

# MicroRNA-524-5p suppresses the growth and invasive abilities of gastric cancer cells

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**Abstract.** Previous studies have demonstrated that microRNAs (miRNAs) are associated with tumor development and progression. miRNA-524-5p (miR-524-5p) has been reported to be involved in the development and progression of several types of cancer, but its role in gastric cancer has not been fully elucidated to date. Therefore, the aim of the present study was to investigate the expression levels and function of miR-524-5p in human gastric cancer. The expression levels of miR-524-5p were assessed in gastric cancer specimens and cell lines, including MKN-45, SGC-7901 and MGC-803 cell lines and gastric epithelial mucosa GES-1 cells, using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cell proliferation and cell apoptosis assays and invasion analysis in gastric cancer cell lines were performed to evaluate the effects of miR-524-5p on gastric cancer cells *in vitro*. The expression levels of matrix metalloproteinase (MMP)-2 and MMP-9 were determined by RT-qPCR and western blot analysis. The expression of miR-524-5p was significantly decreased in gastric cancer tissues and cell lines. Additionally, the results of the *in vitro* experiments demonstrated that overexpression of miR-524-5p inhibited cell proliferation and invasion, and promoted cell apoptosis in gastric cancer cells. Human gastric cancer SGC-7901 and MGC-803 cell lines transfected with miR-524-5p exhibited reduced expression levels of MMP-2 and MMP-9. Taken together, the results of the present study indicated that miR-524-5p may function as a novel tumor suppressor gene in gastric cancer, and may serve as a biomarker and therapeutic target for the treatment of gastric cancer.

## Introduction

Gastric cancer is one of the most common types of cancer, and the second leading cause of cancer-associated mortality worldwide (1). Almost half of the patients with gastric cancer are Chinese, the majority of whom are diagnosed when the disease has progressed to advanced stages, due to the non-specific symptoms, including epigastric pain, anorexia and vomiting, that present during the early stages of the disease (2). As a result, the overall 5-year survival rate of gastric cancer is ~20% (3). Numerous studies have investigated the etiology of gastric cancer (4-6). However, the molecular mechanisms that underlie the pathogenesis and progression of gastric cancer remain undefined. Therefore, additional studies are required to elucidate the molecular mechanisms that lead to the metastasis and progression of gastric cancer, and to identify novel markers for the diagnosis, prognosis and treatment of patients with gastric cancer.

MicroRNAs (miRNAs) are short non-coding RNAs of 19-24 nucleotides in length that control the translation and stability of their target messenger (m) RNA by binding to regulatory sites located in the 3'-untranslated region of the transcripts (7). It is widely accepted that miRNAs play pivotal roles in various biological processes, including development, metabolism, cell proliferation, differentiation and apoptosis (8). Numerous studies suggest that there is an association between altered miRNA expression and cancer, since aberrant expression of miRNAs appears to be involved in several important processes that occur during carcinogenesis. Previous studies have investigated the role of miRNA-524-5p (miR-524-5p) in various types of cancer. Chen *et al* (9) demonstrated that miR-524-5p was associated with overall survival rate and pathological grade of patients with glioma, and Liu *et al* (10) revealed that the expression of miR-524-5p was reduced in human melanoma, while overexpression of miR-524-5p effectively inhibited melanoma cell proliferation and migration. Furthermore, Liu *et al* (10) demonstrated that tumors overexpressing miR-524-5p were significantly smaller than those displayed by negative control mice. However, the role of miR-524-5p in gastric cancer remains unclear.

The present study investigated the expression levels of miR-524-5p in human gastric cancer tissues and cell lines, including MKN-45, SGC-7901 and MGC-803 cell lines and gastric epithelial mucosa GES-1 cells. In addition, cell

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proliferation and migration assays, as well as a cell apoptosis analysis, were performed using human gastric cancer SGC-7901 and MGC-803 cell lines to explore the *in vitro* effects of miR-524-5p in gastric cancer cells.

