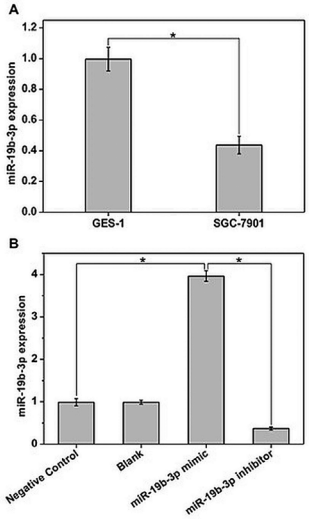

Figure 1. Downregulation of miR19b-3p in SGC-7901 cells.
(**A**) The miR19b-3p expression in normal gastric epithelial cells and gastric cancer cell line detected by RT-qPCR. (**B**) The miR19b-3p expression after transfection with negative control, blank, miR19b-3p mimic, and miR19b-3p inhibitor in SGC-7901 cells. *p<0.01.

transfection with miR19b-3p-mimic, a miR19b-3p inhibitor or negative control in SGC7901 cells and transfection efficiency was measured by RT-qPCR. Figure 1B shows that miR19b-3p mimics markedly elevated miR19b-3p expression levels (p<0.01) in SGC-7901 cells, while it decreased dramatically (p<0.01) with the miR19b-3p inhibitor transfected into SGC -7101 cells.

The morphology of SGC-7901 cells treated with a miR19b-3p mimic or its inhibitor or NCs was examined under inverted microscopy (Fig. 2A–E). Cells in control groups (NCs) appeared round shaped and were densely packed. In the miR19b-3p mimic group, the cells showed a gradual rounding with significantly less densely packed compared to those seen in the control groups. Furthermore, an increased number of floating cells in the miR19b-3p mimic-group indicated a decrease in cell viability. These findings indicated that SGC-7901 cells showed a significant decrease (p<0.01) in their viability in the presence of a miR19b-3p mimic, but viability was significantly restored (p<0.01) in response to miR19b-3p inhibitor. Cell proliferation analysis studied by the MTT assay showed the substantial (p<0.05) inhibitory effects (p<0.05) of miR19b-3p mimic on cell viability compared to negative controls, as shown in Fig. 2F.

The expression level of miR19b-3p was reversely correlated with the expression of the endothelin B receptor in SGC-7901 cells

To study the function of miR19b-3p in the angiogenesis of gastric cancer cells, the relationship between miR19b-3p and ET_BR was studied. The relationship between angiogenesis and endothelin receptor B is well documented. Endothelin-1 (ET-1) and its two receptors, endothelin receptor A (ET_AR) and ET_BR, are overexpressed in tumour cells. ET_BR has been reported to be a key regulator of tumour angiogenesis by being directly involved in VEGF-A expression. Changes in miR19b-3p expression levels and its effect on ET_BR expression were evaluated in SGC-7901 cells. We used Western blot to study ET_BR expression in SGC-7901 cells and GES-1cells. According to our findings, higher ET_BR levels were found in cancerous gastric cells compared to normal cells (Fig. 3A). Based on the results, it was evident that ET_BR was negatively correlated with miR19b-bp expression, as shown in Fig. 3B (p<0.01). From these results, we hypothesise that miR19b-3p might negatively regulate ET_BR expression in gastric cancer cells.

ET_BR is one of the target genes for miR19b-3p

To identify putative targets for miR19b-3p, we used an online bioinformatics tool, Target Human Scan. Figure 4A shows that ET_BR is presumed to be the target

