MicroRNA 495 Inhibits Proliferation and Metastasis and Promotes Apoptosis by Targeting Twist1 in Gastric Cancer Cells

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Recently, microRNAs (miRNAs) have been reported to participate in multiple biological processes. However, the effects of miR-495 on gastric cancer (GC) remain unclear. The purpose of this study was to explore the functions of miR-495 in GC cell proliferation, metastasis, and apoptosis. SGC-7901 and BGC-823 cell lines were transfected with miR-495 mimic, miR-495 inhibitor, and negative controls (mimic control and inhibitor control). The expressions of miR-495, cell viability, migration, apoptosis, and apoptosis-related factors were examined by qRT-PCR, trypan blue staining, Transwell, flow cytometry, and Western blot, respectively. Simultaneously, key factor expression levels of EMT were detected by qRT-PCR and Western blot. The direct target of miR-495 was confirmed by dual-luciferase assay. Additionally, sh-Twist1, pc-Twist1, and corresponding controls were transfected into SGC-7901 and BGC-823 cells, and the protein levels of EMT-associated factors were detected by Western blot. miR-495 was downregulated in GC cells. miR-495 expression level was effectively overexpressed or suppressed in SGC-7901 and BGC-823 cells. Overexpression of miR-495 significantly decreased cell viability and migration, increased apoptosis, and inhibited the EMT process. Suppression of miR-495 showed contrary results. Twist1 was clarified as a target gene of miR-495, and Twist1 silencing obviously reduced the promoting effect of miR-495 suppression on these biological processes. Twist1 silencing significantly blocked the EMT process in both SGC-7901 and BGC-823 cells. miR-495 inhibited proliferation and metastasis and promoted apoptosis by targeting Twist1 in GC cells. These data indicated that miR-495 might be a novel antitumor factor of GC and provide a new method for the treatment of GC.

Key words: Gastric cancer (GC); MicroRNA-495; Proliferation; Apoptosis; Epithelial–mesenchymal transition (EMT); Twist1

INTRODUCTION

Gastric cancer (GC) is a common malignant tumor and is the second leading cause of mortality after lung cancer in the world[1]. More than 70% of all new cases of GC occur in developing countries, especially in China. Approximately 400,000 people have been diagnosed with GC, and the mortality rate is as high as 70%–75% annually in China[2,3]. Although the standard of diagnosis and treatment of GC have continuously improved, the percentage of 5-year survival is still unsatisfactory[4]. The mechanism of GC is complex and multifactorial, and many factors are implicated in these processes[5]. Due to lack of adequate elucidation of the key mechanisms of tumor development and metastasis, there is still a great obstacle for the treatment of GC[6]. Therefore, the further investigation of the effective diagnostic and therapeutic methods of GC is urgently necessary.

MicroRNAs (miRNAs), a kind of small noncoding RNAs that are 20–24 nucleotides in length, have opened a new approach as tumor biomarkers for early cancer diagnosis[7]. In recent years, accumulating evidence has demonstrated that multiple miRNAs are closely related to the occurrence, development, and metastasis of GC[8,9]. For example, miR-233 was found to promote cell invasion and metastasis by targeting EPB41L3 in GC[10]. Furthermore, miR-146a was downregulated in GC and inhibited cell proliferation and induced apoptosis[11]. Xia et al. reported that miR-362 could induce cell proliferation and suppress apoptosis in GC by activation of the nuclear factor B (NF- B) signaling pathway[12].

In previous studies, miR-495 has been reported as a tumor suppressor in acute myeloid leukemia (AML)[13]. However, the roles of miR-495 in GC have not been fully reported.

In the present study, we aimed to explore the effect of miR-495 in GC cell proliferation, metastasis, and apoptosis. The human GC cells SGC-7901 and BGC-823 were transfected with miR-495 mimic, miR-495 inhibitor, sh-Twist1, and pc-Twist1 to regulate miR-495 or Twist1 expressions. Cell viability, migration, apoptosis, and apoptosis-related factors were detected by qRT-PCR, trypan blue staining, Transwell, flow cytometry, and Western blot, respectively. Simultaneously, the expression of key factors in epithelial-mesenchymal transition (EMT) was detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The direct target gene of miR-495 was confirmed by dual-luciferase assay. Our study might provide a new therapeutic method for GC.

MATERIALS AND METHODS

Cell Culture

The four human GC cell lines SGC-7901, BGC-823, MGC803, and AGS, and the human fetal gastric epithelial cell line GES-1 were obtained from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, P.R. China). These cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco BRL, Gaithersburg, MD, USA) that contained 10% fetal bovine serum (FBS; Gibco BRL) and 1% antibiotic antimycotic (Gibco BRL), at 37°C in an atmosphere of 5% CO_2 and 95% air. In addition, 10 ng/ml of transforming growth factor-β (TGF-β) was used for inducing the EMT process.