## Materials and methods

**Tissue samples.** A total of 15 gastric cancer and adjacent non-cancerous tissue samples were obtained from patients that had undergone surgical treatment for gastric cancer at The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) between March 2011 and March 2013. The patients were diagnosed independently by two experienced pathologists from The First Affiliated Hospital of Xinxiang Medical University, according to the Cancer Staging Manual published by the American Joint Committee on Cancer. The present study was approved by the Ethics Committee of the Medical College of Zhengzhou University and informed consent was obtained from the patients prior to sample collection, conforming to the Declaration of Helsinki and the local legislation. Written informed consent was obtained from all patients prior to the start of the study.

**Cell culture.** Human gastric cancer MKN-45, SGC-7901 and MGC-803 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), while the human gastric epithelial mucosa GES-1 cell line was purchased from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (BioTeke Corporation, Beijing, China) supplemented with 10% heat-inactivated Invitrogen fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified cell incubator with 5% CO<sub>2</sub> at 37°C.

**Plasmids and cell transfection.** A miR-524-5p mimic and inhibitor, alongside their corresponding negative controls (scramble miRNA), were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The SGC-7901 and MGC-803 cells were seeded in 6-well plates at 30% confluence one day prior to transfection. The cells were transfected with miR-524-5p mimic, miR-524-5p inhibitor and control miRNA using Invitrogen Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the cell lines and the tissue samples using Invitrogen TRIzol reagent (Thermo Fisher Scientific, Inc.) and DNase (catalog no. ab32124; Abcam, Cambridge, MA, USA). RT was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. The expression of miR-524-5p was verified by stem-loop RT-qPCR with specific RT and PCR primers. The primer sequences (Sangon, Shanghai, China) were: Matrix metalloproteinase (MMP)-2, sense, 5'-CCCCAG ACAGGTGATCTTGAC-3' and antisense, 5'-GCTTGCGAG GGAAGAAGTTG-3'; and MMP-9, sense, 5'-CGCTGGGCT TAGATCATTCC-3' and antisense, 5'-AGGTTGGATACA

TCACTGCATTAGG-3'. U6 small nuclear RNA was used as an internal control. RT-qPCR for MMP-2 and MMP-9 was performed using the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. qPCR was performed on an Applied Biosystems® 7500 thermocycler (Thermo Fisher Scientific, Inc.) using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. The comparative CT method,  $\Delta\Delta C_t$ , was used to quantify the data. Briefly,  $\Delta C_t$  was calculated by subtracting the CT of U6 or GAPDH mRNA from the mRNA of interest, and  $\Delta\Delta C_t$  was calculated by subtracting the  $\Delta C_t$  of the negative control from the  $\Delta C_t$  of the samples. The data was normalized according to the study conducted by Schmittgen and Livak (11).

**Western blot analysis.** Transfected cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed using a cell lysis buffer consisting of 50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 50 mM NaF and 1 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Haimen, China). The cells were centrifuged at 10,000 x g for 15 min at 4°C. The protein concentration was measured by BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Cell protein lysates were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel with the Mini-PROTEAN® II Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using Mini-Protean precast gels (Bio-Rad Laboratories, Inc.) at 200 V for 1 h, and subsequently transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were next blocked with 5% skimmed milk in Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBST), and incubated with primary antibodies, including anti-MMP-2 (mouse; monoclonal; dilution, 1:10; catalog no. ab80737; Abcam, Cambridge, UK), anti-MMP-9 (mouse; monoclonal; dilution, 1:25; catalog no. ab51203; Abcam) and anti-β-actin (mouse; monoclonal; dilution, 1:5000; catalog no. ab75373; Abcam) overnight at 4°C. The membranes were washed three times in TBST for 10 min, and subsequently incubated with HRP conjugated goat anti-rabbit IgG secondary antibodies (catalog no. MBS560261; Mybiosource, San Diego, CA, USA). Chemiluminescence detection was performed using Pierce™ Fast Western Blot kit (Thermo Fisher Scientific, Inc.) and ECL Substrate (GE Healthcare Life Sciences, Chalfont, UK). The densitometry analysis was analyzed using Image-Pro Plus software, version 1.61.

**Cell proliferation assays.** The transfected cells were seeded onto 96-well plates (Corning Incorporated, Corning, NY, USA) at a density of 1x10<sup>4</sup> cells/well. In total, 20 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma-Aldrich) at a concentration of 5 mg/ml was added to the transfected cells, and incubated at 37°C for 4 h. Following the removal of the culture medium, the remaining crystals were dissolved in dimethyl sulfoxide (Sigma-Aldrich), and the absorbance at 570 nm was measured. All experiments were performed in triplicate.

