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## MicroRNA-6884-5p Regulates the Proliferation, Invasion, and EMT of Gastric Cancer Cells by Directly Targeting S100A16

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S100 binding protein A16 (S100A16) expression levels are closely associated with microRNA (miRNA) processing. Higher levels of S100A16 are reported during the progression of many cancers. Our study mainly explored the interaction between S100A16 and miR-6884-5p in gastric cancer (GC). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the level of S100A16 and miR-6884-5p in GC tissues and cell lines. The si-S100A16, pcDNA-S100A16, miR-6884-5p mimic or inhibitor was transfected into GC cells, and the effects of S100A16 and miR-6884-5p on the proliferation, invasion, and epithelial-mesenchymal transition (EMT) were explored by qRT-PCR and Western blot assays. Luciferase assays were performed to validate S100A16 as an miR-6884-5p target in GC cells. In our study, we found that the level of miR-6884-5p was significantly decreased and the expression of S100A16 was significantly increased in GC tissues and cell lines. There was a close association between these changes. Knockdown of S100A16 significantly inhibited the proliferation, invasion, and EMT of GC cells. The bioinformatics analysis predicted that S100A16 is a potential target gene of miR-6884-5p, and the luciferase reporter assay confirmed that miR-6884-5p could directly target S100A16. Introduction of miR-6884-5p to GC cells had similar effects to S100A16 silencing. Overexpression of S100A16 in GC cells partially reversed the inhibitory effects of the miR-6884-5p mimic. miR-6884-5p inhibited the proliferation, invasion, and EMT of GC cells by directly decreasing S100A16 expression.

**Key words:** Gastric cancer (GC); MicroRNA-6884-5p; S100 binding protein A16; Proliferation; Invasion; Epithelial-mesenchymal transition (EMT)

### INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide. Despite its declining incidence, GC remains as the second leading cause of cancer-related deaths<sup>1,2</sup>. Although the diagnosis and treatment for GC have made great progress, and basic and clinical studies have been conducted in recent years, its long-term outcome remains poor<sup>3</sup>. The etiology of GC is a complex process that involves the activation of oncogenes and the inactivation of tumor suppressor genes at different stages. However, its exact pathogenesis remains unclear<sup>4</sup>. Hence, there is still an urgent need to explore novel and special promising predictive factors to improve the prognosis of GC.

The S100 protein family consists of at least 25 distinct members and is composed of EF-hand-type calcium-binding proteins and plays roles in intra- and extracellular functions involved in the regulation of diverse cellular

processes such as contraction, motility, cell growth, differentiation, cell cycle progression, and secretion<sup>5,6</sup>. Recently, the S100 protein family has been implicated in multiple stages of tumor progression such as cell proliferation, migration, invasion, and apoptosis. Moreover, expression of the S100 protein family is detected in many human cancers and has been correlated with a poorer prognosis<sup>7</sup>. High expression of S100A16 was found in several cancers such as breast cancer, oral squamous cell carcinoma, prostate cancer, colorectal cancer, lung adenocarcinoma, and cervical carcinoma<sup>8-13</sup>. However, the expression and its role of S100A16 in GC are still unknown.

Many studies have reported that microRNAs (miRNAs) can regulate cellular proliferation, differentiation, apoptosis, and invasion, as well as cancer initiation and progression<sup>14-16</sup>. Actually, a number of reports demonstrated that miRNAs were differentially expressed in GC17-19. miR-17-5p acted as an oncogene, and knockdown of

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miR-17-5p significantly inhibited proliferation, migration, invasion, and epithelial–mesenchymal transition (EMT) of GC cells through regulating phosphatase and tensin homolog (PTEN) signaling<sup>17</sup>. Overexpression of miR-448 suppressed the proliferation of GC cells by directly decreasing ADAM10 expression<sup>18</sup>. Introduction of miR-199a-5p significantly promoted migration and invasion of GC cells by directly targeting klotho expression<sup>19</sup>. In this study, we found that miR-6884-5p could directly regulate the expression of S100A16. However, the expression and the role of miR-6884-5p are still unknown in GC.

Therefore, our study aimed to elucidate the expression of S100A16 and its effects on the biological behavior of GC cells according to gain-of-function or loss-of-function cell models by overexpression or silencing of S100A16. Combined with bioinformatics prediction and dual-luciferase assay, we verified whether miR-6884-5p directly targets S100A16, and investigated the effect of miR-6884-5p on the proliferation, invasion, and EMT of GC cells. The miR-6884-5p/S100A16 axis may provide theoretical basis for application in the diagnosis and treatment of GC.

## MATERIALS AND METHODS

### *Human Tissue Samples*

A total of 30 GC tissue samples and matched adjacent nontumor tissues were obtained from patients with histologically confirmed GC at the Department of Oncology, Henan Cancer Hospital between 2017 and 2019. The eligibility criteria for the present study were as follows: (1) patients with no history of gastrectomy or other malignancies, (2) patients with no distant metastasis or peritoneal dissemination, and (3) the number of dissected lymph nodes was not less than 15. The exclusion criteria were as follows: (1) patients who underwent palliative surgery, and (2) patients who received treatment such as chemotherapy, or radiation therapy, prior to radical surgery. The tissue samples were immediately frozen using liquid nitrogen. The study was performed with the approval of the Clinical Research Ethics Committee of Henan Cancer Hospital, and all patients gave written informed consent for the use of the tissue samples for research purposes.

### *Cell Lines and Transfection*

Five human GC cell lines (AGS, MKN45, BGC-823, SGC-7901, and MGC-803) and an immortalized normal human fallopian tube epithelial cell line FTE187 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). All cell lines were incubated in a humidified chamber with 5% CO<sub>2</sub> at 37°C.

The miR-6884-5p mimics, inhibitor, and corresponding negative controls were synthesized and purchased from RiboBio Company (Guangzhou, China). The specific small interfering RNA (siRNA) for S100A16 and corresponding negative control were synthesized and purchased from GenePharma Company (Shanghai, China). Transfection was performed by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### *Cell Counting Kit-8 (CCK-8) Assay*

Cell growth was measured using the cell proliferation reagent WST-8 (Roche Biochemicals, Indianapolis, IN, USA). Cells ( $1.0 \times 10^3$ /well) were plated in 96-well microtiter plates (Corning Costar, Corning, NY, USA), and then the CCK-8 agent (10  $\mu$ l/well) was added to each well at the time of harvest according to the manufacturer's protocols. One hour after adding the CCK-8, cellular viability was determined by measuring the absorbance of the converted dye at 450 nm.