Cell Transfection

SGC-7901 and BGC-823 cells were incubated in six-well plates for 24 h at 37°C. Then miR-495 mimic, miR-495 inhibitor, mimic control, and inhibitor control were synthesized by GenePharma Co. (Shanghai, P.R. China) and transfected into these cell lines. To explore the functions of Twist1, the full-length Twist1 sequences and short hairpin RNA directed against Twist1 were constructed in pcDNA3.1, and they were named as pc-Twist1 and sh-Twist1. All these cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Cell Viability

Cell viability of SGC-7901 and BGC-823 cells was examined by trypan blue assay. In brief, transfected cells were seeded in duplicate in 60-mm dishes at a density of 1×10^5 cells and cultured for 24 h at 37°C and 5% CO_2. After this, cells were stained with 0.4% trypan blue (Invitrogen) for 3 min. The viable cells refused staining blue and were counted by using an optical microscope with a hemocytometer (Hausser Scientific, Horsham, PA, USA).

Cell Migration Assay

For cell migration assay, 5×10^4 transfected cells were resuspended in 200 μl of serum-free medium and were seeded on the upper compartment of a 24-well Transwell culture chamber (Millipore, Bedford, MA, USA). Then 600 μl of complete medium was added to the lower compartment. After incubation at 37°C for 12 h, cells were fixed with methanol (4%; NIST, Gaithersburg, MD, USA) for 30 min. A wet cotton swab was used to gently remove the nonmigratory cells from the upper surface of the filter. Traversed cells on the lower side of the filter were stained with 0.1% crystal violet for 20 min and then counted by a microscope (Leica Microsystems, Wetzlar, Germany).

Apoptosis Assay

Cell apoptosis was measured by using a flow cytometry assay. Briefly, transfected SGC-7901 and BGC-823 cells were grown in six-well plates for 24 h and then washed in phosphate-buffered saline (PBS). These cells were double stained with 10 μl of fluorescein isothiocyanate (FITC)-conjugated annexin V and 5 μl of propidium iodide (PI) and incubated for 1 h at room temperature in the dark. The apoptotic cells were detected by using flow cytometry analysis (Beckman Coulter, Fullerton, CA, USA). These data were analyzed by using the FlowJo software (Tree Star, Ashland, OR, USA).

Dual-Luciferase Activity Assay

The Twist1 3′-untranslated region (3′-UTR) segments containing a putative miR-495 binding site were inserted into pMiR-report vector and were amplified by PCR. Cells were cotransfected with the reporter construct or control vector, and miR-495 mimic or mimic control. After transfection for 48 h using Lipofectamine 3000 (Invitrogen), the luciferase activity was analyzed by using the Dual-Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instruction.

qRT-PCR

In this study, the miR-495 and mRNA expressions were examined by qRT-PCR assay. The total RNA was isolated from the transfected cells by using TRIzol reagent (Invitrogen) and treatment with DNaseI (Promega). The complementary DNAs (cDNAs) were obtained by RT using the MultiScribe RT Kit (Applied Biosystems, Austin, TX, USA) and random hexamers or oligos

(dT; Applied Biosystems). The RT conditions were 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C. The expression of miR-495 was examined using a miScript SYBR Green PCR Kit (Qiagen, Germantown, MD, USA). The expression of Twist1 was detected by using an RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Dalian, P.R. China). These data were normalized to U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta Ct}$ method[14].

Western Blot

The transfected SGC-7901 and BGC-823 cells were washed with PBS, and the proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, P.R. China) supplemented with protease inhibitors (Roche, Basel, Switzerland). Equal amounts of the cell proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated at 4°C overnight with primary antibodies of Bax (ab14796), cleaved caspase 3 (ab2302), cleaved caspase 9 (ab2324), E-cadherin (ab76055), N-cadherin (ab76011), Slug (ab27568), vimentin (ab16700), zinc finger E-box binding homeobox 1 (ZEB1) (ab124512), Twist1 (ab50581), and GAPDH (ab181602; all dilutions of 1:1,000; Abcam, Cambridge, UK). Subsequently, the membranes were washed and incubated with the horseradish peroxidase-conjugated (HRP) goat anti-rabbit immunoglobulin G (IgG) (ab205718) and goat anti-mouse IgG (ab6789) (1:5,000; Abcam) as appropriate for 1 h at room temperature. The signals were detected by an ECL system (Amersham Pharmacia, Piscataway, NJ, USA).

Statistical Analysis

All these results were obtained from experiments repeated at least three times. The results of the multiple experiments are presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 6.0 statistical software (GraphPad Software, La Jolla, CA, USA). The multiple-group comparisons were calculated using a one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to indicate a statistically significant result.

