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Effects of microRNA-378 on epithelial-mesenchymal transition, migration, invasion and prognosis in gastric carcinoma by targeting BMP2

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Abstract. – OBJECTIVE: Gastric cancer (GC) is a common malignancy. Recent studies have suggested that microRNAs are crucial factors in tumorigenesis. Thus, we investigated the effect of miR-378 on GC metastasis and further explored the underlying mechanism.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was carried out to measure the miR-378 expressions in GC and adjacent normal tissue samples. MiR-378 expressions in human GC cells were determined using qRT-PCR and Western blots. Moreover, transwell assays were conducted to measure the invasion and migration capacities of GC cells. Additionally, the regulating effects on BMP2 by miR-378 were assessed by luciferase reporter assays and western blots. Western blot was also carried out to observe the protein expressions of epithelial-mesenchymal transition (EMT) related genes.

RESULTS: MiR-378 expressions in GC tissues were downregulated. In the meantime, reduced miR-378 expression was associated with poor prognosis and malignant clinicopathologic features of GC patients. MiR-378 overexpression repressed GC cell invasion, migration and EMT. Furthermore, BMP2 was a direct target of miR-378 and implicated in miR-378-mediated suppressive functions in GC invasion, migration and EMT.

CONCLUSIONS: We showed that miR-378 served as a tumor suppressor in GC via modulating BMP2, suggesting that miR-378/BMP2 axis might be therapeutic targets and promising biomarkers for GC treatment.

Key Words

Gastric carcinoma, miR-378, BMP2, Epithelial-mesenchymal transition, Migration, Invasion, Prognosis.

Introduction

Gastric carcinoma (GC), a common tumor of the digestive system, has a high mortality rate[1]. Although with the in-depth studies on pathogenesis, there have been some improvements in the diagnosis and therapy of GC; however, it is still an important global health burden[2]. As GC is an asymptomatic tumor in the early stages, it is often diagnosed at advanced stages[3]. Most GC mortalities are mainly caused by metastasis of tumor cells, and additionally, the 5-year survival rate for GC is 20-30%[4]. GC is a complex tumor, which could be regulated by various tumor suppressors, or oncogenes, which are in charge of the GC initiation and progression[5]. As the progression of GC is a multifactorial and multistep process, the studies of the potential mechanisms in the process of GC progression seem to be conducive to the development of GC therapies. Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells adopt mesenchymal phenotypes and proceed to colonize[6]. EMT is implicated in the process of tumor-related metastasis[7]. EMT results in mesenchymal cells and is characterized by up-regulation of mesenchymal markers such as vimentin and decreased expressions of epithelial markers such as E-cadherin, leading to tumor metastasis and recurrence[8]. Although literature[9,10] also demonstrated the significance of EMT in GC progression, the underlying mechanism which regulates EMT is not fully understood. MicroRNAs (miRNAs) are a class of small noncoding RNAs which could partially bind to the complementary sites in the target mRNA 3'-UTRs, negatively regulating gene expressions[11]. Accumulating studies[12] have confirmed that miRNAs have important functions in cancer development, including cell metastasis, apoptosis and proliferation. In addition, recent research has provided novel insights into the correlation between multiple tumors and miRNAs. For example, miR-129-5p was found to suppress lung carcinoma cell invasion and proliferation *via* targeting vimentin, E-cadherin and microspherule protein 1[13]. Wei et al[14] found that miR-223 functioned as a tumor marker by suppressing FOXO1 in breast cancer. Lu et al[15] reported that miR-186

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inhibited tumor growth and cell proliferation in prostate cancer by modulating YY1 and CDK6. However, little is known about the roles of miR-378 in GC. Here, considering the significance of miR-378, we focused on the ectopic expressions and the regulatory mechanisms of miR-378 in GC cells. Bone morphogenetic proteins (BMPs) are cytokines, which play important roles in various cellular functions. BMP2 is the most effective inducer of osteoblast differentiation among the BMPs[16]. Accordingly, there have been many researches which focused on the effects of BMP2 on tumor progression. For instance, BMP2/BMPR1A was reported to be associated with dedifferentiated liposarcomas progression[17]. Rajski et al[18] found that response pattern of BMP2 in human lung fibroblasts was a potential marker of lung adenocarcinomas; the identification of BMP2 was an inhibitor of rhabdomyosarcoma[19]. However, the functions of BMP2 in GC have received little attention. Here, we measured BMP2 expressions and studied the correlations between miR-378 and BMP2 in GC.

Patients and Methods

Patients and Specimens

Fifty GC tissues and adjacent non-tumor tissues were collected from Shenzhen People's Hospital between July, 2015 and October, 2017. All GC patients provided written informed consent prior to the collection of tissue samples. Tissue fragments were immediately frozen in liquid nitrogen and stored at −80°C for further use. Current study was approved by the Ethic Committee of Shenzhen People's Hospital.

Cell Culture

Five human GC cell lines (BGC-823, MKN-28, MGC-803, SGC-7901and AGS) and human gastric epithelial cell line GES-1 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). All cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

Cell Transfection

MiR-378 mimics, inhibitor and scrambled miRNA control were obtained from GenePharma (Shanghai, China). BMP2 vector and siRNA were purchased from GeneCopoeia (Guangzhou, China). The transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufactures' instructions. Forty-eight hours after the transfection, the cells were harvested for further assays.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate the total RNA from GC tissues and cells. Then, TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA with isolated total RNA in line with the manufacturer's protocols. The qPCR analysis was carried out using SYBR Premix Ex Taq™ (TaKaRa Biotechnology Co., Ltd., Dalian, China) on an Applied Biosystems 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative expressions of genes were analyzed by the $2^{-\Delta\Delta CT}$ relative quantification method with human U6 as an internal control in the case of miR-378, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control for the target gene and EMT related genes. The sequences of the primers were described in Table I.