### *In Vitro Invasion Assay*

For invasion assays,  $1 \times 10^5$  cells in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). Medium containing 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, cells remaining on the upper membrane were removed, whereas cells that had migrated or invaded to the lower membrane were stained with 0.1% crystal violet. The membranes were then rinsed with 30% glacial acetic acid. Finally, the washing solution was measured by a microplate reader (Bio-Rad, Hercules, CA, USA) at 540 nm for the counting of the number of GC cells.

### *Measurement of MMP-2, MMP-9, and TIMP-1 Levels by ELISA Assay*

According to the protocol, the supernatants of MKN45 and SGC-7901 cells were collected after treatment, and the concentrations of matrix metalloproteinase-2 (MMP-2), MMP-9, and tissue inhibitor of metalloproteinase-1 (TIMP-1) were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (USCN Life Science, Wuhan, China) according to the manufacturer's instruction. Briefly, the primary antibody was coated onto ELISA plates and incubated for 2 h at room temperature. Samples and standards were added to the wells and incubated for 1 h. The wells were then washed, and a biotinylated antibody was added for 1 h. The plates were washed again, and streptavidin conjugated to horseradish peroxidase (HRP) was added for 10 min. After washing, tetramethylbenzidine was added for color development, and the reaction was terminated with 1 mol/L H<sub>2</sub>SO<sub>4</sub>.

Absorbance was measured at 490 nm. Values were expressed as ng/ml.

#### RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted from cervical cancer tissues and cell lines by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription and quantitative real-time PCR (qRT-PCR) were performed by using the One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa Biotechnology, Tokyo, Japan) according to the manufacturer's protocol on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal controls for normalization and quantification, respectively, of S100A2, S100A4, S100A6, S100A7, S100A8, S100A14, S100A16, and miR-6884-5p expressions. The relative expression of genes was analyzed and calculated by using the  $2^{-\Delta\Delta C_t}$  method. Primer sequences are shown in Table 1.

#### Luciferase Reporter Assay

The 3'-untranslated region (3'-UTR) of S100A16 containing the wild-type or mutant miR-6884-5p binding site was designed and cloned into the pGL3 plasmid (Invitrogen) according to the manufacturer's protocol. Cells were seeded in 24-well plates and cotransfected with plasmid pGL3-S100A16-3'-UTR-WT or pGL3-S100A16-3'-UTR-MUT and miR-6884-5p mimics or negative control by using Lipofectamine 3000. Cells were

collected 48 h after transfection, and relative luciferase activities were measured by a dual-luciferase reporter assay (Promega, Madison, WI, USA) according to the manufacturer's instruction. *Renilla* luciferase was used for normalization.

#### Western Blot

Proteins were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer, and the concentrations were detected by using BCA Protein Assay Kit (Beyotime, Shanghai, China). Equal amounts of protein samples were fractionated by using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membranes, and blocked with 5% nonfat milk for 30 min at room temperature. Membranes were probed with primary antibodies against S100A16 (ab130419), proliferating cell nuclear antigen (PCNA) (ab92552), CDK2 (ab32147), cyclin E1 (ab33911), p21 (ab109520), and GAPDH (ab181602) (Abcam, Cambridge, MA, USA); E-cadherin (#14472), N-cadherin (#13116), and vimentin (#5741) (Cell Signaling Technology Inc., Danvers, MA, USA) at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies. GAPDH was used as an endogenous protein for normalization. Results were detected by using the Odyssey Scanning system (Li-Cor, Lincoln, NE, USA).

#### Statistical Analysis

The data are expressed as the mean  $\pm$  standard error of the mean (SEM). The number of independent

**Table 1.** Sequence of Primers for qRT-PCR

Gene	Primer Sequence
S100A2	F: 5'-TGCCAAGAGGGCGACAAGTTCA-3' R: 5'-AAGTCCACCTGCTGGTCACTGT-3'
S100A4	F: 5'-CAGAACTAAAGGAGCTGCTGACC-3' R: 5'-CTTGGAAAGTCCACCTCGTTGTC-3'
S100A6	F: 5'-GTGACAAGCACACCCTGAGCAA-3' R: 5'-GGAAGTTCACCTCCTGGTCCTT-3'
S100A7	F: 5'-AGAAGCCAAGCCTGCTGACGAT-3' R: 5'-GTCCTTTTTCTCAAAGACATCGGC-3'
S100A8	F: 5'-ATGCCGTCTACAGGGATGACCT-3' R: 5'-AGAATGAGGAACTCCTGGAAGTTA-3'
S100A14	F: 5'-CCTCATCAAGAACTTTCACCAGTA-3' R: 5'-GGTTGGCAATTTCTCTTCCAGG-3'
S100A16	F: 5'-GCTCCAGAAAGAGCTGAACCAC-3' R: 5'-ATGCCGCCTATCAAGGTCCAGT-3'
miR-6884-5p	F: 5'-AGAGGCTGAGAAGGTGATGT-3' R: 5'-GAACATGTCTGCGTATCTC-3'
U6	F: 5'-CTCGCTTCGGCAGCAC-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	F: 5'-GAGTCAACGGATTTGGTCTGATTG-3' R: 5'-CCTGGAAGATGGTGATGGGATT-3'

experiments is represented by “*n*.” Correlations between miR-6884-5p and S100A16 mRNA levels were analyzed using Pearson’s correlation coefficient. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test. Other comparisons were analyzed using two-tailed Student’s *t*-test. A value of  $p < 0.05$  was considered a statistically significant difference.

## RESULTS

### *The Expression of S100A16 Was Upregulated in GC Tissues and Cell Lines*

It has been reported that S100 proteins such as S100A2, S100A4, S100A6, S100A7, S100A8, S100A14, and S100A16 were closely associated with cancers<sup>7</sup>. In this study, we detected these seven S100 genes in GC tissues. The results showed that the mRNA level of S100A16 was the highest in GC tissues among these seven S100 genes compared with the adjacent tissues (Fig. 1A). We also found that the protein expression of S100A16 was significantly increased in tumor tissues compared with the adjacent tissues (Fig. 1B). Subsequently, we also determined the mRNA and protein levels of S100A16 in GC cell lines such as AGS, BGC-823, MKN45, MGC-803, SGC-7901, and in immortalized normal human fallopian tube epithelial cell line FTE187 cells. Compared with FTE187, the expressions of S100A16 in MKN45 and SGC-7901 cells were higher than those in the other three GC cell lines (Fig. 1C and D). Therefore, MKN45 and SGC-7901 cells were used in the following experiments.