**Cell apoptosis.** Cell apoptosis analyses were performed as previously described, with certain modifications (12). Briefly, SGC-7901 and MGC-803 cells were detached using trypsin

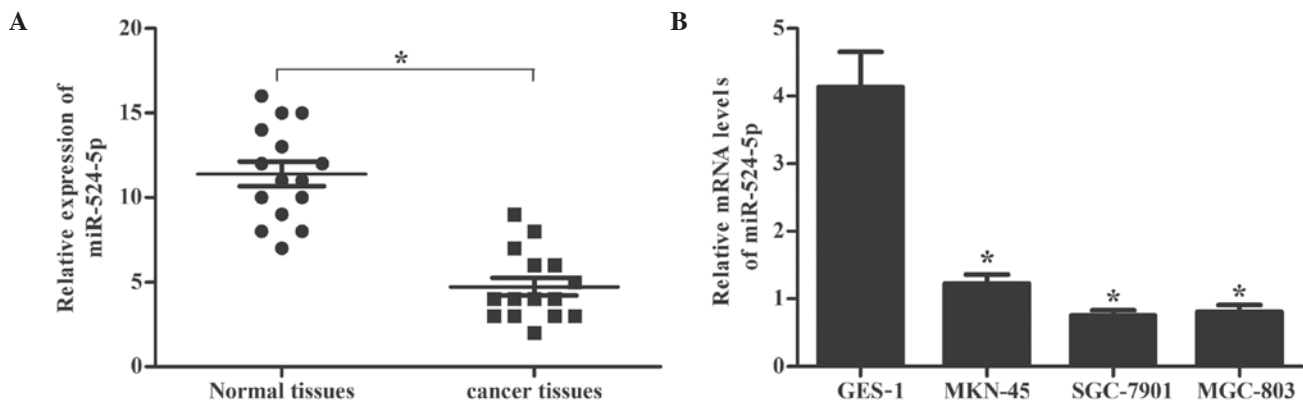


Figure 1. Expression of miR-524-5p in human gastric cancer tissues and cell lines. (A) miR-524-5p expression in 15 pairs of gastric cancer and adjacent non-cancerous tissues was assessed by RT-qPCR. \* $P < 0.05$  vs. normal tissues. (B) miR-524-5p expression in human gastric cancer MKN-45, SGC-7901 and MGC-803 cell lines, and human normal gastric epithelial mucosa GES-1 cell line was evaluated using RT-qPCR. \* $P < 0.05$  vs. GES-1 cells. All data are reported as the mean  $\pm$  standard deviation of 3 independent experiments. miR, microRNA; mRNA, messenger RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

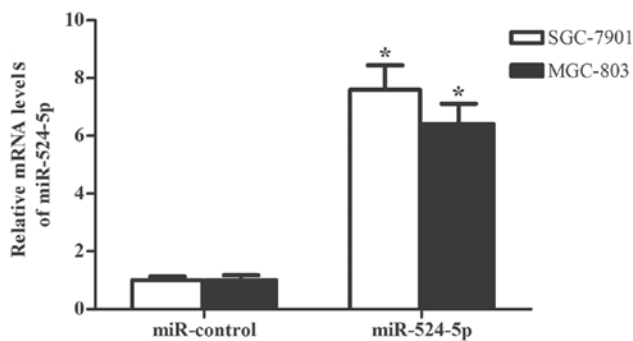


Figure 2. Determination of transfection efficiency. miR-524-5p expression was detected in human gastric cancer SGC-7901 and MGC-803 cells using reverse transcription-quantitative polymerase chain reaction 48 h following transfection with miR-control and miR-524-5p mimic. All experiments were repeated  $\geq 3$  times and all data are reported as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. miR-control. miR, microRNA; mRNA, messenger RNA.

(Sigma-Aldrich), washed twice in PBS, centrifuged at 1,000  $\times$  g for 5 min and resuspended in 195  $\mu$ l Annexin V-fluorescein isothiocyanate (FITC) binding buffer (Sigma-Aldrich). Subsequently, 5  $\mu$ l Annexin V-FITC (BD Biosciences, Erembodegem, Belgium) was added to the cells. Following an incubation period of 15 min in the dark at room temperature, 400  $\mu$ l binding buffer was added. The percentage of apoptotic cells was analyzed using flow cytometry (FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA) and CellQuest Pro™ Software (version 5.2.1; BD Biosciences).