Western Blot

Iced lysis buffer containing protease and phosphatase inhibitors (Roche, Basel, Switzerland) were used to lyse GC cells and extract total proteins. Bicinchoninic acid (BCA) Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA.) was used to detect the concentrations of total proteins. Next, protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk at room temperature in Tris buffered saline and Tween (TBST) for 2 hours and then incubated overnight at 4°C with the respective primary antibodies against BMP2 (1:1000, ab14933, Abcam, Cambridge, MA, USA), E-cadherin (1:1000, ab40772, Abcam, Cambridge, MA, USA), Vimentin (1:1000, ab45939, Abcam, Cambridge, MA, USA) and GAPDH (1:1000, ab9485, Abcam, Cambridge, MA, USA). Membranes were then exposed to the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000, ab6721, Abcam, Cambridge, MA, USA) at room temperature for 2 h.

Table I. Primer sequences for qRT-PCR

Primer	Sequence
miR-378 forward	5'-GGGACTGGACTTGGAGTCA-3'
miR-378 reverse	5'-GTGCGTGTCGTGGAGTCG-3'
U6 forward	5'- CTCGCTTCGGCAGCACA-3'
U6 reverse	5'- AACGCTTCACGAATTTGCGT-3'
BMP2 forward	5'-AAGCGTCAAGCCAAACACAAAC-3'
BMP2 reverse	5'-GCCACGATCCAGTCATTCCAC-3'
GAPDH forward	5'- GGAGCGAGATCCCTCCAAAAT-3'
GAPDH reverse	5'- GGCTGTTGTCATACTTCTCATGG-3'
E-cadherin forward	5'-GCTGCTGCAGGTCTCCTCTTG-3'
E-cadherin reverse	5'-CCTTTGTCGACCGGTGCAATCT-3'
vimentin forward	5'-AGCTGCAGGCTCAGATTCAGGA-3'
vimentin reverse	5'-CGGTTGGCAGCCTCAGAGAGGT-3'

U6: small nuclear RNA, snRNA
BMP2: Bone morphogenetic protein 2
GAPDH: glyceraldehyde-3-phosphate dehydrogenase

An enhanced chemiluminescence system (Thermo Fisher Scientific, Waltham, MA, USA) was then used to visualize the protein bands. GAPDH was used as an internal control.

Transwell Assays

Cell invasion and migration of GC cells were assayed by a transwell chamber (8.0 μm pore size, Corning Incorporated, Corning, NY, USA). Transwell chamber coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used for invasion assay, while transwell chamber without Matrigel were used for the migration assay. GC cells resuspended in serum-free medium were seeded into the top chamber of the transwell inserts while the medium containing 10% fetal bovine serum (FBS) was placed in the bottom chambers. The cells were incubated at 37°C for 48 h in a 5% CO_2 atmosphere. After that, cells remaining on the top surface were removed carefully using cotton swabs when the cells adhered to the bottom surface were fixed (10% methanol, 37°C, 15 min) and stained (0.1% crystal violet, 37°C, 10 min) to detect the results under an inverted Microscope (Olympus, Tokyo, Japan) From Five Randomly Selected Visual Fields.

Luciferase Reporter Assay

Wild-type BMP2 3'-UTR containing target sites of miR-378 or the mutated BMP2 3'-UTR was inserted into the pGL3 plasmids (Promega, Madison, WI, USA), to get the wild-type BMP2-3'UTR or mutant BMP2-3'UTR, respectively. GC cells were cotransfected with wild-type or mutant 3'-UTR of BMP2 along with miR-378 mimics. At 48 h after co-transfection, the luciferase activities for the wild-type or mutant BMP2 3'-UTR was determined by luciferase reporter assays (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) statistical software, version 17.0 (SPSS Inc., Chicago, IL, USA) with Student's *t*-test or ANOVA and Scheffe post-hoc test. $p<0.05$ was considered statistically significant difference. Correlation between mRNA and miRNA were estimated using the Spearman's correlation method. Survival rate was assessed by the Kaplan-Meier method, and survival curves were compared by the log-rank test.

Results

Expression Levels of miR-378 and BMP2 in GC

To examine miR-378 expressions in GC, firstly, we measured the expressions of miR-378 in GC tissues and adjacent normal tissues. Compared to adjacent non-tumor tissues, lower miR-378 expressions were found in GC tissues (Figure 1A). Then, we further detected the miR-378 expressions in the GES-1 and five GC cells by qRT-PCR. The data also demonstrated that the miR-378 expressions in GC cells were significantly reduced (Figure 1B). In addition, the expressions of BMP2 in the GC cells were also measured and the results demonstrated higher expression levels of BMP2 in GC cells than the normal GES-1