RESULTS

miR-495 Was Downregulated in GC Cell Lines

To explore the effect of miR-495 on GC, the expression levels of miR-495 in GC cell lines SGC-7901, BGC-823, MGC803, and AGS were examined by qRT-PCR. The results in Figure 1A showed that the expression of miR-495 was significantly decreased in SGC-7901, BGC-823,
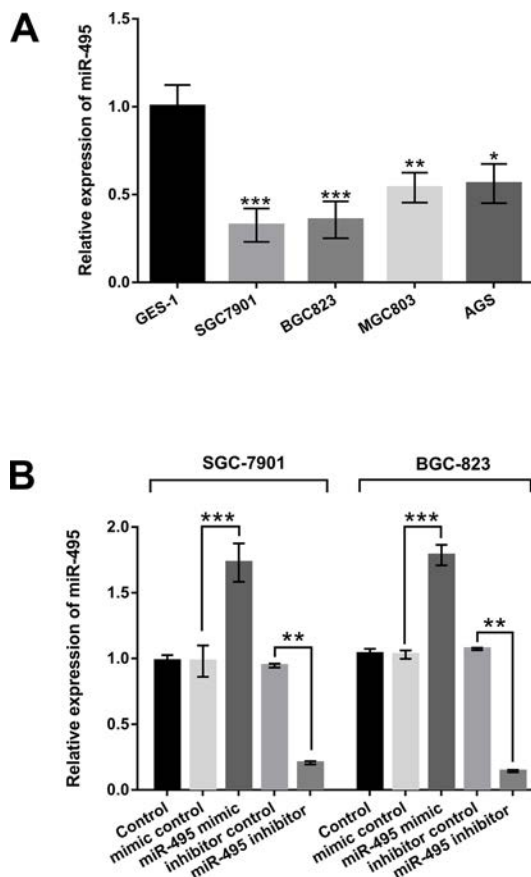

Figure 1. MicroRNA-495 (miR-495) was downregulated in gastric cancer (GC) cell lines. (A) The expression levels of miR-495 in different GC cell lines (SGC-7901, BGC-823, MGC803, and AGS) were examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR assay). (B) Transfection efficiency of miR-495 mimic and miR-495 inhibitor in SGC-7901 and BGC-823 cells. miR-495 mimic and miR-495 inhibitor were transfected into SGC-7901 and BGC-823 cells, and the expression level of miR-495 in these transfected cells was detected by the qRT-PCR assay. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

MGC803, and AGS compared with that in GES-1 cells. The maximum reductions were observed in the SGC-7901 and BGC-823 cell lines. Therefore, these two cell lines were used in the subsequent experiments. Then miR-495 mimic, miR-495 inhibitor, and their corresponding controls were transfected into SGC-7901 and BGC-823 cells. The transfection efficiency was measured by qRT-PCR. As shown in Figure 1B, the expression of miR-495 was significantly upregulated in SGC-7901 and BGC-823 cells transfected with miR-495 mimic compared to the mimic control group ($p < 0.001$). However, after transfection with miR-495 inhibitor, the expression level of miR-495 was remarkably downregulated in these two cell lines compared to inhibitor control group ($p < 0.01$).

The above results demonstrated that miR-495 was lowly expressed in the GC cells, and the transfection efficiency of miR-495 mimic and miR-495 inhibitor was strong and thus could be used for the following experiments.

miR-495 Inhibited Cell Viability and Migration and Promoted Apoptosis of SGC-7901 and BGC-823 Cells

To detect the relationship of miR-495 and cell viability, migration, and apoptosis of GC cells, trypan blue staining, Transwell, and flow cytometry assays were performed. As shown in Figure 2A–C, miR-495 overexpression significantly decreased cell viability and migration but increased apoptosis compared to the mimic control group in both SGC-7901 and BGC-823 cells ($p < 0.05$). However, suppression of miR-495 prominently reversed the results in both SGC-7901 and BGC-823 cells ($p < 0.05$). Western blot assay was used to reveal the expression of the apoptosis-associated factors Bax, cleaved caspase 3, and cleaved caspase 9. The results shown in Figure 2D demonstrated that miR-495 overexpression notably upregulated the protein levels of Bax, cleaved caspase 3, and cleaved caspase 9 in both SGC-7901 and BGC-823 cells. These results suggested that miR-495 could inhibit cell viability and migration and induce apoptosis of SGC-7901 and BGC-823 cells.