**Cell invasion assays.** Cell invasion was determined using 24-well Transwell chambers coated with Matrigel™ (BD Biosciences, Bedford, MD, USA) in order to determine the effect of miR-524-5p on gastric cancer cell invasion. Briefly, 8- $\mu$ m pore size filters (Sigma-Aldrich) were coated with 100  $\mu$ l Matrigel™ (1 mg/ml; dissolved in serum-free RPMI-1640). A total of 600  $\mu$ l RPMI-1640 containing 10% Invitrogen FBS (Thermo Fisher Scientific) was added to the lower chamber, while single cell suspensions ( $1 \times 10^5$  cells/well) were added to the upper chamber. Cells were incubated for 48 h at 37°C, and non-invading cells were removed using cotton swabs (Takara

Biotechnology Co. Ltd.). Invaded cells were stained with 0.1% crystal violet (Takara Biotechnology Co., Ltd.) for 10 min at room temperature and examined using the Olympus BX51 fluorescence microscope (Olympus Optical Co., Tokyo, Japan).

**Statistical analysis.** The results are presented as the mean  $\pm$  standard deviation. The Student's *t*-test was used to assess the statistical significance of differences between groups. The data were analyzed by SPSS version 11.0 software (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of miR-524-5p in gastric cancer tissues and cell lines.** In order to determine the expression levels of miR-524-5p in gastric cancer, the present study evaluated the expression levels of miR-524-5p in human gastric cancer tissues and cell lines using RT-qPCR. As demonstrated by Fig. 1A, the expression levels of miR-524-5p were markedly lower in gastric cancer tissues, compared with normal gastric mucosa tissue. Similarly, the expression levels of miR-524-5p were significantly decreased in MKN-45, SGC-7901 and MGC-803 cells, compared with human normal gastric epithelial mucosa GES-1 cells (Fig. 1B). These results demonstrate that miR-524-5p is downregulated in gastric cancer tissues and cell lines. SGC-7901 and MGC-803 cells were selected for the subsequent *in vitro* experiments, since the mRNA expression levels of miR-524-5p were lower in these cells than in MKN-45 cells.

**Effects of miR-524-5p on gastric cancer cell proliferation.** Since miR-524-5p was significantly downregulated in gastric cancer, the present study concluded that miR-524-5p may inhibit growth, promote apoptosis and enhance invasion in gastric cancer cells. To verify this hypothesis, the expression levels of miR-524-5p in SGC-7901 and MGC-803 cells were upregulated by transfecting the cells with miR-524-5p mimics, as detected by RT-qPCR (Fig. 2). Subsequently, an MTT assay was performed to evaluate the effects of miR-524-5p on the