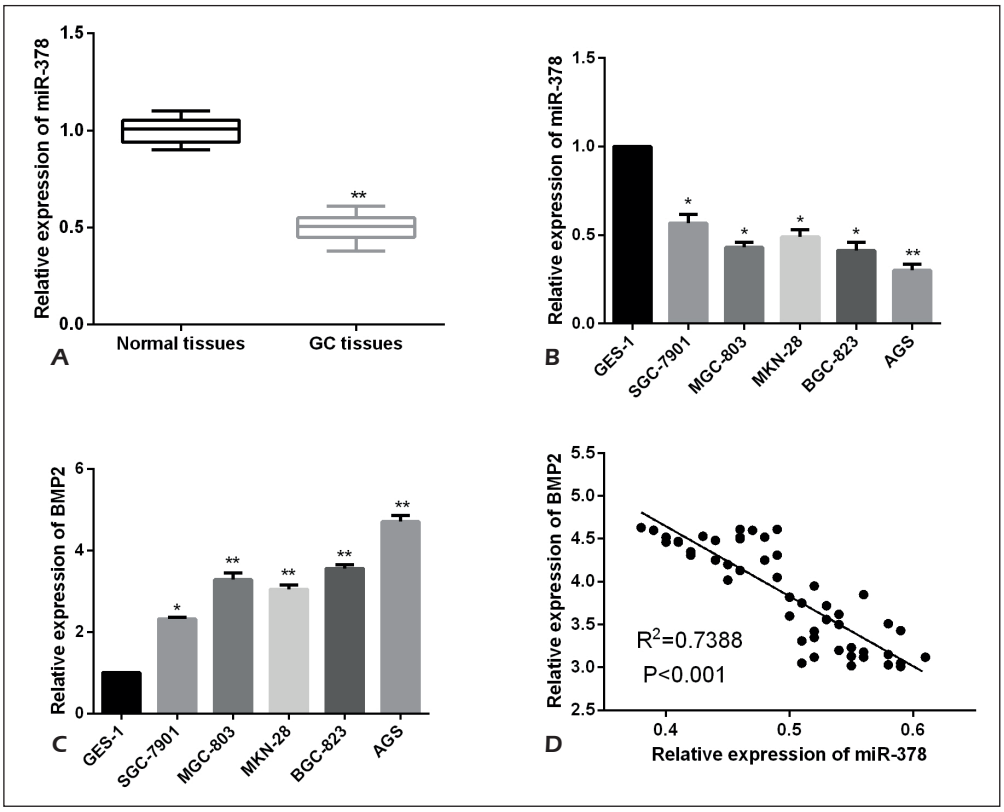

Figure 1. The expressions of miR-378 and BMP2 in GC. **A**, qRT-PCR results of miR-378 expressions in GC tissue samples and matched non-tumor tissues. **B**, qRT-PCR results of miR-378 expressions in GC cells. **C**, Relative expressions of BMP2 in GC cells. **D**, miR-378 expressions were negatively correlated with BMP2 expressions in GC tissues. *p<0.05, **p<0.01.

cell (Figure 1C). Furthermore, we analyzed the correlation between BMP2 and miR-378 in GC tissues. As expected, the data revealed that, in GC tissues, miR-378 expressions were negatively correlated to the BMP2 expressions (Figure 1D).

MiR-378 and BMP2 Expressions Were Correlated With Prognosis and Clinicopathological Features of GC Patients

As we confirmed miR-378 and BMP2 expressions in GC, subsequently, we further studied the clinical significance about miR-378 as well as BMP2 in GC. Firstly different subgroups were plotted according to miR-378 expressions, the mean level of which was defined as the cutoff. Clinical association analysis (Table II) revealed that the decreased miR-378 expressions were related to advanced TNM stage (p=0.0025), poor tumor differentiation (p=0.0013) and lymph node metastasis (p=0.0011). Furthermore, Kaplan-Meier analysis indicated that GC patients with relatively lower miR-378 expressions had significantly decreased OS (Figure 2A, p=0.0121) and DFS (Figure 2B, p=0.0157). In addition, BMP2 high-expressing GC patients showed a shorter OS and DFS compared to BMP2 low-expressing cases (Figure 2C and 2D). The findings indicated that miR-378 and BMP2 may function as potent biomarkers for predicting GC patient prognosis.

MiR-378 Overexpression Inhibited Invasion and Migration in GC Cells

As described in Figure 1B, endogenous miR-378 expression in AGS cells was the lowest while the one in SGC-7901 cells was the highest. Therefore, AGS and SGC-7901 cells were selected for overexpression assay and knockdown assay respectively. As examined by qRT-PCR, miR-378 was effectively upregulated in AGS (Figure 3A) or downregulated in SGC-7901 cells (Figure 3B). Transwell assays revealed that, upon miR-378 restoration, AGS presented dramatically decreased invasion and migration capacities (Figure 3C). On the other hand, miR-378 inhibition facilitated SGC-7901 cell migration and invasion (Figure 3D).

Table II. Correlation of miR-378 expression with the clinicopathological characteristics of the GC patients.

Clinicopathological features	Cases (n=50)	miR-378[a] expression		*p*-value
		High (n=20)	Low (n=20)	
Age (years)				0.2417
>60	26	10	16	
≤60	24	10	14	
Gender				0.4011
Male	26	9	17	
Female	24	11	13	
Tumor size (cm)				0.0814
≥ 5.0	23	5	18	
<5.0	27	15	12	
TNM stage				0.0105*
I-II	25	16	9	
III	25	4	21	
AFP (ng/mL)				0.2794
<400	27	12	15	
>400	23	8	15	
Tumor differentiation				0.0014*
Well and Moderate	23	15	8	
Poor	27	5	22	
Lymph node metastasis				0.0017*
Present	25	4	21	
Absent	25	16	9	

TNM: tumor-node-metastasis;
AFP: alpha-fetoprotein;
[a]The mean expression level of miR-378 was used as the cutoff
*Statistically significant.