miR-495 Inhibited the EMT Process of SGC-7901 and BGC-823 Cells

To further examine the effect of miR-495 on the EMT process, 10 ng/ml of TGF- was used for inducing EMT. According to Figure 3A and B, miR-495 suppression downregulated E-cadherin, as well as upregulated N-cadherin ($p < 0.05$), Slug, and vimentin compared with the inhibitor control group in both SGC-7901 and BGC-823 cells ($p < 0.001$). On the contrary, miR-495 overexpression upregulated E-cadherin and downregulated N-cadherin, Slug, and vimentin compared with the mimic control group ($p < 0.001$). Western blot analysis showed consistent change of miR-495 suppression or overexpression in the above four factor protein expressions in both SGC-7901 and BGC-823 cells (Fig. 3C and D). Overall, these data revealed that miR-495 inhibited the EMT process of SGC-7901 and BGC-823 cells.
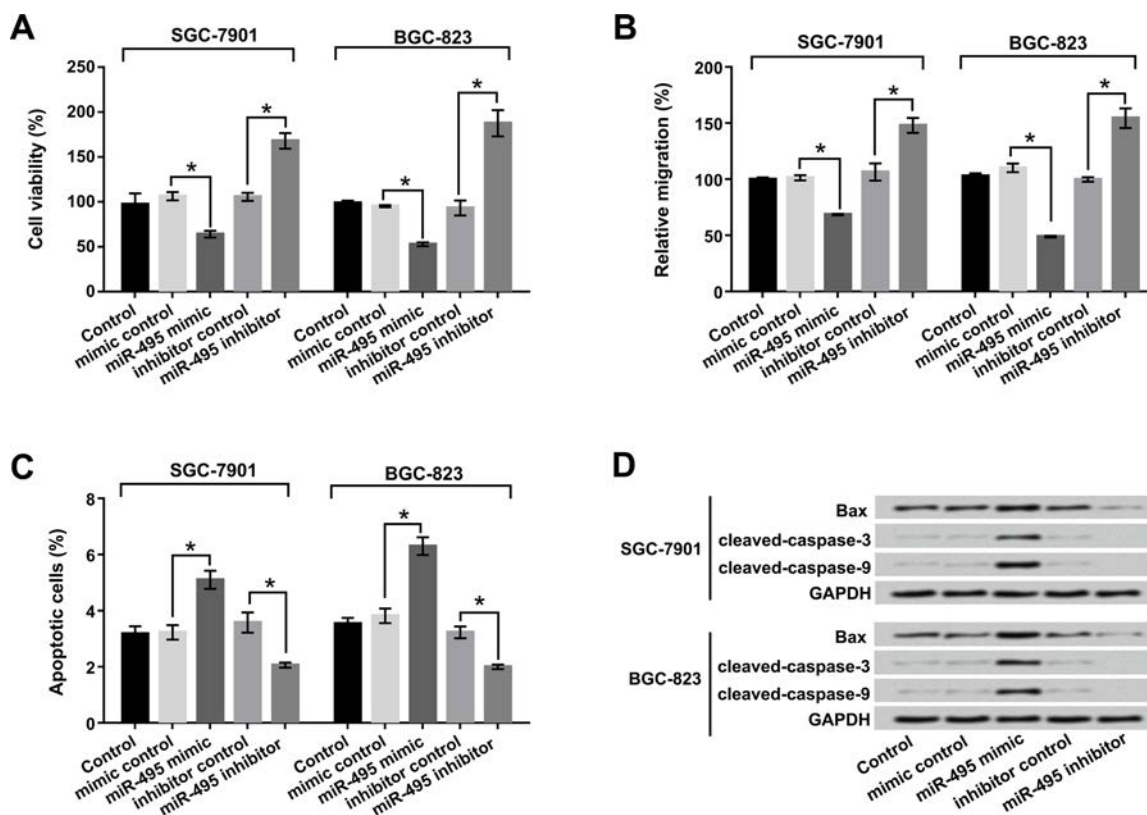