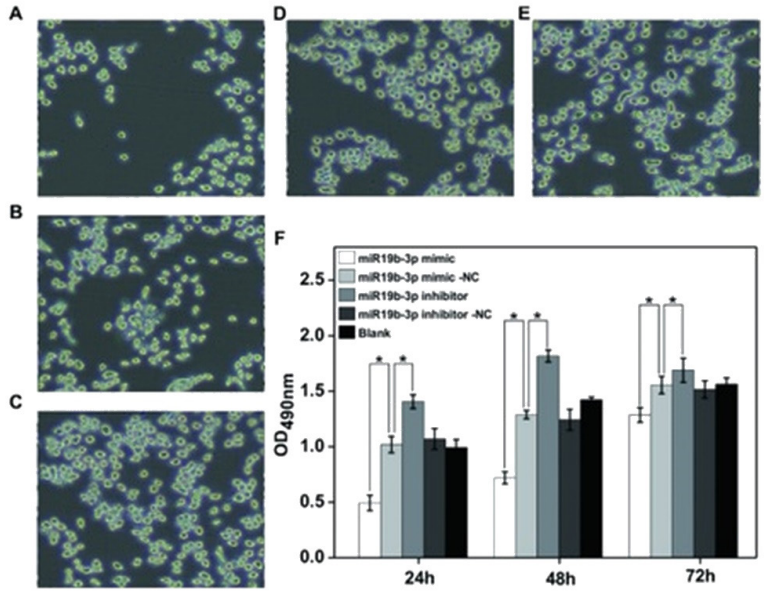

Figure 2. Transfection of miR19b-3p mimics decreases SGC-7901 cell proliferation.
(**A**) Observation of cell morphology under inverted microscope. The cells transfected with miR19b-3p mimic. (**B**) Cells transfected with miR19b-3p mimic negative control. (**C**) Cells transfected with miR19b-3p inhibitor. (**D**) Cells transfected with miR19b-3p inhibitor negative control. (**E**) Cells without transfection (blank). (**F**) Cell proliferation by MTT assay of various transfected groups at 24, 48 and 72 h. *p<0.01.

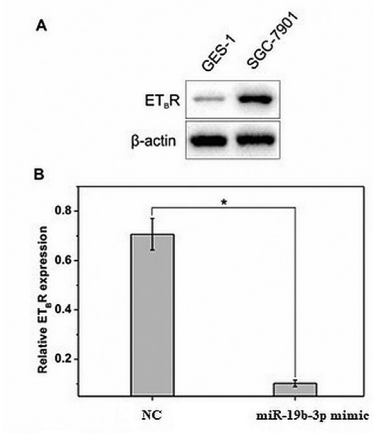

Figure 3. Enhanced expression of ETBR in human gastric cancer cells compared to normal gastric epithelial cells showing reverse correlation to miR19b-3p expression.
(**A**) The results from Western blot showing the relative ETBR expression. (**B**) Changes in the expression of ETBR after transfecting with miR19b-3p mimic or negative control into SGC-7901 cells. The data is presented from the average of three independent experiments. *p<0.01.

site for miR19b-3p and the possible binding site between miR19b-3p and the predicted 3'-UTR of ET_BR. We used a luciferase reporter assay to verify whether miR19b-3p plays a role in ET_BR regulation through the binding site at the 3'-UTR of ET_BR. The predicted miR19b-3p binding site was cloned along with its wild- or mutant-type ET_BR. SGC-7901 cells were transfected with wild ET_BR or mutant ET_BR together with precursor and control. Cell luciferase transfection was performed 48 h after cell harvesting followed by total protein extraction. Figure 4B shows the significant expression of ET_BR in SGC-7901 cells compared to the reporter vector. However, a luciferase reporter assay was performed to identify aberrant cells in SGC-7901 cells. It was determined that luciferase activity decreased significantly (p<0.01, Fig. 4C) when miR-196-5p mimics were co-transfected