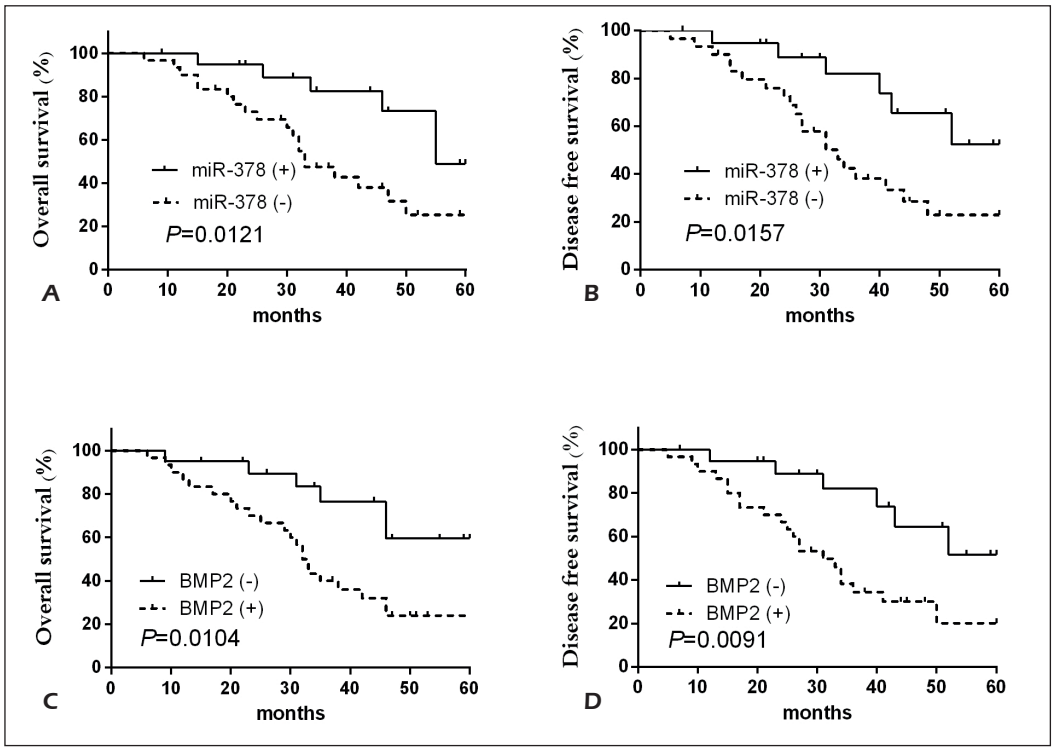

Figure 2. The influence of miR-378 and BMP2 on survival rate for GC patients. **A**, OS and **B**, DFS of GC patients with high and low miR-378 expressions. **C**, OS and **D**, DFS of GC patients with high and low BMP2 expressions.

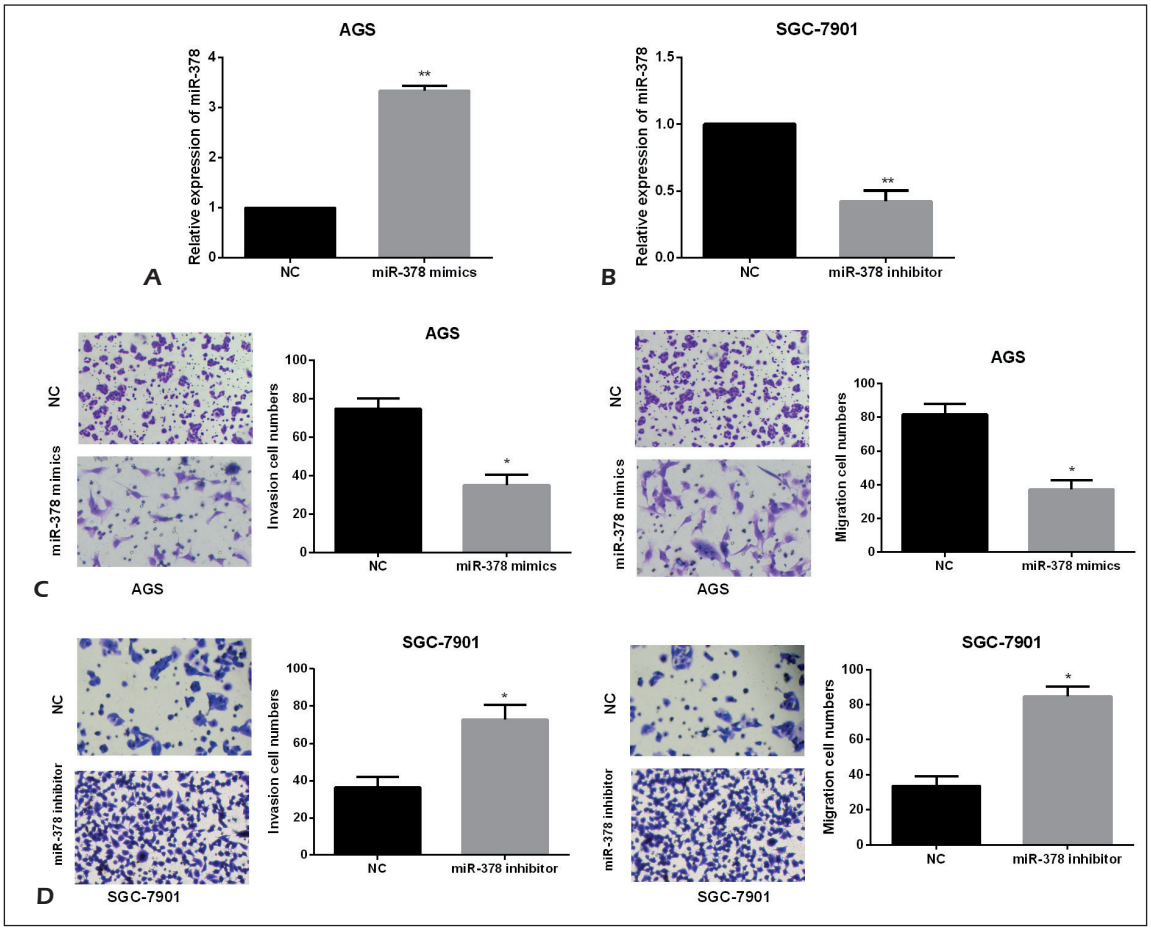

Figure 3. MiR-378 inhibited GC cell invasion and migration. **A**, The miR-378 expression in AGS transfected with miR-378 mimics. **B**, miR-378 expressions in SGC-7901 with transfection of miR-378 inhibitor. **C-D**, The invasion and migration abilities of AGS or SGC-7901 cells with transfection of miR-378 mimics or inhibitor were measured by transwell assays. *$p<0.05$, **$p<0.01$.