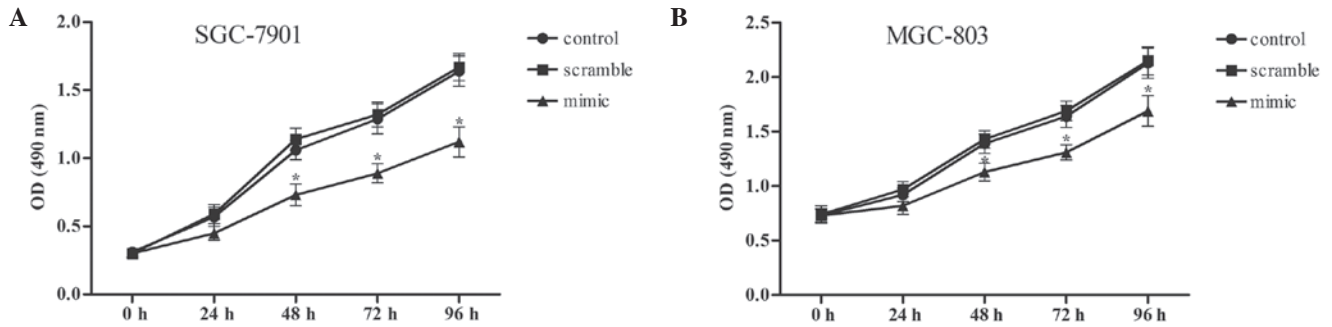


Figure 3. Effects of miR-524-5p on gastric cancer cell proliferation. To evaluate the effects of miR-524-5p on the growth of human gastric cancer SGC-7901 and MGC-803 cells, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay was conducted every 24 h from 0 to 96 h following transfection. miR-524-5p overexpression inhibited the proliferation of (A) SGC-7901 and (B) MGC-803 cells, compared with control and scramble miRNA groups. All experiments were repeated  $\geq 3$  times and all data are reported as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. miR-control. miR, microRNA; mRNA, messenger RNA; OD, optical density.

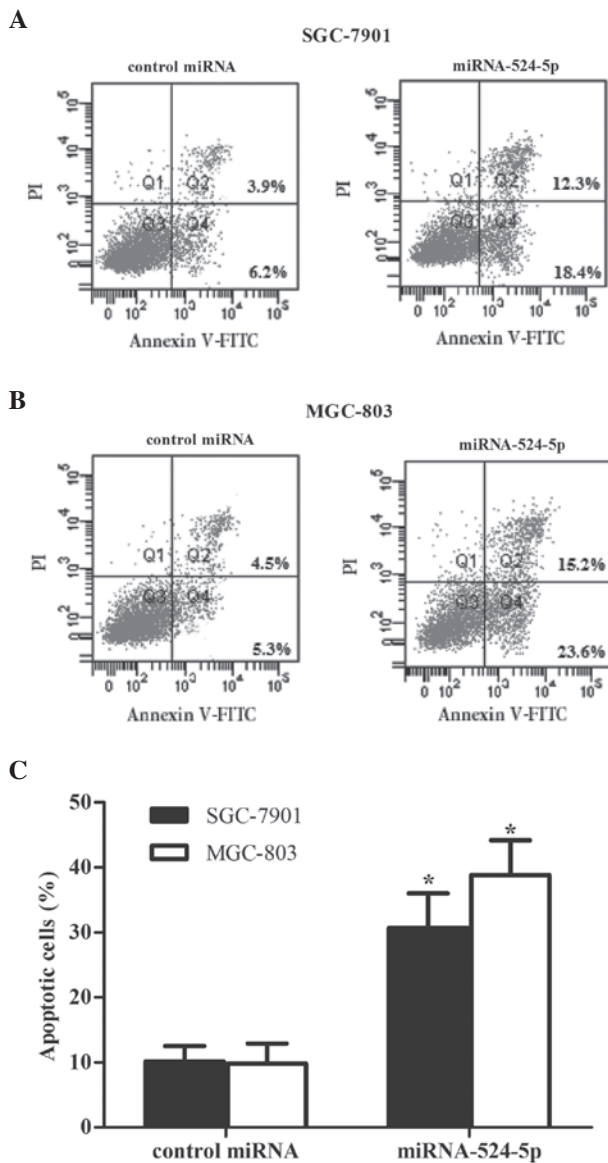


Figure 4. Effect of miR-524-5p on gastric cancer cell apoptosis. The percentage of apoptotic cells in human gastric cancer (A) SGC-7901 and (B) MGC-803 cells was detected by flow cytometry using Annexin V-FITC and PI staining following 48 h of miR-524-5p mimic transfection. (C) Data are reported as the mean  $\pm$  standard deviation of 3 independent experiments. \* $P < 0.05$  vs. miR-control. miR, microRNA; FITC, fluorescein isothiocyanate; PI, propidium iodide.

growth of SGC-7901 and MGC-803 cells. As demonstrated by Fig. 3, miR-524-5p overexpression inhibited the growth of SGC-7901 and MGC-803 cells, and the inhibitory effect of miR-524-5p was markedly increased with increasing transfection times.

*Effect of miR-524-5p on gastric cancer cell apoptosis.* Cell apoptosis was detected using propidium iodide and Annexin V-FITC staining following 48 h of miR-524-5p mimic transfection. The percentage of cells undergoing apoptosis was measured using flow cytometry. Following transfection with miR-524-5p mimics, the percentage of apoptosis significantly increased to 30.7% in SGC-7901 cells (Fig. 4A) and 38.8% in MGC-803 cells (Fig. 4B), compared with 10.1 and 9.8%, respectively, in the control groups. These results demonstrate that miR-524-5p is critical in promoting the apoptosis of gastric cancer cells.