### *Knockdown of S100A16 Inhibits Proliferation, Invasion, and EMT of GC Cells*

To explore the roles of S100A16 in GC cells, the MKN45 and SGC-7901 cells were transfected with si-NC or si-S100A16. The Western blot analysis showed that expression of S100A16 was significantly decreased at the mRNA and protein levels in MKN45 and SGC-7901 cells transfected with si-S100A16 (Fig. 2A). The results from the CCK-8 assay demonstrated that downregulation of S100A16 restrained proliferation of GC cells (Fig. 2B). The Transwell assay revealed that downregulation of S100A16 suppressed invasion of GC cells (Fig. 2C). Moreover, decreased S100A16 expression could significantly reduce total expressions of MMP-2 and MMP-9 and enhance expression of TIMP-1 in MKN45 and SGC-7901 cells (Fig. 2D). In addition, silencing of S100A16 contributed to the upregulation of E-cadherin and the downregulation of N-cadherin and vimentin (Fig. 2E).

### *miR-6884-5p Directly Targets S100A16 in GC Cells*

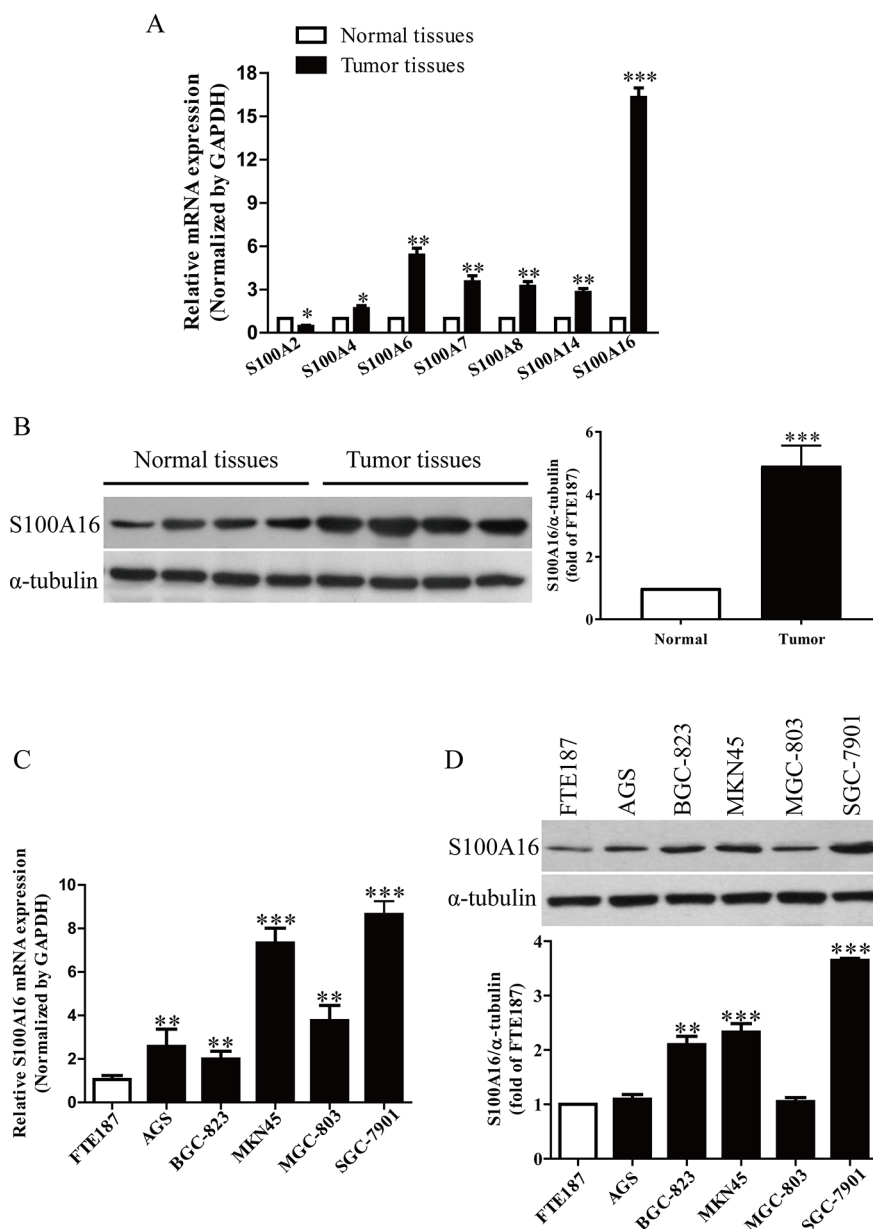
Based on the TargetScan 7.2 database, we predicted that miR-485-5p, miR-873-5p, and miR-6884-5p might directly target S100A16 (Fig. 3A). We found that only

overexpression of miR-6884-5p could significantly decrease S100A16 expression in MKN45 and SGC-7901 cells after transfection with miR-485-5p, miR-873-5p, or miR-6884-5p mimic, respectively (Fig. 3B). Moreover, luciferase reporter assays also confirmed that miR-6884-5p directly targeted S100A16. S100A16 3′-UTR was cloned into a luciferase reporter vector, and the putative miR-6884-5p binding site in the S100A16 3′-UTR was mutated (Fig. 3A). Our data showed that upregulation of miR-6884-5p markedly inhibited the luciferase activity of pGL3-S100A16-3′-UTR-WT (Fig. 3C). Mutation of the miR-6884-5p-binding site in the S100A16 3′-UTR abolished the effect of miR-6884-5p (Fig. 3C), which indicated that miR-6884-5p directly and negatively regulated the expression of S100A16. Next, our results showed that the level of miR-6884-5p in the GC tissues was significantly lower in comparison to the adjacent tissues (Fig. 3D). We also demonstrated that miR-6884-5p expression was markedly downregulated in MKN45 and SGC-7901 cells compared to that in FTE187, as shown in Figure 3E. To determine whether the S100A16 expression was closely related to the miR-6884-5p level in GC tissues, the Pearson’s correlation analysis revealed a significant negative correlation between S100A16 and miR-6884-5p (Fig. 3F).