Figure 2. miR-495 inhibited cell viability and migration and promoted apoptosis of SGC-7901 and BGC-823 cells. miR-495 mimic and miR-495 inhibitor were transfected into SGC-7901 and BGC-823 cells. (A) Cell viability, (B) migration, (C) apoptosis, and (D) apoptosis-related factors were determined by trypan blue staining, Transwell, flow cytometry, and Western blot respectively. *$p < 0.05$.
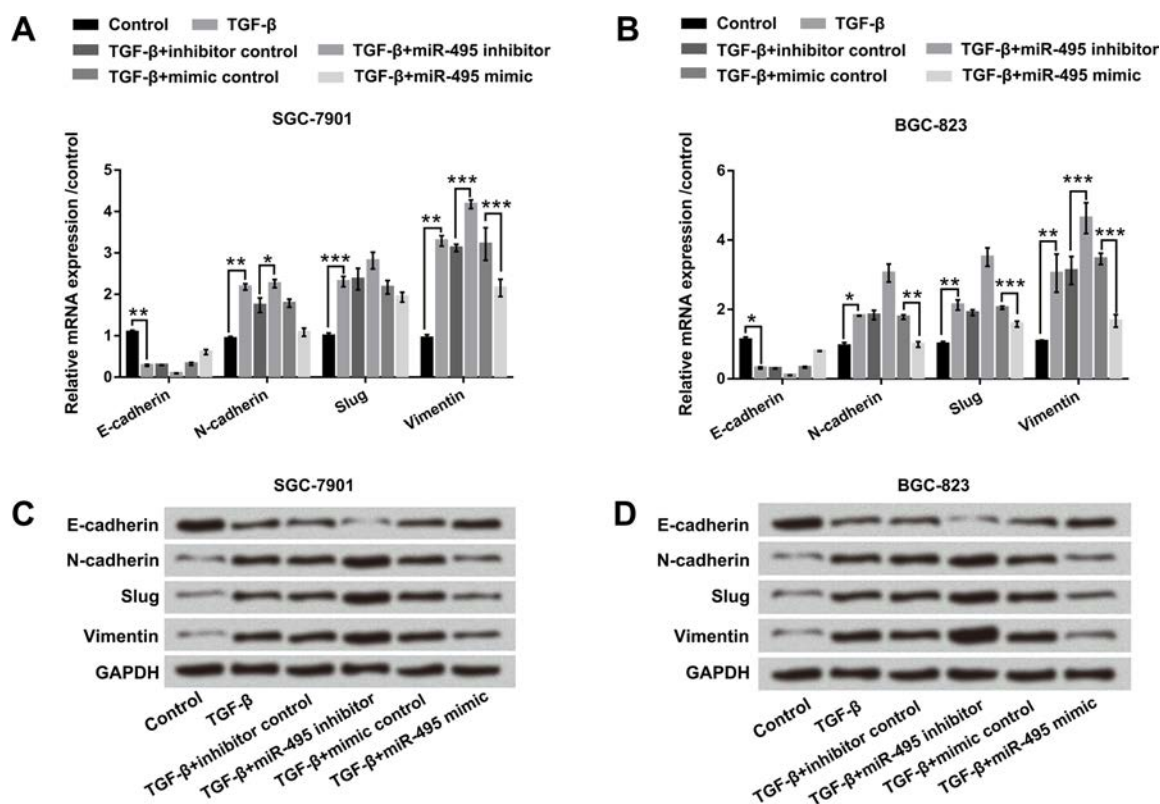
Figure 3. miR-495 inhibited the epithelial–mesenchymal transition (EMT) process of SGC-7901 and BGC-823 cells. miR-495 mimic and miR-495 inhibitor were transfected into SGC-7901 and BGC-823 cells. After treatment with 10 ng/ml of transforming growth factor-β (TGF-β) for 24 h, the mRNA expressions of E-cadherin, N-cadherin, Slug, and vimentin in (A) SGC-7901 and (B) BGC-823 cells were examined by qRT-PCR. The protein levels of E-cadherin, N-cadherin, Slug, and vimentin in (C) SGC-7901 and (D) BGC-823 cells were examined by Western blot. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Twist1 Was a Direct Target Gene of miR-495

We next tested the relationship between Twist1 and miR-495 in GC cells. The bioinformatics software of TargetScan (http://www.targetscan.org), miRNA database (http://www.microrna.org), and National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) were used to predict the sequence relationship between miR-495 and Twist1. The potential binding domain was predicted as shown in Figure 4A. Additionally, the mRNA and protein levels of Twist1 were determined by qRT-PCR and Western blot. As displayed in Figure 4B, the mRNA and protein levels of Twist1 were significantly downregulated by miR-495 overexpression, as well as upregulated by miR-495 suppression in both SGC-7901 and BGC-823 cells. Furthermore, dual-luciferase reporter assay results revealed that overexpression of miR-495 significantly reduced the luciferase activity of Twist-wt, but did not influence Twist-mt compared to mimic control group ($p < 0.05$) (Fig. 4C). The data described above indicated that Twist1 was a direct target gene of miR-495 and that the expression of Twist1 was negatively regulated by miR-495.

miR-495 Inhibited Cell Viability, Migration, and EMT Process by Regulation of Twist1