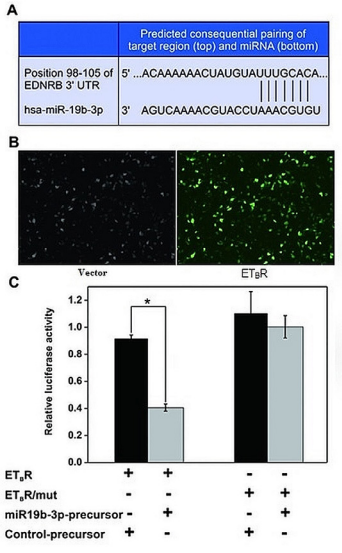

Figure 4. The ETBR is a target gene of miR19b-3p.
(**A**) Predicted binding site for miR19b-3p in ETBR. (**B**) Luciferase reporter assay for ETBR in human gastric cancer cells, SGC-7901. (**C**) Down-regulation of miR19b-3p precursor affect luciferase activity by ETBR 3′ UTR in wild type but no effect was noticed on luciferase activity in mutant ETBR 3′ UTR. The data presented is the average of three independent experiments. *p<0.01.

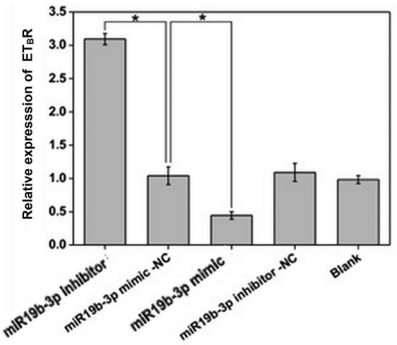

Figure 5. Effect of miR19b-3p on ETBR relative expression.
RT-qPCR results show decrease in the expression of ETBR with miR19b-3p mimic and increased expression of ETBR with miR19b-3p inhibitor when compared with miR19b-3p mimic negative control in the transfected SGC-7901 cells. *p<0.01.

with luciferase plasmids containing ET_BR 3' UTRs with native binding sites. Figure 4C shows that the expression of the miR19b-3p precursor with ET_BR wild type significantly reduced luciferase activity.

Inhibition of ET_BR expression in gastric cancer cells by restoring miR19b-3p expression

To study the role of ET_BR regulation in human gastric cancer, SGC-7901 cells were transfected with miR19b-3p mimic, miR19b-3p inhibitor, miR19b-3p mimic negative control, miR19b-3p inhibitor negative control or mock transfection. Real-time qPCR was performed to measure relative expression between the various groups of transfected cells. Figure 5 shows that ET_BR expression in the miR19b-3p mimic group was substantially decreased (p<0.01) whereas the expression was significantly restored when treated with miR19b-3p inhibitor relative to the control group.

Furthermore, we studied ET_BR expression levels in SGC-7901 cells with Western blot experiments. As shown in Fig. 6, ET_BR expression was reduced in the miR-19b-3p mimic group and elevated with the miR-19b-3p inhibitor, thus restoring its expression.

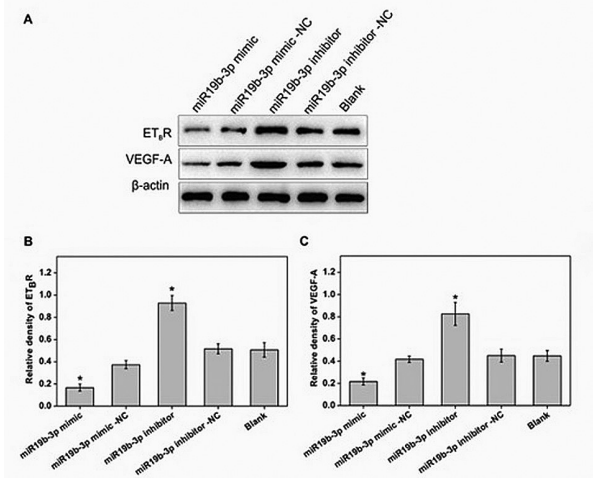

Figure 6. The miR19b-3p overexpression regulates ETBR expression in SGC-7901 cells using Western blot analysis.
(**A**) Western blot analysis of ETBR and VEGF-A expressions. (**B**) Relative densitometry analysis of ETBR analysis after transfecting with miR19b-3p mimic or inhibitor or controls or blank in SGC-7901 cells. (**C**) Relative densitometry analysis of VEGF-A after transfecting with miR19b-3p mimic or inhibitor or controls or blank. The data presented is an average of three independent experiments. *p<0.01.