### *Effects of miR-6884-5p on the Proliferation and Invasion of GC Cells*

After transfection with miR-6884-5p mimic and inhibitor, the qRT-PCR analysis showed that the level of miR-6884-5p was significantly upregulated and downregulated, respectively (Fig. 4A). These data demonstrated that we efficiently enhanced and reduced miR-6884-5p expression in MKN45 and SGC-7901 cells. Next, overexpression of miR-6884-5p significantly inhibited the proliferation of GC cells, whereas knockdown of it could promote the proliferation of GC cells (Fig. 4B). To study the role of miR-6884-5p in the invasion of GC cells, we evaluated the invasive capacities of MKN45 and SGC-7901 cells transfected with miR-6884-5p mimic or inhibitor by Transwell invasion assays. Transwell assays illustrated that the invasion of MKN45 and SGC-7901 cells was remarkably suppressed in the miR-6884-5p mimic group compared to the miR-NC group, but was significantly promoted in the miR-6884-5p inhibitor group compared to the anti-miR-NC group (Fig. 4C). These findings showed that miR-6884-5p might play a critical role in the inhibition of GC cell invasion. The balance between MMPs and TIMP-1 is demonstrated to play an important role of invasion by stimulating degradation of the ECM in GC cells and is associated with enhanced tumor metastatic potential. Our ELISA and qRT-PCR assays indicated that total secretion of MMP-2 and MMP-9 in the culture supernatants and mRNA expressions of MMP-2





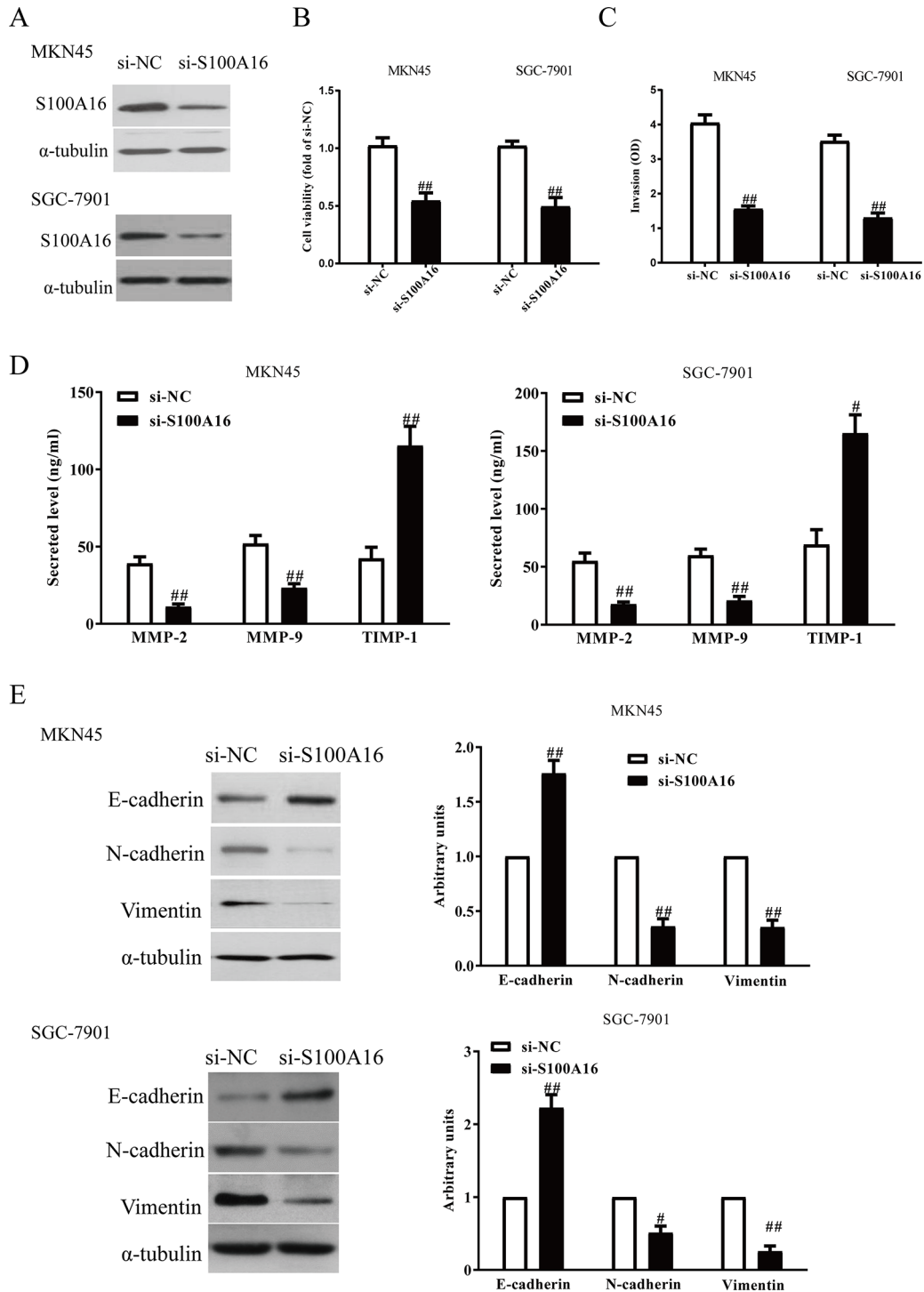
**Figure 1.** The levels of S100A16 in gastric cancer (GC) tissues and cell lines. (A) The mRNA levels of S100A2, S100A4, S100A6, S100A7, S100A8, S100A14, and S100A16 in GC tissues and their corresponding adjacent normal tissues. (B) The protein expressions of S100A16 in GC tissues and their corresponding adjacent normal tissues. The mRNA (C) and protein (D) expressions of S100A16 analyzed by real-time quantitative polymerase chain reaction (qRT-PCR) in GC cell lines (AGS, BGC-823, MKN45, MGC-803, and SGC-7901) and an immortalized normal human fallopian tube epithelial cell line FTE187 cells. All data are presented as mean  $\pm$  standard error of the mean (SEM),  $n = 6$ . \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus normal tissues or FTE187.

and MMP-9 were evidently decreased by overexpression of miR-6884-5p in MKN45 and SGC-7901 cells, and total secretion of TIMP-1 and mRNA expression of TIMP-1 were significantly increased (Fig. 4D). However, knock-down of miR-6884-5p could enhance the secretion and mRNA expressions of MMP-2 and MMP-9 and reduce the secretion and mRNA expression of TIMP-1 (Fig. 4D). Taken together, our findings suggested that downregulated