MiR-378 Suppressed GC Cell EMT

We next investigated the regulation of miR-378 on GC EMT to explore the potential mechanism implicated in the anti-metastatic functions of miR-378 using qRT-PCR and Western blots analysis. As expected, miR-378 overexpression resulted in elevated E-cadherin expressions and reduced Vimentin expressions in AGS (Figure 4A). Results of western blot also verified the alterations of Vimentin and E-cadherin expressions with miR-378 restoration (Figure 4B). On the other hand, miR-378 inhibition promoted SGC-7901 cell EMT with elevated Vimentin and declined E-cadherin expressions, as determined by qRT-PCR and western blot results (Figure 4C and 4D). The data demonstrated that miR-378 inhibited GC cell EMT.

BMP2 was a Downstream Target of miR-378 in GC Cells

To explore the underlying molecular mechanism of miR-378 in GC, TargetScan was used to predict the potential target of miR-378. The data showed that BMP2 had complementary binding sites for miR-378 (Figure 5A). Then, we performed luciferase assays to validate that BMP2 was a direct target of miR-378. The results indicated that miR-378 overexpression notably decreased the wild-type (WT) BMP2 3'-UTR luciferase activity while had no influence on the mutant (MUT) BMP2 3'-UTR luciferase activity (Figure 5B). Additionally, miR-378 overexpression significantly reduced the BMP2 expressions in AGS cells (Figure 5C). On the contrary, the expression of BMP2 was significantly elevated by the downregulation of miR-378 in SGC-7901 (Figure 5 D).

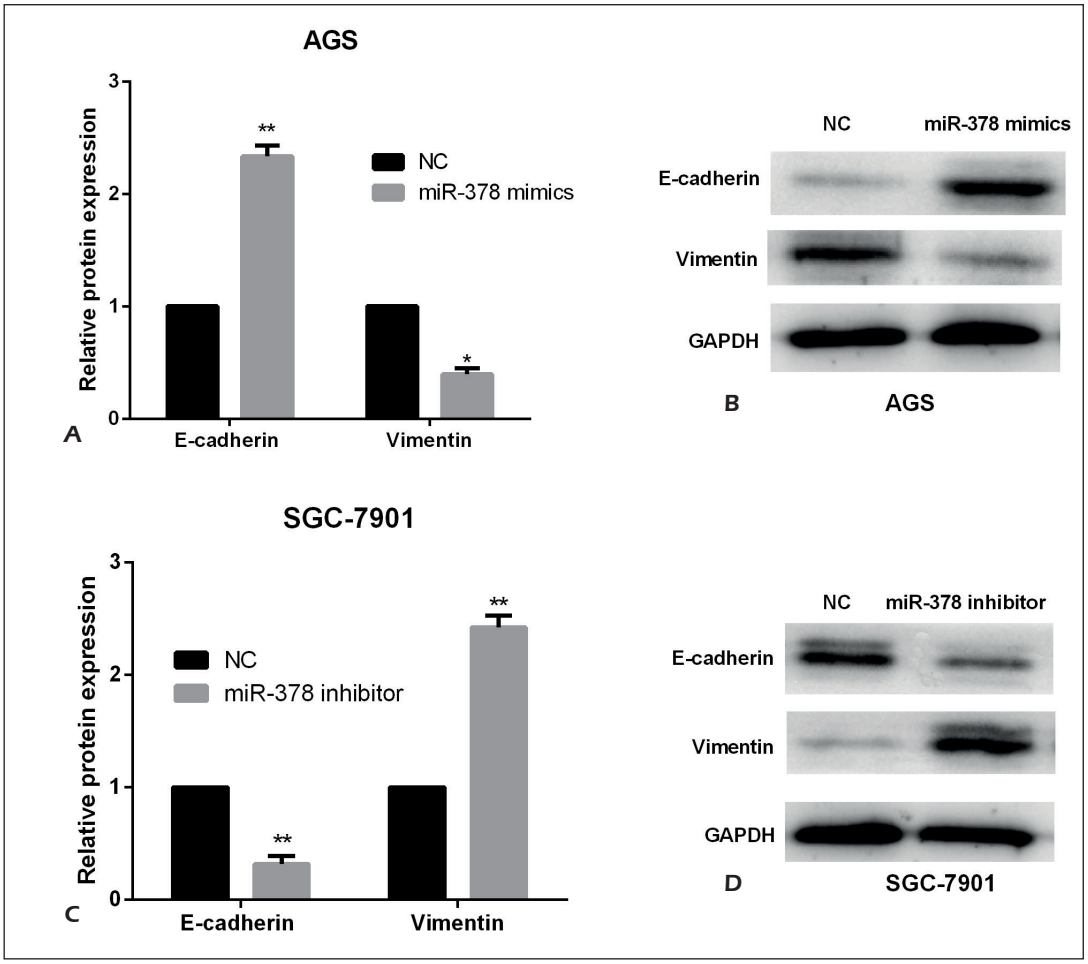

Figure 4. MiR-378 suppressed GC cell EMT. **A-B**, miR-378 overexpression significantly increased the expression of E-cadherin while decreased the expression of vimentin in AGS cells. **C-D**, miR-378 downregulation significantly decreased the expression of E-cadherin while increased the expression of vimentin in SGC-7901. *p<0.05, **p<0.01.