To further investigate the effect of Twist1 on GC, sh-Twist1, pc-Twist1, and corresponding controls were transfected into SGC-7901 and BGC-823 cells. qRT-PCR and Western blot results showed that the mRNA and protein levels of Twist1 were significantly decreased by Twist1 silencing, while being increased by Twist1 overexpression ($p < 0.01$) (Fig. 5A). Then SGC-7901 and BGC-823 cells were transfected with miR-495 inhibitor alone or accompanied with sh-Twist1. Cell viability, migration, and expression levels of EMT-related factors were determined in these transfected cells. As revealed in Figure 5B and C, suppression of miR-495 significantly promoted cell viability and migration compared with its corresponding control in both SGC-7901 and BGC-823 cells ($p < 0.05$ or $p < 0.01$). The promoting effects of miR-495 suppression were reduced by cotransfection of sh-Twist1 ($p < 0.05$ or $p < 0.01$). Additionally, qRT-PCR and Western blot results revealed that cotransfection of miR-495 inhibitor and sh-Twist1 obviously upregulated E-cadherin, as well as downregulated N-cadherin
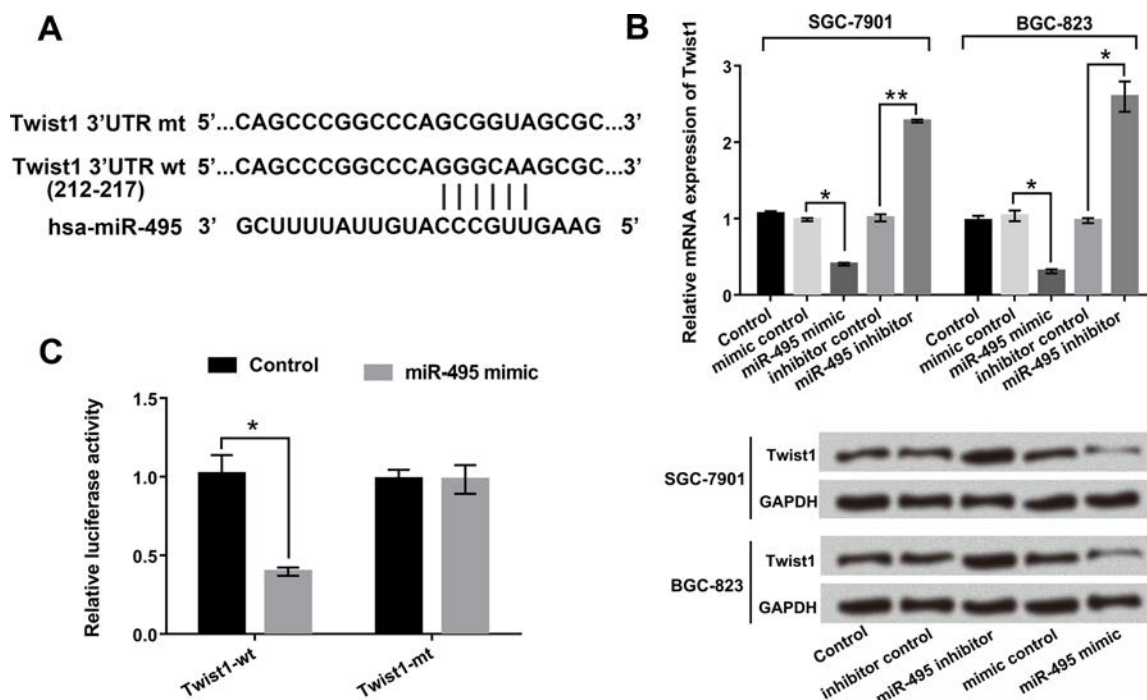
Figure 4. Twist1 was a direct target gene of miR-495. miR-495 mimic and miR-495 inhibitor were transfected into SGC-7901 and BGC-823 cells. (A) The sequence relationship between miR-495 and Twist1. (B) The mRNA and protein levels of Twist1 in the transfected cells were detected by qRT-PCR and Western blot. (C) Relative luciferase activity in cells after cotransfection of miR-495 mimic or mimic control with Twist1-wt or Twist1-mt vector was examined by dual-luciferase activity assay. $*p < 0.05$; $**p < 0.01$.

($p < 0.05$), Slug ($p < 0.01$), and vimentin ($p < 0.01$) compared with the miR-495 inhibitor group (Fig. 5D–G). Overall, these data indicated that miR-495 inhibited cell viability, migration, and the EMT process by regulation of Twist1 in both SGC-7901 and BGC-823 cells.

Twist1 Promoted the EMT Process in SGC-7901 and BGC-823 Cells

It is known that the Twist1 transcription factor is able to promote tumor metastasis and induce the EMT process[15]. However, whether Twist1 also promoted EMT in GC remains unclear. Therefore, sh-Twist1 and pc-Twist1 were transfected into SGC-7901 and BGC-823 cells, and the protein levels of EMT-associated factors including E-cadherin, N-cadherin, vimentin, ZEB1, and snail were detected by Western blot. As shown in Figure 6, Twist1 silencing notably upregulated the protein level of E-cadherin, but downregulated N-cadherin, vimentin, ZEB1, and snail protein levels both in SGC-7901 and BGC-823 cells. However, Twist1 overexpression showed opposite results in the protein levels of E-cadherin, N-cadherin, vimentin, ZEB1, and snail. These data demonstrated that Twist1 promoted the EMT process of SGC-7901 and BGC-823 cells.