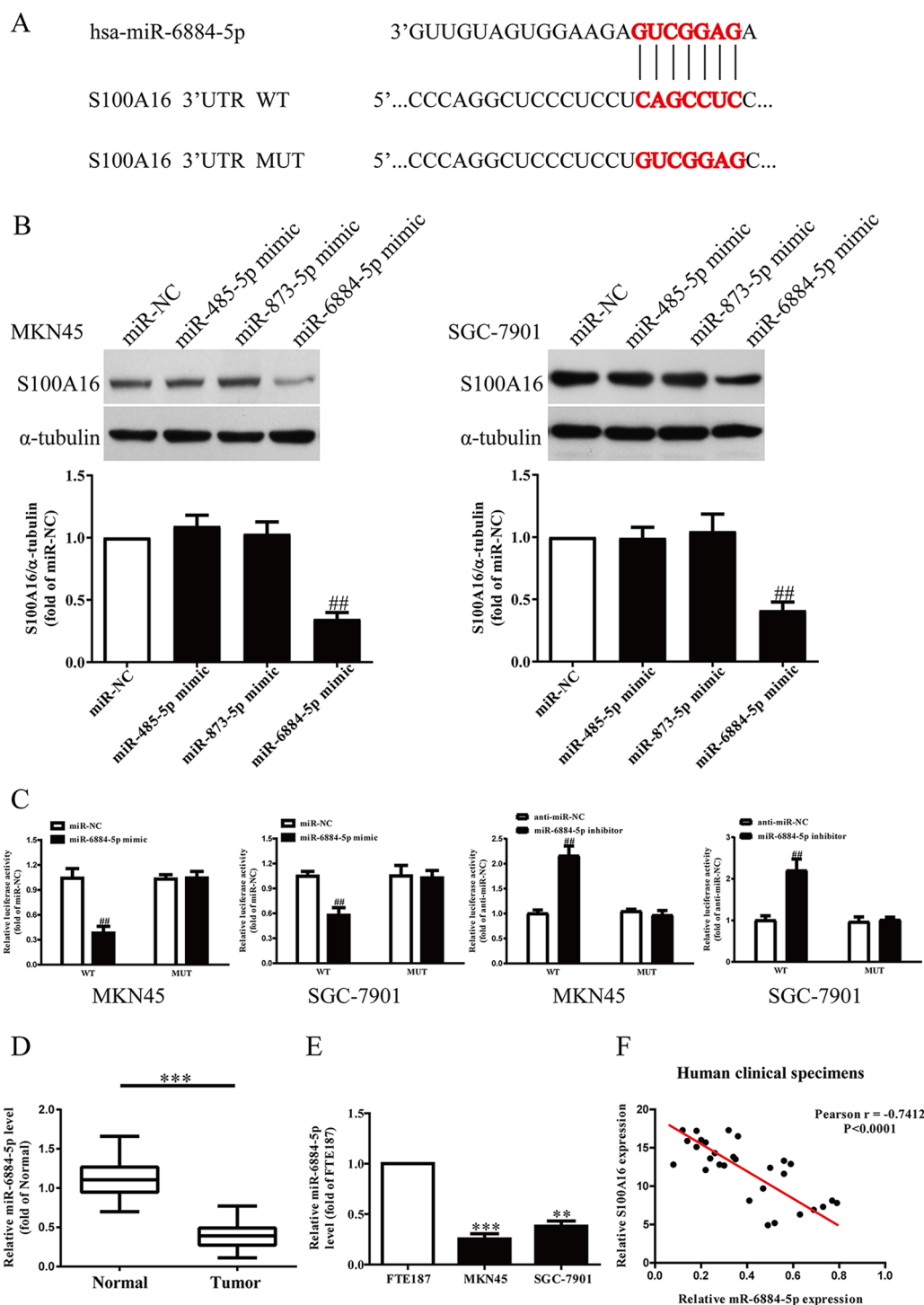
MMP-2 and MMP-9 expressions and upregulated TIMP-1 expression might be the possible mechanisms that contributed to the inhibitory effect of miR-6884-5p mimic on the invasion of MKN45 and SGC-7901 cells.

#### Effects of miR-6884-5p on EMT of GC Cells

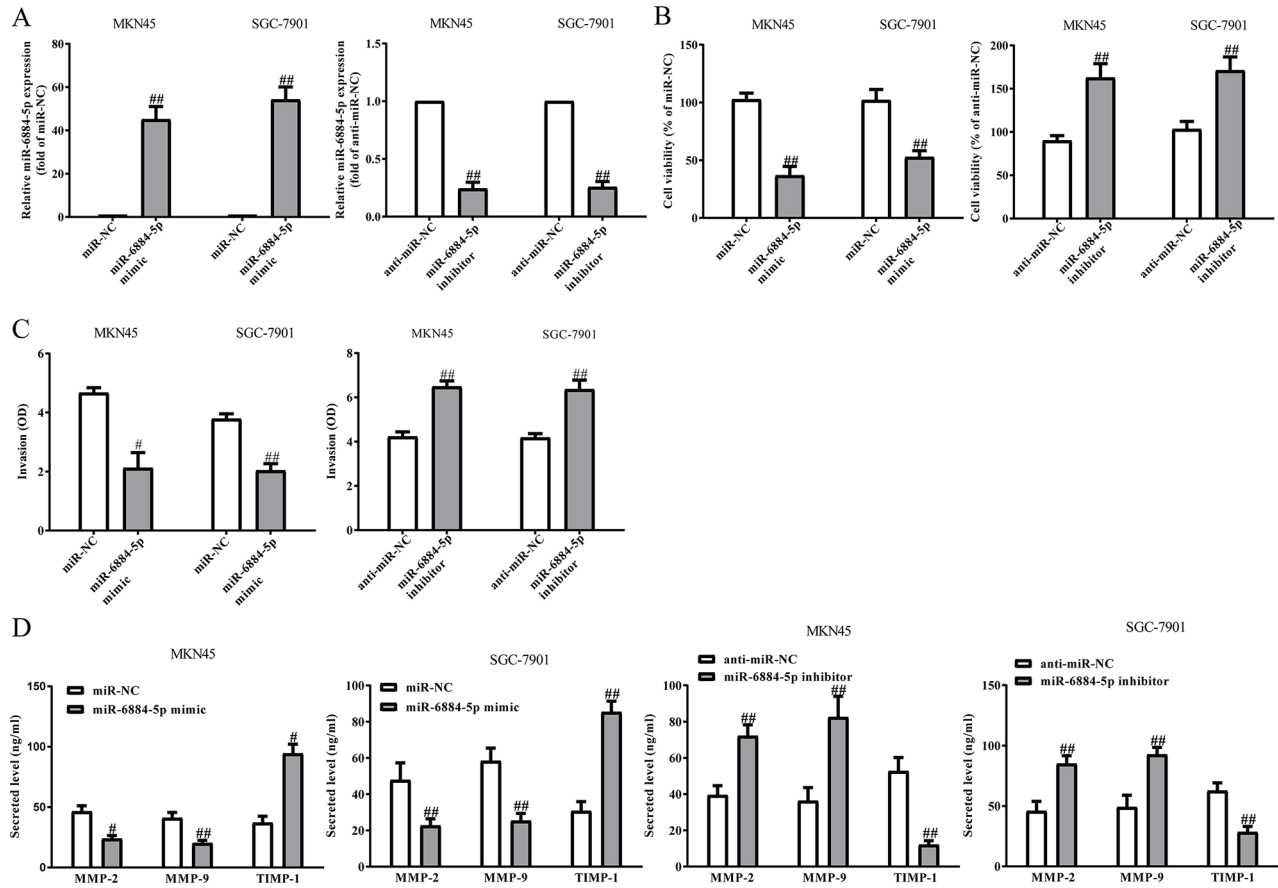
Next, we explored the effects of miR-6884-5p on the expressions of EMT markers in MKN45 and SGC-7901