The study aims to confirm the function of ET_BR in cancer angiogenesis, and we also studied the expression level of VEGF-A along with ET_BR. The results showed that the increase in ET_BR regulation is positively correlated with VEGF-A expression. When miR19b-3p expression increased, ET_BR and VEGF-A expression levels decreased (Fig. 6, $p<0.01$). The results showed that miR19b-3p played an active role in gastric cancer angiogenesis by regulating ET_BR expression, which is positively correlated with VEGF-A.

DISCUSSION

The miRNA has been shown to play a key role in tumour progression by regulating various genes after transcription. It is identified as a potential marker for cancer diagnosis and treatment (Blandino *et al*., 2014). miR19b-3p has been shown in the regulation of various cancers (Jin *et al*., 2018; Song *et al*., 2019). Down-regulation of miR19b-3p was previously reported in patients with gastric cancer at different stages (Zhang *et al*., 2015b). The precise role of miR19b-3p in gastric cancer and its angiogenesis remains unexplored.

The study findings showed that miR19b-3p could be involved in gastric carcinoma cells and healthy gastric epithelial cells. In this study, miR19b-3p was negatively regulated in SGC-7901 cells relative to GES-1 cells, which is consistent with previous reports (Wei *et al*., 2020b). Our findings showed that miR19b-3p mimic transfections reduced the proliferation of SGC-7901 cells, indicating the role of miR19b-3p as a tumour suppressor by inhibiting the progression of gastric cancer cells. Similar results from a study were reported, but using a different miRNA in gastric cancer through EMT (epithelial-mesenchymal transitions) regulation, showing that miR-423-5p overexpression suppressed gastric cancer cell invasion, colony formation, and proliferation through the Smad3 pathway (Li *et al*., 2022). Another showed that miR19b-3p negatively controlled the expression of NRP1 by targeting its 3'-UTR, thus suppressing gastric cancer cell growth, migration, and invasion (Zhang *et al*., 2018). Previous studies have established that miR19b-3p is capable of inhibiting breast cancer cell proliferation through PI3K/Akt signaling (Jin *et al*., 2018). Reduction in the accumulation of the non-small cell lung carcinoma (NSCLC) cell line A549 was observed when miR-19b was overexpressed (Li *et al*., 2015). However, some findings have also been reported to contradict the idea that miR19b-3p promotes proliferation of NSCLC cells (Baumgartner *et al*., 2018), colon cancer (Jiang *et al*., 2017), and pancreatic cancer (Song *et al*., 2019), which contrasts with our findings. The findings imply that miR-19b-3p plays a different role due to the untargeted effect of increased expression or cell-specific effects.

Our findings showed elevated expression of ET_BR in gastric cancer cells, SGC-7901. The similar results in the study were demonstrated the elevated levels of ET-1 and ET_BR expression in pancreatic adenocarcinoma relative to normal pancreatic samples (Cook *et al*., 2015). To investigate the interaction of miR19b-3p, we used bioinformatic analysis to look for miR19b-3p target genes. The results showed that miR19b-3p interacts with ET_BR at its 3'UTR binding site. The luciferase reporter assay confirmed ET_BR as the target gene for miR19b-3p in human gastric cancer cells. The role of ET-1 and its receptors, ET_AR and ET_BR, in cancer angiogenesis has been investigated and reviewed (Olender *et al*., 2016).

Tumour angiogenesis is directly promoted by ET-1 by inducing endothelial cell survival and proliferation and by upregulating ET_BR expression that causes the progression of breast cancer, and invasion and bone metastasis of triple negative breast cancer cells (Gu *et al*., 2019). Our findings revealed that increased expression of miR19b-3p is reversely correlated with a lower expression of ET_BR, resulting in decreased angiogenesis in gastric cancer cells. When miR-19b-3p expression was restored in SGC-7901 cells, it reduced ET_BR expression and angiogenesis. Similarly, to our findings, a recent study reported that up-regulation of miR-449a-5p, miR-19b-3p, miR-223-3p, miR-511-3p, and miR-130b-3p are likely involved in flow-stimulated angiogenesis *in vivo* through modulation of the expression of the mitochondrial membrane protein SYNJ2BP, oxygenation-associated genes (HIF1A, HMOX1), cytokines (e.g., IL1A, CXCL2) as well as the embryonic transcription factor FOXC1 (Henn *et al*., 2019).