*Effects of miR-524-5p on gastric cancer cell invasion.* The present study examined the effect of miR-524-5p on gastric cancer cell invasion. As demonstrated by Fig. 5, the number of invasive cells was significantly decreased in SGC-7901 and MGC-803 cells transfected with miR-524-5p mimics, compared with miR-control-transfected cells (SGC-7901 miR-524-5p-transfected vs. control cells, 85 vs. 173 cells; MGC-803 miR-524-5p-transfected vs. control cells, 139 vs. 222 cells). These results indicate that overexpression of miR-524-5p inhibits the invasion ability of gastric cancer cells *in vitro*.

*miR-524-5p transfection attenuates cell invasion by inhibiting the expression of MMP-2 and MMP-9.* Numerous studies have demonstrated that extracellular MMP-2 and MMP-9 are overexpressed in various types of cancer (13-15). As demonstrated in Fig. 6, the protein expression levels of MMP-2 and MMP-9 were significantly decreased in SGC-7901 (Fig. 6A) and MGC-803 (Fig. 6B) cells transfected with miR-524-5p mimics. Similarly to the results of western blot analysis, a decrease in the mRNA levels of MMP-2 and MMP-9 was observed using RT-qPCR (Fig. 6C and D). These results demonstrate that transfection with miR-524-5p attenuates gastric cancer cell invasion by inhibiting the expression of MMP-2 and MMP-9.



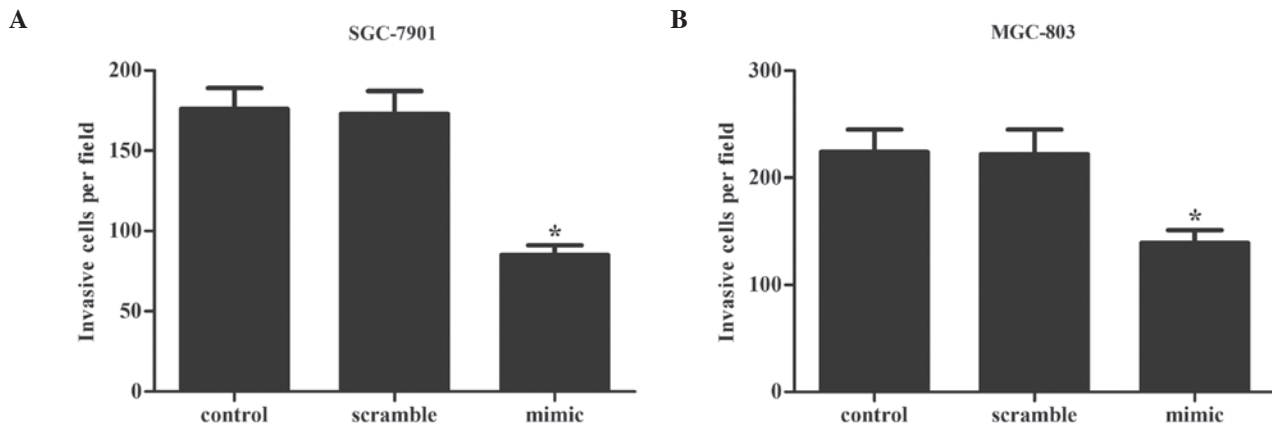


Figure 5. Effects of miR-524-5p on gastric cancer cell invasion. Cell invasion was measured using a Transwell assay 48 h following miR-524-5p transfection. miR-524-5p overexpression inhibited the invasion of human gastric cancer (A) SGC-7901 and (B) MGC-803 cells, compared with control cells. All experiments were repeated  $\geq 3$  times and the data are reported as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. miR-control. miR, microRNA.