BMP2 Was Implicated in miR-378-Induced Effects on GC Cell Migration and Invasion

Rescue experiments were carried out to confirm whether miR-378 regulated GC metastasis by targeting BMP2. BMP2 overexpression vector was transfected into miR-378 overexpressed AGS cells, which was treated with miR-378 mimics. The transfection efficiencies were confirmed by performing qRT-PCR and Western blots. Results indicated that BMP2 expression was dramatically increased in miR-378 overexpressed AGS cells (Figure 6A). In the meantime, transwell assays revealed that BMP2 restoration could reverse the suppressive functions of miR-378 in AGS cell invasion and migration (Figure 6B). On the other hand, BMP2 siRNA was transfected into miR-378 suppressed SGC-7901 cells (Figure 6C). Inhibition of BMP2 prevented the promoting effects on SGC-7901 cell migration and invasion mediated by miR-378 knockdown (Figure 6D).

BMP2 Regulated the Functions of miR-378 in GC EMT

We further explored the effects of BMP2 on mediating the functional roles of miR-378 in EMT of GC cells. As expected, the E-cadherin expression was dramatically declined and the Vimentin expression was significantly enhanced in miR-378 overexpressed AGS cells by BMP2 restoration (Figure 7A and 7B). Furthermore, knockdown of BMP2 abrogated the functions of miR-378 inhibitor in SGC-7901 cell EMT (Figure 7C and 7D). Hence, the results showed that miR-378 inhibited GC EMT by regulating BMP2.

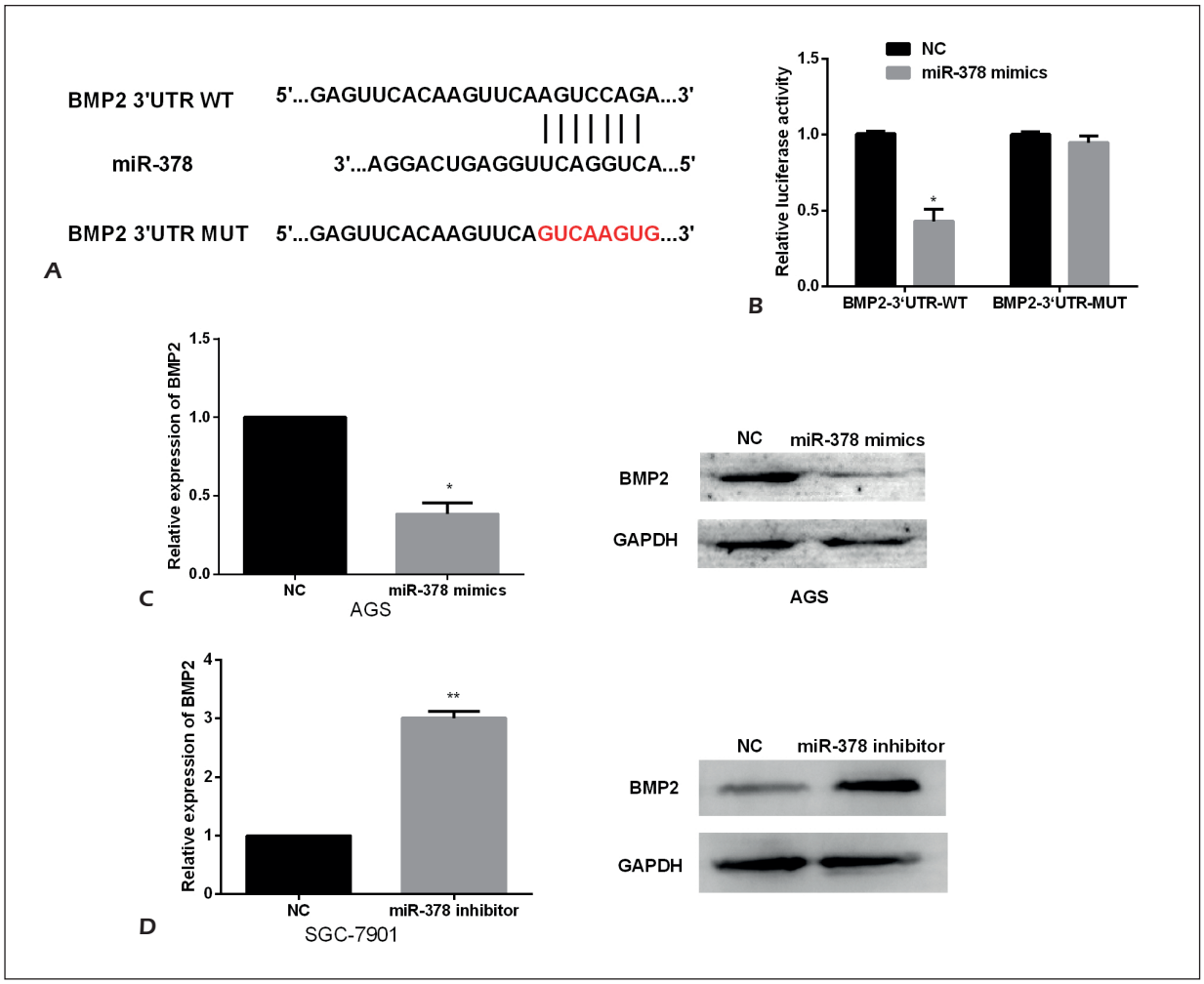

Figure 5. BMP2 was a direct target of miR-378 in GC. **A**, The putative miR-378-binding sequence in BMP2 3'-UTR. **B**, The relative luciferase activities of GC cells which were co-transfected with WT or MUT BMP2 3'UTR and miR-378 mimics were detected. **C-D**, The relative BMP2 expressions in AGS or SGC-7901 cells were detected after cells were treated with miR-378 mimics or inhibitor respectively. *p<0.05, **p<0.01.