DISCUSSION

In the present study, we found that miR-495 was a tumor suppressor in GC cells by inhibiting cell viability and migration, as well as inducing apoptosis and blocking the EMT process. Twist1 was clarified as a target gene of miR-495, and Twist1 silencing obviously reduced the promoting effect of miR-495 suppression on these biological processes. In addition, Twist1 silencing significantly blocked the EMT signaling pathway in both SGC-7901 and BGC-823 cells.

In the tumor-related miRNAs, the mechanism of miR-495 in cancer development and progression attracted our attention. miR-495 has been reported as a tumor suppressor in various diseases, including GC[2,16]. Li et al. reported that miR-495 could inhibit cell migration and invasion of GC cells by directly interacting with PRL-3[17]. Similarly, Wang et al. reported that miR-495 suppressed GC cell migration and invasion potentially by its direct inhibition on HMGA2[18]. However, relying on these few studies is not sufficient to elucidate the functions of miR-495 in GC. Therefore, in our study, we further explored the effect of miR-495 on cell proliferation, metastasis, and apoptosis of GC cells. The results revealed that overexpression of miR-495 could obviously suppress cell
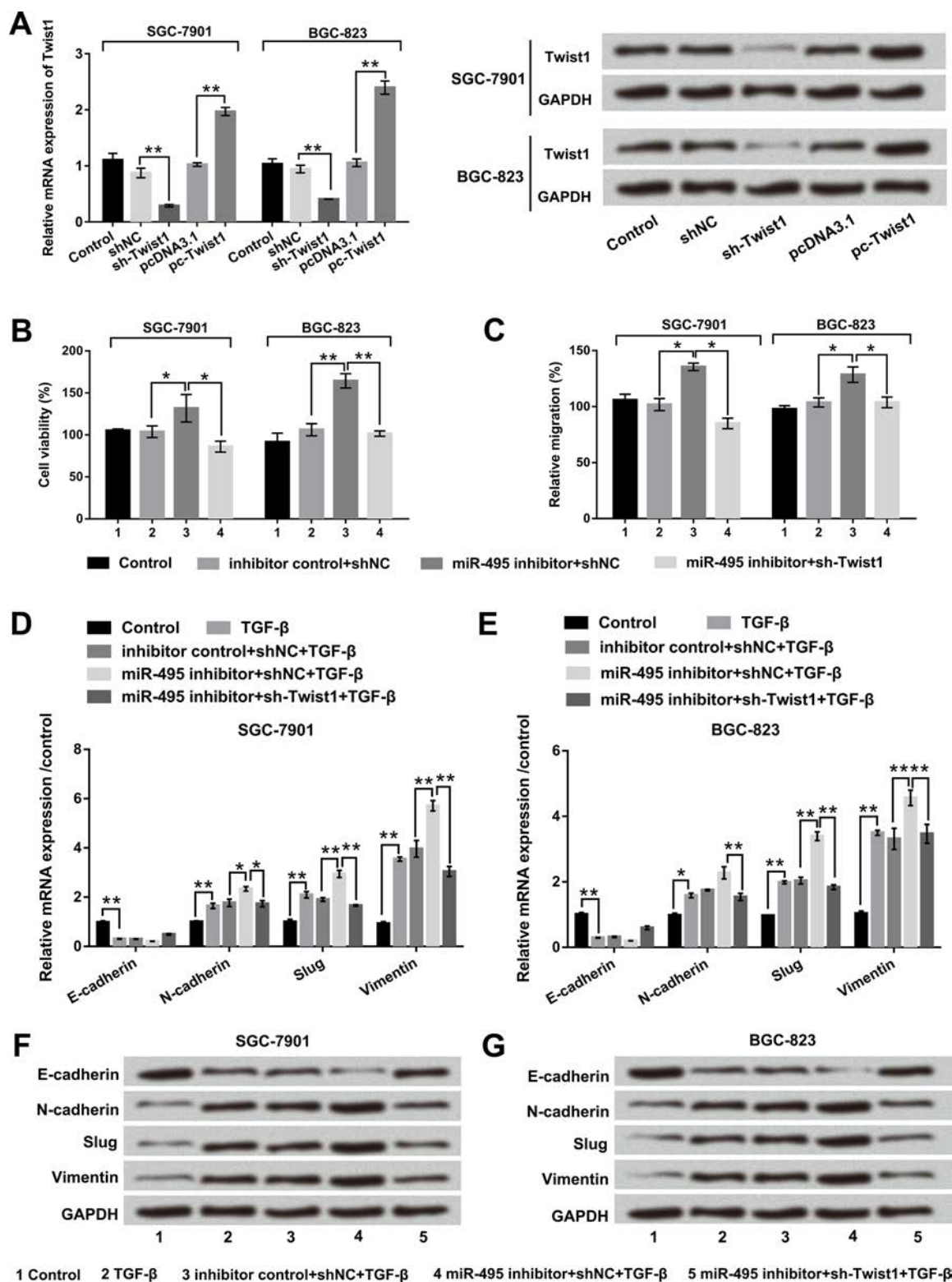
Figure 5. miR-495 suppression promoted cell viability, migration, and EMT process by regulation of Twist1. SGC-7901 and BGC-823 cells were transfected with sh-Twist1, pc-Twist1, and corresponding controls. (A) The mRNA and protein levels of Twist1 were determined by RT-PCR and Western blot. (B) Cell viability was examined by trypan blue assay. (C) Cell migration was examined by Transwell assay. (D, E) the mRNAs and (F, G) protein levels of E-cadherin, N-cadherin, Slug, and vimentin were measured by qRT-PCR and Western blot.*$p<0.05$; **$p<0.01$.
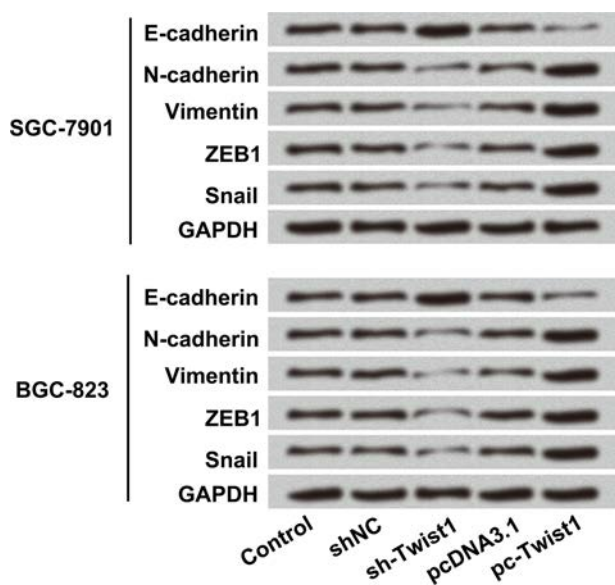
Figure 6. Twist1 promoted the EMT process of SGC-7901 and BGC-823 cells. The vectors of sh-Twist1, pc-Twist1, or appropriate controls were transfected into SGC-7901 and BGC-823 cells. The protein levels of EMT-associated factors including E-cadherin, N-cadherin, vimentin, zinc finger E-box binding homeobox 1 (ZEB1), and snail were detected by Western blot.