The relationship between ET_BR expression and VEGF expression was studied by Western blotting. When miR19b-3p down-regulated ET_BR expression, VEGF expression was also reduced, leading to reduced angiogenesis in gastric cancer cells. The findings showed that angiogenesis indirectly reduced the up-regulation of VEGF by ET-1 through the down-regulation of ET_BR. Our findings are supported by the study results that demonstrate inhibited angiogenesis and permeability of blood vessels through the VEGF or VEGFR2 signalling pathway by antiangiogenic medicine (asiatic acid) significantly suppresses breast cancer growth and metastasis (Tian *et al*., 2021). In another study, it was found that miR-638 overexpression inhibited the processes of tumour angiogenesis *in vitro* and *in vivo*, suppressing angiogenesis and tumour growth of hepatocellular carcinoma by inhibiting VEGF signaling (Cheng *et al*., 2016). However, there are contrasting reports that ET_BR downregulation leads to upregulation of VEGF, causing an increase in angiogenesis in human melanoma cells (Carpenter *et al*., 2003; Lahav *et al*., 2004) and ET_BR mutant rats showed an increase in VEGF expression compared to wild-type (Li *et al*., 2012).

Regardless of the role of ET_BR in suppressing or repressing VEGF, our results showed that regulation of ET_BR expression plays a considerable role in controlling angiogenesis in human gastric cancer cells. In our investigation, we also tried to find whether miR19b-3p expression directly affects VEGF expression without ET_BR regulation, but there was no significant effect on VEGF expression. This shows that miR19b-3p is not directly involved in the regulation of VEGF expression.

The role of miRNAs in modulating endothelin expression by binding to a 3 untranslated region was previously reported (Lei *et al*., 2017b; Jacobs *et al*., 2013). Several research articles have been published on the possibility of using endothelin receptors as a new approach to cancer treatment (Tocci *et al*., 2019). However, how miRNAs interact and regulate endothelin-1 and its receptors must be clarified. Our findings suggest that miR19b-3p may be able to function as a tumour suppressor by regulation of ET_BR in human gastric cancer cells. Therefore, we propose that miR19b-3p could be used to inhibit the angiogenesis and proliferation of gastric cancer.

LIMITATIONS OF THE STUDY

Our functional experiments were conducted on a single SGC-7901 cell line. In our preliminary study, SGC-

7901 demonstrated greater proliferation ability using miR19b-5p than MKN-45 or AGS.

The *in vivo* expression of miR19b-3p and its regulation in gastric cancer remain unknown. More detailed *in vivo* studies on the role of miR19b-3p in gastric cancer should be explored.

There is a need for further studies exploring the effects of miR19b-3p on cell invasion, apoptosis and apoptotic proteins including cell cycle proteins.

CONCLUSIONS

According to our findings, reduced expression of miR19b-3p was found to be instrumental in the development and angiogenesis of cancerous cells in gastric tissues. However, overexpression of miR19b-3p could significantly inhibit ET_BR expression, leading to a reduction in angiogenesis and proliferation through the reduced expression of VEGF *in vitro*. Further studies on the role of gastric cancer will help develop potential therapies for gastric cancer.

Declarations

Authors' contributions. XH and C.L designed the study plan and performed experiments. B.X and T.W drafted the original manuscript. WG and BP were involved in the formal analysis. T.W supervised the whole study.

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Conflict of interest. There is no conflict of interest to declare.

11.0 Ethics statement (including the committee approval number) for animal and human studies – Not applicable

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