Discussion

GC is one frequent tumor all around the world and its prognosis is poor because of lack of effective therapies and late detections. In recent years, the significance of the tumor microenvironment in regulating tumor growth and progress has received extensive attentions[20-22]. EMT is a fundamental, complex and developmental, process that is crucial for pathophysiological events, especially for metastasis and progression of tumors[23]. In this study, we clinically studied the significance of miR-378 and BMP2 in the regulation of GC EMT and metastasis. Increasing evidence has revealed that the abnormal miR-378 expressions play critical roles in different malignancies. For example, studies by Li et al[24] revealed that miR-378 repressed glioma cell invasion and correlated with prognosis of glioma patients. Qian et al[25] reported that miR-378 overexpression may affect the treatment outcomes of acute myeloid leukemia patients. In current research, miR-378 expressions were prominently lower in GC tissues when compared to the adjacent normal tissues. Reduced miR-378 expressions were found to be related to poor OS as well as DFS and worse clinicopathological parameters of GC patients. Functional studies showed that GC cell invasion and migration capacities were suppressed by miR-378 overexpression. Furthermore, miR-378 overexpression inhibited GC cell EMT. Therefore, all the data

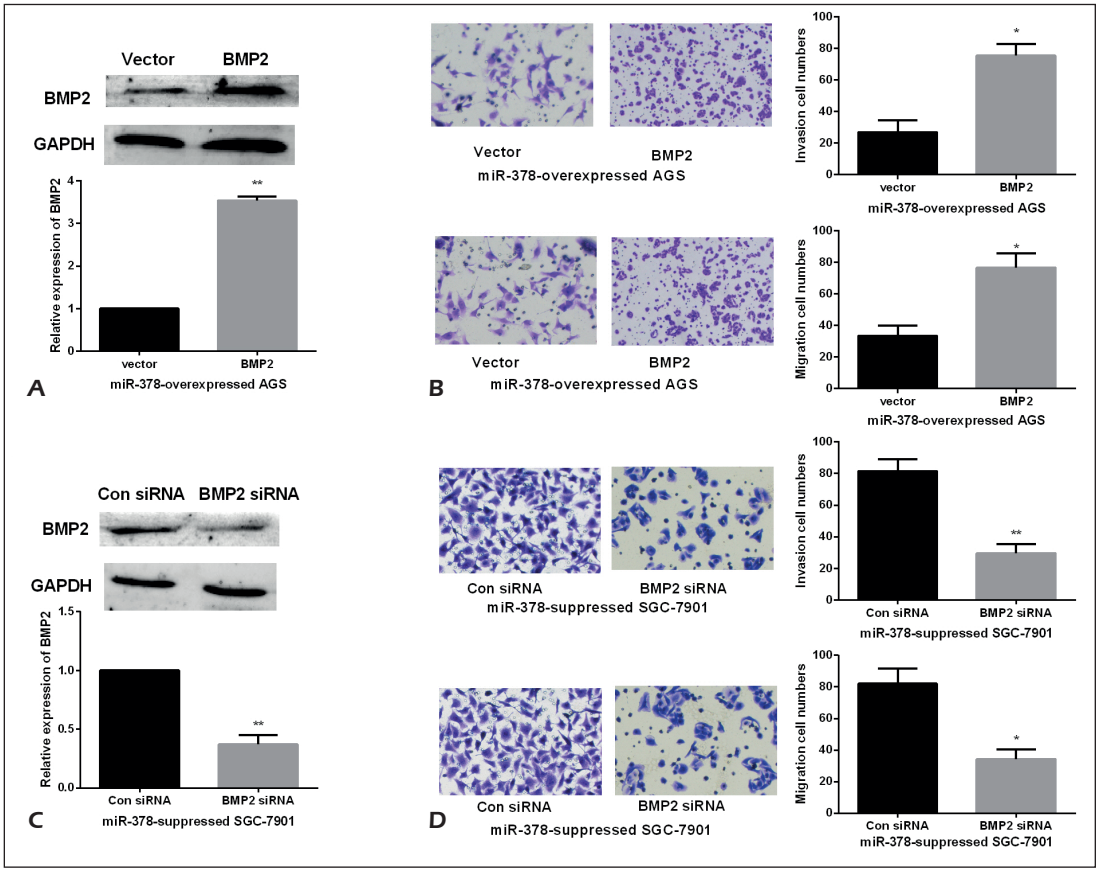

Figure 6. BMP2 was implicated in miR-378-induced effects on GC cell invasion and migration. **A**, The relative BMP2 expressions in miR-378-overexpressed AGS cells that were transfected with BMP2 overexpression plasmid. **B**, Cell invasion and migration abilities of miR-378-overexpressed AGS cells, which were cotransfected with BMP2 overexpression plasmid, were determined by transwell assays. **C**, BMP2 expressions in miR-378-suppressed SGC-7901 cells, which were cotransfected with BMP2 siRNA. **D**, Transwell assays were performed to determine the migration and invasion capacities of miR-378-suppressed SGC-7901, which was cotransfected with BMP2 siRNA. *$p<0.05$, **$p<0.01$.