viability and migration, as well as induce apoptosis and inhibit the EMT process in both SGC-7901 and BGC-823 cells. Consistent with previous studies, our study also indicated that miR-495 might be a tumor suppressor by inhibiting cell proliferation and metastasis and by promoting apoptosis of GC cells.

It has come to be known that miRNAs could negatively regulate target genes by directly degrading mRNA or inhibiting protein synthesis, thereby affecting cell growth, proliferation, invasion, and metastasis[19,20]. Several studies have demonstrated that Twist1 was a direct target gene of various miRNAs, such as miR-106b, miR-720, miR-186, and miR-32[21–24]. It participated in modulation of cell proliferation and metastasis and affected the EMT process in different kinds of cancers[25,26]. However, whether Twist1 was a direct target gene of miR-495 was not previously reported. In the present study, we first used dual-luciferase activity assay to detect the relationship between Twist1 and miR-495. We found that Twist1 was a direct target gene of miR-495 and was negatively regulated by miR-495. Furthermore, we also found that Twist1 silencing significantly reduced the promoting effect of miR-495 suppression on cell proliferation, migration, and the EMT process. According to these data, we speculated that miR-495 exerted an antitumor effect through the regulation of Twist1 expression in GC cells.

It is well known that Twist1 is a basic helix–loop–helix transcription factor, which is a key regulator in tumor metastasis and the EMT process[27]. Recent evidence has demonstrated that Twist1 could increase the EMT-related factors of N-cadherin and decrease those of E-cadherin, thereby inducing the EMT process[28,29]. Dong et al. also reported that Twist1 contributed to the EMT process via downregulation of the epithelial marker E-cadherin and upregulation of the mesenchymal marker N-cadherin in endometrial cancer[21]. In view of existing research, we further explored the effect of Twist1 on the EMT signaling pathway. Similar with these previous studies, we found that overexpression of Twist1 upregulated E-cadherin levels but downregulated N-cadherin, vimentin, ZEB1, and snail protein levels both in SGC-7901 and BGC-823 cells. These data indicated that Twist1 could promote the EMT process in GC cells. However, further study is still needed to explore the cross-regulation effect of miR-495 and Twist1 on the EMT process.

In conclusion, these results of the study demonstrated that miR-495 might be a tumor suppressor, as evidenced by inhibition of proliferation, migration, and EMT as well as promotion of apoptosis by targeting Twist1 in GC cells. These data indicated that miR-495 might be a tumor suppressor of GC, and hopefully the results could supplement potential therapies for the treatment of GC.

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