**Figure 2.** S100A16 silencing could inhibit the invasion and epithelial–mesenchymal transition (EMT) in GC cells. MKN45 and SGC-7901 cells were transfected with si-S100A16 or si-NC. (A) The protein levels of S100A16 were determined by Western blot. (B) Cell proliferation was detected by cell counting kit-8 (CCK-8). (C) The invasion of GC cells was assessed by Transwell assay. (D) Levels of total matrix metalloproteinase-2 (MMP-2), MMP-9, and tissue inhibitor of metalloproteinase-1 (TIMP-1) were detected in the culture supernatants of cultured GC cells by enzyme-linked immunosorbent assay (ELISA) assays. (E) The expressions of E-cadherin, N-cadherin, and vimentin were determined by qRT-PCR. All data are presented as mean  $\pm$  SEM,  $n = 4$ . # $p < 0.05$ , ## $p < 0.01$  versus si-NC.



**Figure 3.** S100A16 was a direct target of miR-6884-5p. (A) Schematic representation of S100A16 3'-untranslated regions (3'-UTRs) showing putative microRNA (miRNA) target site. (B) The protein levels of S100A16 were determined by Western blot in MKN45 and SGC-7901 cells transfected with miR-NC, miR-485-5p, miR-873-5p, or miR-6884-5p mimic, respectively. (C) The analysis of the relative luciferase activities of S100A16-WT and S100A16-MUT in GC cells. (D) Relative miR-6884-5p level in GC tissues and their corresponding adjacent normal tissues. (E) Relative miR-6884-5p levels analyzed by qRT-PCR in MKN45, SGC-7901, and FTE187 cells. (F) Pearson's correlation analysis of the relative miR-6884-5p levels of and the relative S100A16 mRNA levels in GC tissues. All data are presented as mean  $\pm$  SEM,  $n = 4$ .  $\#\#p < 0.01$  versus miR-NC or anti-miR-NC.  $**p < 0.01$ ,  $***p < 0.001$  versus normal tissues or FTE187.



**Figure 4.** The effects of miR-6884-5p on the proliferation and invasion of GC cells. MKN45 and SGC-7901 cells were transfected with miR-6884-5p mimic or inhibitor. (A) The level of miR-6884-5p analyzed by qRT-PCR. (B) Cell proliferation was detected by CCK-8. (C) The invasion of GC cells was assessed by Transwell assay. (D) Levels of total MMP-2, MMP-9, and TIMP-1 were detected in the culture supernatants of cultured GC cells by ELISA assay. All data are presented as mean  $\pm$  SEM,  $n = 4$ . # $p < 0.05$ , ## $p < 0.01$  versus miR-NC or anti-miR-NC.

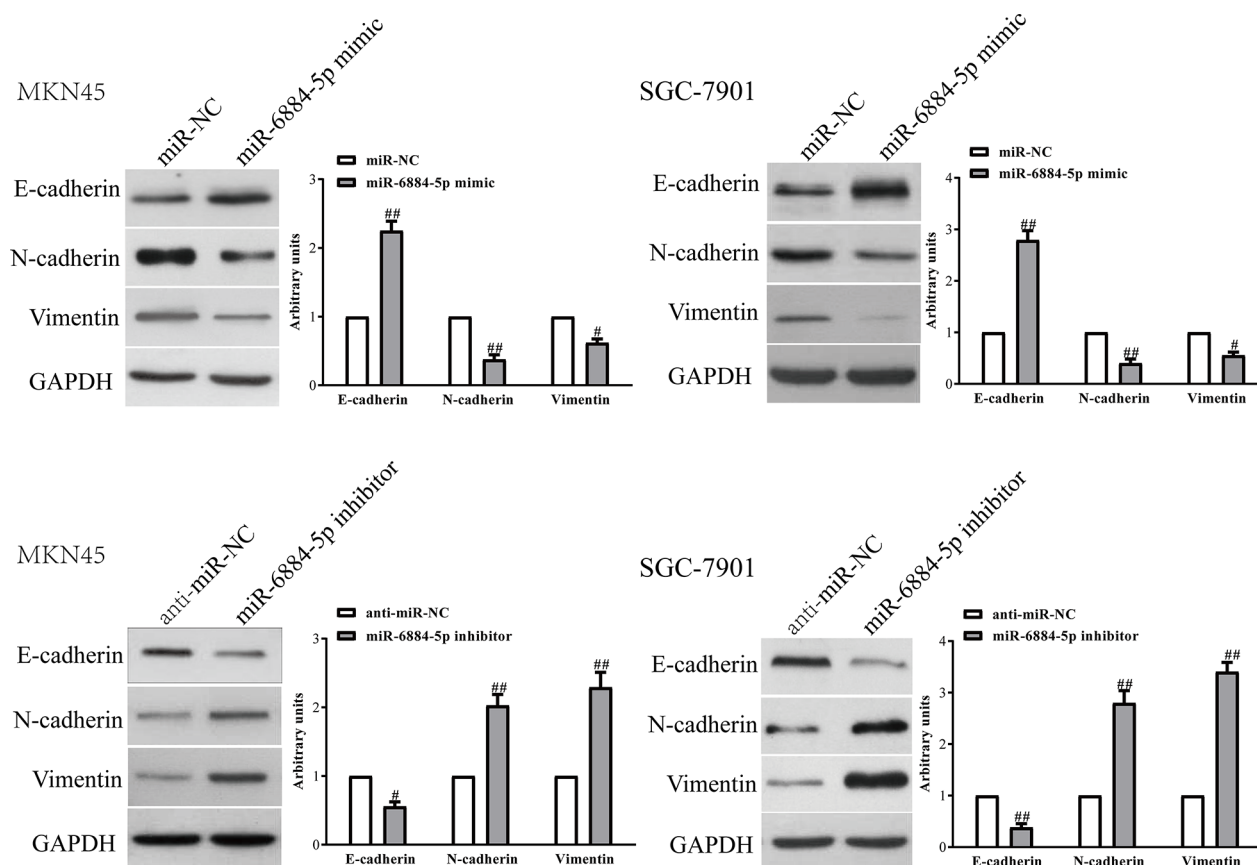
cells using qRT-PCR and Western blot. Introduction of miR-6884-5p in MKN45 and SGC-7901 cells led to increasing the expression of the epithelial marker E-cadherin and to decreasing the expressions of the mesenchymal markers N-cadherin and vimentin at the mRNA and protein levels (Fig. 5). However, knockdown of miR-6884-5p had opposite effects of miR-6884-5p up-regulation on EMT of GC cells (Fig. 5). Altogether, our findings revealed that overexpression of miR-6884-5p could inhibit EMT of GC cells.