suggested that miR-378 may be a biomarker for GC treatment, acting as a tumor suppressor in GC. BMP2 acts as the key regulator of bone formation and repair, embryogenesis and osteogenic differentiation[26,27]. BMP2 has recently been identified as a crucial regulator in tumor progression, including colorectal cancer[28], breast cancer[29] and nasopharyngeal carcinoma[30]. In our research, results indicated that elevated BMP2 expressions may be prognostic indicators for GC patients. Moreover, our data from qRT-PCR, luciferase assays and western blots solidly demonstrated that BMP2 was a direct target of miR-378 in GC cells. Importantly, rescue functional assays revealed that the suppressive functions of miR-378 in GC cell metastasis and EMT were regulated by BMP2. These data indicated that miR-378 suppressed GC cell metastasis and EMT *via* targeting BMP2.

Conclusions

MiR-378 was down-regulated in GC cells and tissue samples. Decreased miR-378 expression was relevant to the poor prognosis and malignant clinicopathological features of GC patients. Moreover, miR-378 overexpression could suppress GC cell invasion, migration and EMT. Furthermore, we detected that BMP2 was a target of miR-378 in GC, and mediated the functions of miR-378 in GC metastasis and EMT. The above data demonstrated that miR-378/BMP2 axis was attractive biomarkers and therapeutic targets for GC patients.

Conflict of Interests

The authors declared no conflict of interest.

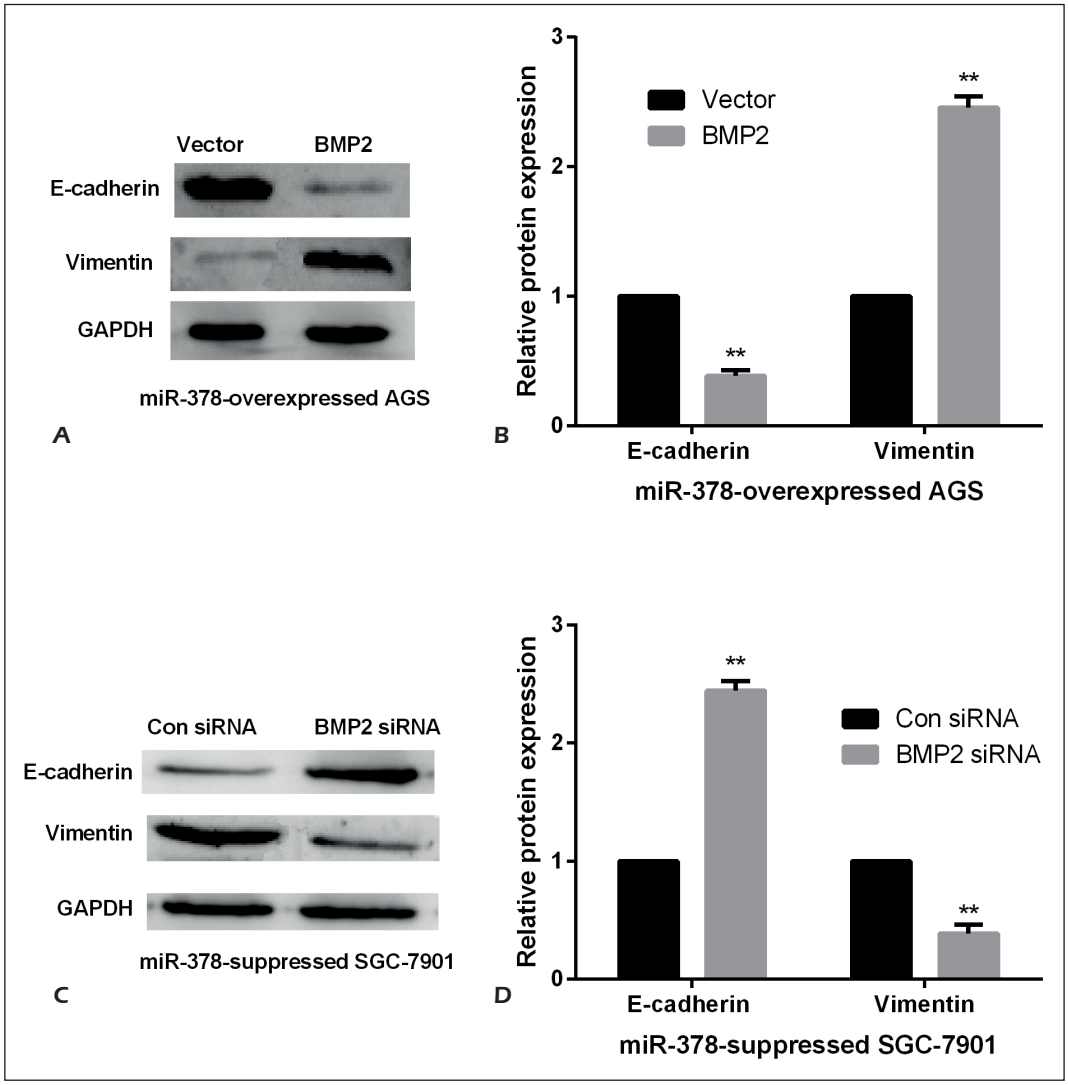

Figure 7. BMP2 regulated the functions of miR-378 in GC EMT. **A**-**B**, BMP2 overexpression in miR-378-overexpressed AGS cells declined the E-cadherin expression and elevated the Vimentin expression. **C**-**D**, BMP2 knockdown in miR-378-suppressed SGC-7901 increased the E-cadherin expression and reduced the Vimentin expression. $^{**}p<0.01$.

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