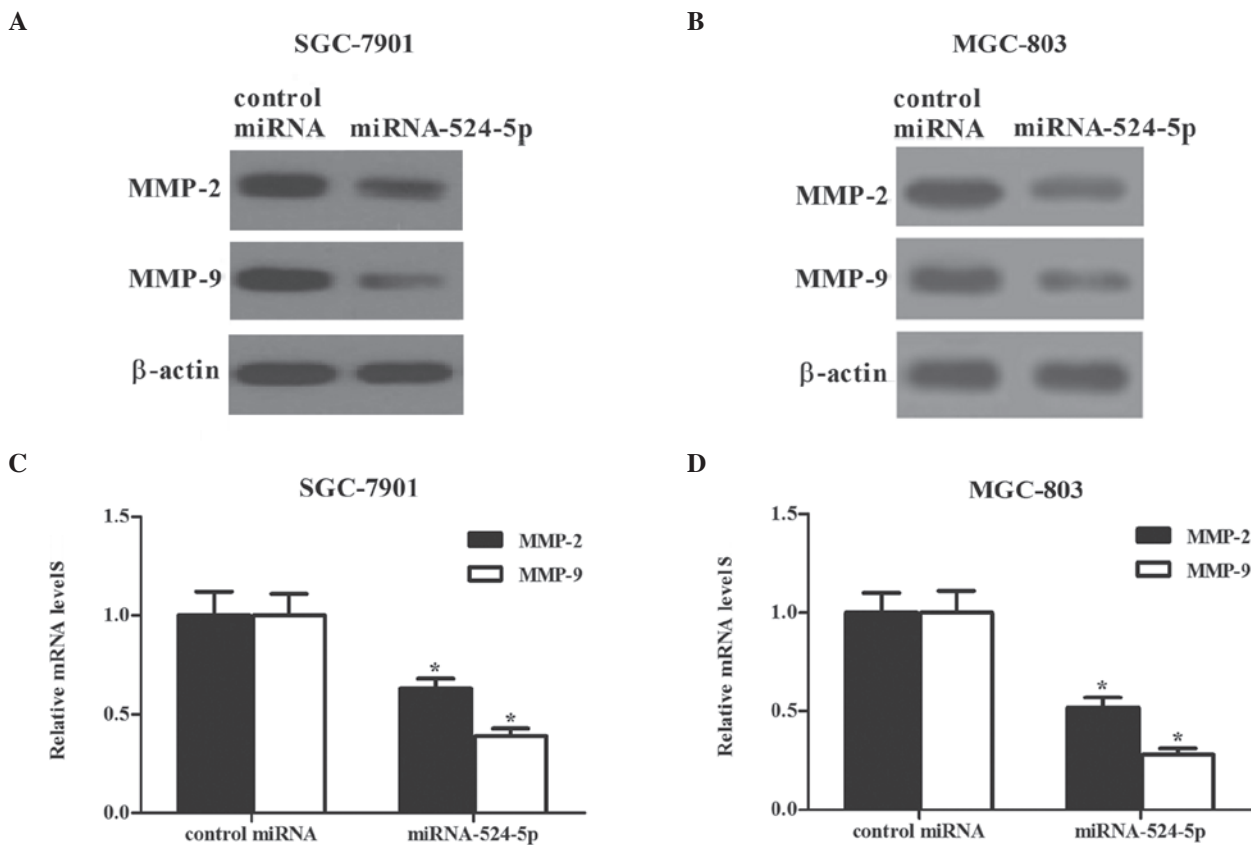


Figure 6. miR-524-5p transfection attenuates cell invasion by inhibiting the expression of MMP-2 and MMP-9. miR-524-5p overexpression decreased the protein levels of MMP-2 and MMP-9 in human gastric cancer (A) SGC-7901 and (B) MGC-803 cells. miR-524-5p overexpression decreased the messenger RNA levels of MMP-2 and MMP-9 in (C) SGC-7901 and (D) MGC-803 cells. All experiments were repeated  $\geq 3$  times and the data are reported as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. miR-control; miR, microRNA; miRNA, microRNA; mRNA, messenger RNA; MMP, matrix metalloproteinase.

**Discussion**

Previous studies have demonstrated that the aberrant expression of miRNAs is associated with the development of various types of human cancer. These studies have also indicated that miRNAs may function as oncogenes or tumor-suppressor genes (16,17). In addition, previous studies have identified numerous dysregulated miRNAs, including miR-30b,

miR-372, miR-126 and miR-21, that modulate growth, apoptosis, migration and invasion in gastric cancer cells (18-23). The present study revealed that the expression levels of miR-524-5p were downregulated in gastric cancer tissues and cell lines, and overexpression of miR-524-5p inhibited gastric cancer cell proliferation and invasion and promoted cell apoptosis. Therefore, the present study suggests that miR-524-5p functions as a tumor suppressor in human gastric cancer.