#### *miR-6884-5p Suppressed the Proliferation, Invasion, and EMT of GC Cells Through Inhibition of S100A16*

To confirm whether miR-6884-5p inhibited the proliferation, invasion, and EMT of GC cells through S100A16-dependent mechanism, we cotransfected MKN45 and SGC-7901 cells with miR-6884-5p mimic and pcDNA-S100A16 vector. Our data showed that the expressions of S100A16 were dramatically increased after transfection with miR-6884-5p mimic and pcDNA-S100A16

compared with miR-6884-5p mimic and pcDNA3.1 vector in MKN45 and SGC-7901 cells (Fig. 6A). S100A16 upregulation abrogated the inhibitory effect of miR-6884-5p mimic on cell proliferation (Fig. 6B). At the same time, expressions of PCNA, CDK2, and cyclin E1 were decreased and expression of p21 was increased in miR-6884-5p-overexpressing GC cells after exogenous upregulation of S100A16 (Fig. 6C). Our results showed that upregulation of S100A16 could reverse the inhibitory effect of miR-6884-5p overexpression on invasion of GC cells (Fig. 6D) by increasing expressions of MMP-2 and MMP-9 and decreasing the expression of TIMP-1 (Fig. 6E). Moreover, introduction of S100A16 decreased the expression of E-cadherin and increased the expressions of N-cadherin and vimentin in MKN45 and SGC-7901 cells after transfection with miR-6884-5p mimic (Fig. 6F). Therefore, our data clearly confirmed that upregulation of miR-6884-5p inhibited the proliferation, invasion, and EMT of GC cells by directly targeting S100A16, and knockdown of S100A16 was essential for





**Figure 5.** The effects of miR-6884-5p on expressions of EMT-related molecules in GC cells. MKN45 and SGC-7901 cells were transfected with miR-6884-5p mimic or inhibitor. The protein levels of E-cadherin, N-cadherin, and vimentin were determined by Western blot. All data are presented as mean  $\pm$  SEM,  $n = 4$ . # $p < 0.05$ , ## $p < 0.01$  versus miR-NC or anti-miR-NC.

the miR-6884-5p mimic-induced inhibition of the proliferation, invasion, and EMT in GC cells.

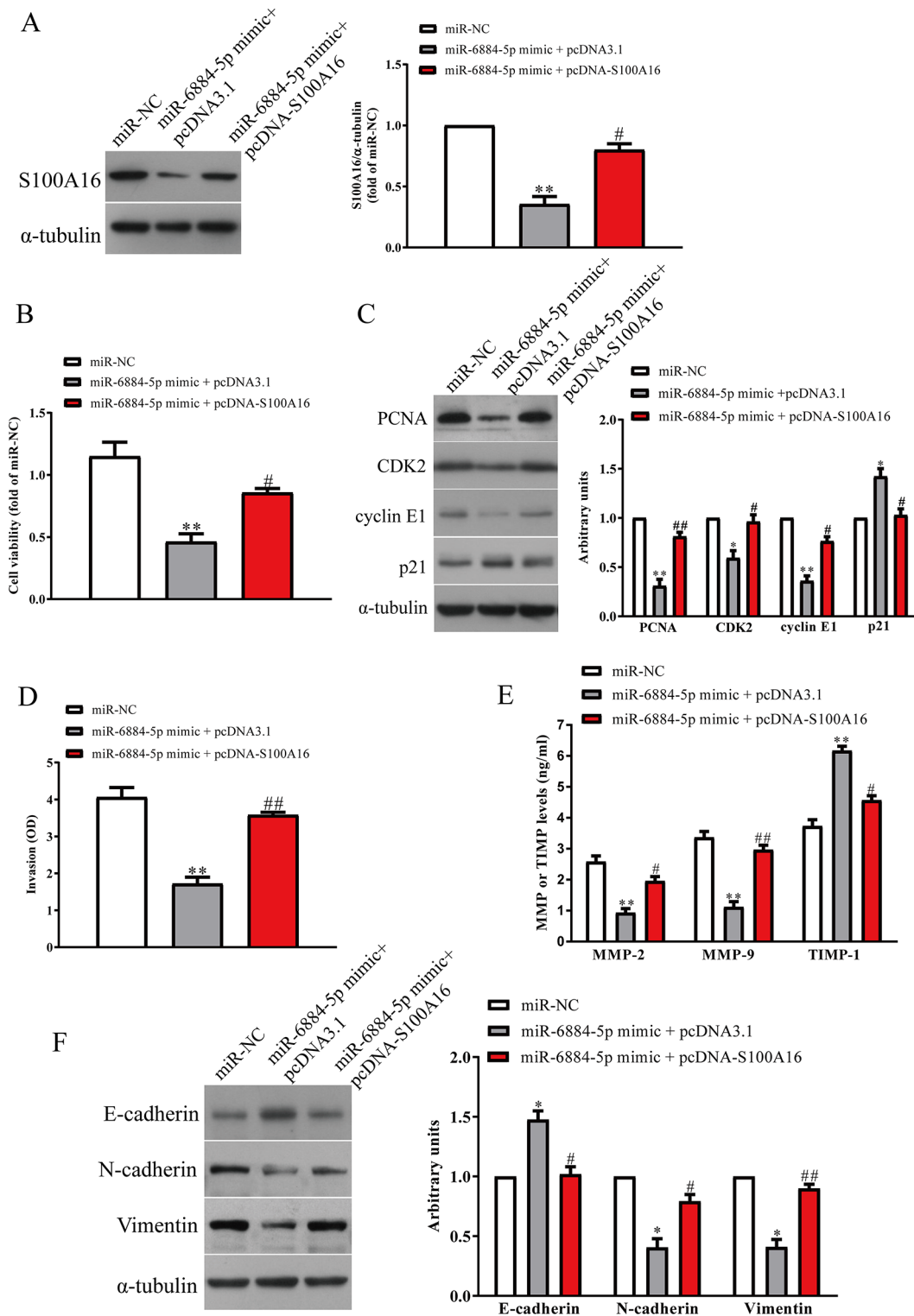
## DISCUSSION

GC is a heterogeneous disease, and there are many ways to treat it, including surgical resection and chemoradiotherapy, or both. However, survival is poor under the current therapeutic regimens. Therefore, there is a great need to find a more effective treatment to improve the therapeutic outcomes. A new biomarker, miR-6884-5p, comes to mind.

More and more studies report the critical roles of S100 proteins in tumorigenesis and cancer metastasis<sup>5,6</sup>. S100 proteins have been confirmed as key regulators of cell cycle progression, cell growth, cell migration, invasion, and so on<sup>7</sup>. Importantly, different expressions of these S100 proteins have been closely associated with multiple human cancers<sup>20</sup>. Recently, an increasing number of researchers have focused on the role of S100A16 in tumorigenesis and tumor progression. Up to now, the results showed that S100A16 acts as an oncogene in several cancers<sup>21–26</sup>. However, the role of S100A16 in GC remained

unclear. Here, we show for the first time an important role of S100A16 in the tumorigenesis and progression of GC. Our PCR and Western blot results showed that S100A16 is overexpressed in human GC tissues and cell lines. Next, we directly evaluated the role of S100A16 in cell growth, cell cycle regulation, and invasion by overexpression and knockdown of S100A16 using a series of functional assays. Overexpression of S100A16 promotes cell growth and invasion. In contrast to S100A16 overexpression experiments, depletion of S100A16 inhibited cell growth and invasion. For further study, we determined the effect of S100A16 on cell proliferation and invasion at the molecular level. Taken together, all these findings highlight the importance of aberrant expression and biological function of S100A16 in cervical cancer.

Metastasis is a main cause of cancer-related death in patients, and EMT is essential for cancer metastasis, which includes local invasion, intravasation, extravasation, and proliferation at distant sites and is a complicated and currently uncontrolled process. A key feature of EMT is the switch from E-cadherin to N-cadherin. Cancer cells undergoing EMT decrease the expression of



**Figure 6.** Overexpression of S100A16 promoted miR-6884-5p-inhibited cell invasion and EMT in GC cells. MKN45 and SGC-7901 cells were transfected with miR-NC and miR-6884-5p mimic with pcDNA3.1 or pcDNA-S100A16 vector. (A) The protein levels of S100A16 were determined by Western blot. (B) Cell proliferation was detected by CCK-8. (C) The expressions of proliferating cell nuclear antigen (PCNA), CDK2, cyclin E1, and p21 were detected by Western blot. (D) The invasion of GC cells was assessed by Transwell assay. (E) Levels of total MMP-2, MMP-9, and TIMP-1 were detected in the culture supernatants of cultured GC cells by ELISA assay. (F) The expressions of E-cadherin, N-cadherin, and vimentin were determined by Western blot. All data are presented as mean  $\pm$  SEM,  $n = 4$ . \* $p < 0.05$ , \*\* $p < 0.01$  versus miR-NC; # $p < 0.05$ , ## $p < 0.01$  versus miR-6884-5p mimic + pcDNA3.1.

E-cadherin and increase expressions of N-cadherin and vimentin<sup>27</sup>. Notably, our results showed that introduction of S100A16 significantly decreased E-cadherin expression and increased N-cadherin and vimentin expressions in GC cell lines at the protein level, which suggested that S100A16 could induce EMT in GC cells. In contrast, knockdown of S100A16 exhibits adverse effect on the regulation of the main components of EMT including E-cadherin, N-cadherin, and vimentin. Our data suggest that S100A16 induces EMT and, therefore, is important in GC progression and metastasis. However, the detailed mechanism of regulation of EMT components by S100A16 needs to be further elucidated. A complete understanding of the role of S100A16 in controlling EMT in cancer is very important for the use of S100A16 as a potential therapeutic target in GC.

Anomaly expression of miRNAs is closely related to tumorigenesis, and different miRNA expression profiles have been sequentially reported in multiple cancers such as breast cancer, prostate cancer, and liver cancer<sup>28</sup>. Abnormal expression of miRNAs participates in many processes including malignant transformation, apoptosis, invasion, and metastasis of GC<sup>17–19</sup>. miRNAs widely participate in the regulation of different pathological processes of carcinogenesis according to downregulating target gene expression; they also can be modulated by upstream regulators, such as transcription factors or epigenetic growth factors. One kind of miRNA may target various genes and involve multiple distinctive signaling pathways. Moreover, various miRNAs can also target a certain gene. Different miRNAs and mRNAs interact at corresponding posttranscriptional levels and form an intricate regulatory network. Actually, we predicted three miRNAs targeting S100A16. Afterward, qRT-PCR, Western blotting, and luciferase reporter assay confirmed that miR-6884-5p directly targeted S100A16. However, the precise mechanism of miR-6884-5p in GC remained unclear. Therefore, we aimed to elucidate the biological functions and mechanism of miR-6884-5p in GC. Our findings confirmed that miR-6884-5p was found to be downregulated in GC cells and tissues. Furthermore, we found that miR-6884-5p overexpression also significantly inhibited the proliferation, invasion, and EMT of GC cells, mimicking the effects of S100A16 knockdown. Importantly, we also demonstrated that overexpression of S100A16 partly blocked the inhibitory effects of miR-6884-5p mimic on the proliferation, invasion, and EMT of GC cells. Therefore, we made a conclusion that miR-6884-5p played critical role in the inhibition of the proliferation, invasion, and EMT of GC cells by directly regulating S100A16 expression.

Taken together, we have identified S100A16 and miR-6884-5p as associated with metastasis in human GC. High expression of S100A16 and low level of miR-6884-5p

might be valuable for the prediction of metastasis. We further showed that miR-6884-5p regulates cell cycle progression, cell proliferation, invasion, and EMT by directly regulating S100A16. Notably, we demonstrated that miR-6884-5p and S100A16 can act as a regulator of EMT. For the first time, this study implicates critical roles for miR-6884-5p and S100A16 in the metastatic process of human GC.

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