Dysregulation of miR-524-5p is a frequent event in several types of cancer, including a reduction in the expression of miR-524-5p in melanoma (10) and glioma cells (9). The present study demonstrated that the expression levels of miR-524-5p were downregulated in gastric cancer tissues and cell lines, suggesting that miR-524-5p functions as a tumor suppressor in human gastric cancer, which is consistent with previous studies (24).

Apoptosis is programmed cell-death that occurs in physiological and pathological conditions (25). A disruption in cell apoptosis may result in the development of cancer (26). The current study revealed that ectopic expression of miR-524-5p induced a greater percentage of gastric cancer cells to undergo apoptosis, compared with control cells *in vitro*. These results suggest that miR-524-5p may be involved in gastric cancer by promoting the apoptosis of cancer cells.

In patients with gastric cancer, metastasis is one of the leading causes of mortality (27). Since miR-524-5p was observed to be downregulated in gastric cancer tissues and cell lines, the present study hypothesized that overexpressing miR-524-5p may suppress the metastasis of gastric cancer cells. The results of the present study demonstrated that overexpression of miR-524-5p in human gastric cancer SGC-7901 and MGC-803 cells significantly inhibited metastasis. These findings suggest an inhibitory role for miR-524-5p in the development of metastasis in gastric cancer, which is, to the best of our knowledge, a novel result. In addition, the results of the present study indicate that decreased expression of miR-524-5p in gastric cells may contribute to the development of gastric cancer.

Degradation of the extracellular matrix (ECM) in blood or lymph vessels is critical to develop metastasis, since the loss of the ECM allows the cancer cells to invade the blood or lymphatic system and spread to distant tissues and organs (28). MMPs are a family of zinc-dependent enzymes that are essential for the progression of cancer (29). MMP-2 is expressed on the surface of tumor cells, and is involved in tumor metastasis by activating pro-MMP-2, which exacerbates the malignancy (30). The activation of pro-MMP-2 by membrane-type 1 matrix metalloproteinase (MT1-MMP) is considered to be a critical event in cancer cell invasion. In the activation step, TIMP metalloproteinase inhibitor 2 bound to MT1-MMP on the cell surface acts as a receptor for pro-MMP-2. MT1-MMP is expressed on the cancer cell surface as an invasion-promoting proteinase. By localizing at the leading edge of invasive cancer cells, MT1-MMP degrades components of the tissue barriers. One of the major targets is type I collagen, the most abundant ECM component. Although MT1-MMP itself cannot degrade type IV collagen in the basement membrane, it binds to and activates proMMP-2, one of the type IV collagenases. However, degradation of the ECM is not the sole function of MT1-MMP (31). MMP-2 is considered an important marker for the presence of distant metastasis in cancer (32). MMP-9 is also responsible for the degradation of the ECM, and is pivotal in tumor invasion and metastasis (33). The activated MMP-9 enhances the invasive phenotype of the cultured cells as their ability to both degrade extracellular matrix and transverse basement membrane is significantly increased following zymogen activation.

Liao *et al* (34) reported that miRNA-196 (miR-196) is highly expressed in gastric cancer, and overexpression of miR-196 significantly induced migration and invasion of gastric cancer cells by decreasing the levels of MMP-2 and MMP-9. By contrast, the present study investigated whether the inhibitory role of miR-524-5p on cell invasion occurs through regulating the expression of MMP-2 and MMP-9. The present study revealed that miR-524-5p downregulated the expression levels of MMP-2 and MMP-9, which explains the inhibition of invasion caused by miR-524-5p in gastric cells during the invasion assay.

In summary, the results of the present study indicate that miR-524-5p is a tumor suppressor gene that is involved in the development of gastric cancer. Overexpression of miR-524-5p inhibits cell proliferation and invasion, and promotes apoptosis in gastric cancer cells. Therefore, miR-524-5p may be a novel therapeutic target for the treatment of gastric cancer.

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