miR-506 Inhibits Epithelial-to-Mesenchymal Transition and Angiogenesis in Gastric Cancer

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Accepted for publication May 26, 2015.

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Gastric cancer is one of the most common malignancies in developing countries. We examined the possible role of miR-506 in gastric cancer, investigated its associations with the clinical outcomes of gastric cancer patients, and explored its potential role in angiogenesis and the metastasis of gastric cancer cells. We found that miR-506 expression was a useful marker for stratifying patients from early to advanced clinical stages and for overall survival prediction. miR-506 overexpression inhibited the epithelial-to-mesenchymal transition of gastric cancer cells; however, depletion of miR-506 promoted it. In addition, miR-506 suppressed gastric cancer angiogenesis and was associated with decreased matrix metalloproteinase-9 expression. We also found that ETS1 was a miR-506 target, and it was expressed in 71.10% of gastric cancer tissue samples. Moreover, ETS1 expression was associated with matrix metalloproteinase-9 expression ($P < 0.001$). In conclusion, miR-506 was identified as an ETS1 targeting suppressor of metastatic invasion and angiogenesis in gastric cancer. (Am J Pathol 2015, 185: 2412–2420; http://dx.doi.org/10.1016/j.ajpath.2015.05.017)

Although the incidence of gastric cancer has declined by >80% in developed countries during the past 50 years, it remains one of the most significant health problems in the developing countries.1–3 In China, gastric cancer is the third leading cause of cancer-related death.4 Gastric cancer patients in China and most other developing countries are usually diagnosed during the advanced stages of the disease. Because of the high likelihood of metastasis in advanced gastric cancer, the characteristically late diagnosis contributes to the high rate of gastric cancer mortality.5 A better understanding of the molecular mechanisms underlying gastric cancer development and progression could help the development of novel approaches for the early detection and treatment of this type of cancer.

In recent years, post-transcriptional regulation has been intensively studied as a phenotype-level control mechanism of gene expression regulation.6 Among the post-transcriptional regulators with metastatic activity, one of the most studied classes is small noncoding RNAs.7–9 miRNAs are a class of small noncoding RNAs that regulate gene expression by binding to the 3′-untranslated region (3′-UTR) of target genes. miRNA regulation results in mRNA degradation and the inhibition of protein translation.10 Although an increasing number of studies examining different miRNAs in various pathophysiological settings have been reported,11,12 only a few of these interactions have been validated experimentally.

In this study, we demonstrate that miR-506 is deregulated in metastatic gastric cancer cell lines and that miR-506 deficiency is associated with poor overall gastric cancer patient survival. In addition, we report that miR-506...
overexpression in gastric cancer inhibits endothelial cell angiogenesis and metastatic invasion by targeting the proto-oncogene *ETS1*, which plays several important roles in the angiopoietic and developmental processes of cancer cells. Therefore, we hypothesized that miR-506 targeting of *ETS1* influences the angiogenic activity of endothelial cells and gastric cancer metastasis.

Materials and Methods

Patients and Tissue Specimens

For miR-506 detection, gastric cancer specimens were obtained from 109 patients who underwent surgery without any radiotherapy, chemotherapy, or biotherapy from The Department of Pathology, The Third Affiliated Hospital, Kunming Medical University (Kunming, China), from January 2008 through November 2013. After collection, samples were immediately frozen and kept in liquid nitrogen until further use. Follow-up data were collected from 84 of the 109 patients. For immunohistochemistry, specimens from 173 gastric cancer patients were collected from The Department of Pathology, The Third Affiliated Hospital, Kunming Medical University, from January 2003 through November 2008. For all patients, detailed clinicopathological data were collected, including sex, age, TNM staging, and clinical staging, as defined according to the criteria of the American Joint Commission on Cancer (seventh edition). Eight normal gastric tissue samples were obtained from macroscopically uninvolved areas 2 to 3 cm away from the benign nodules of patients with stomach leiomyoma (*n* = 5) or adenomyoepithelioma (*n* = 3) who underwent surgical resection. All normal gastric tissues were histopathologically assessed and were morphologically normal. This study was approved by the Ethics Committee of The Third Affiliated Hospital, Kunming Medical University, and all patients provided written informed consent.

Real-Time PCR

For miRNA expression analysis, frozen tissue specimens were ground into powder in liquid nitrogen. Total RNA was extracted from 50 to 100 mg of the powdered tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer’s protocols. Real-time PCR for miR-506 was performed in a reaction mixture containing SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan). Quantitation of miRNAs was performed using an ABI 7500 system (Life Technologies, Grand Island, NY), according to the manufacturer’s protocol. All PCRs were performed in triplicate, and a relative quantitative method was applied using the averaged ΔCₜ from the normal tissues or untreated cells. The endogenous control was U6.

Cell Lines and Culture

Primary cultures of normal gastric epithelial cells were established from fresh specimens of the adjacent noncancerous gastric tissue taken from an area over 10 cm from the cancerous tissue. The human gastric cancer cell lines AGS, BGC-823, HGC-27, Kato-III, SGC-7901, MKN45, and MGC-803 were supplied and authenticated by the Cell Bank of Shanghai Institute of Cell Biology and the Chinese Academy of Sciences (Shanghai, China). Cell lines were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO) in a humidified 5% CO₂ atmosphere at 37°C.

Construction and Transfection of Expression Vectors Containing miR-506 Inhibitors and Mimics

The miR-506 mimics, inhibitors, and negative control were purchased from RiboBio Co Ltd (Guangzhou, China). For gene transfection, gastric cancer cells were grown overnight in the logarithmic growth phase and transiently transfected with miR-506 mimic, inhibitor, or negative control vectors using Lipofectamine 2000 (Invitrogen) for 48 hours, according to the manufacturer’s protocol, before being subjected to analyses.

Protein Extraction and Western Blot Analysis

Total cellular protein was extracted from gastric cancer cells with or without miRNA transfection using a lysis buffer containing protease and phosphatase inhibitors. After protein quantification using a BCA Protein Assay Kit (Pierce 23,227; Thermo Fisher Scientific Inc., Rockford, IL), equal amounts of protein samples (50 g) were separated using SDS-PAGE and then transferred onto a 0.22-μm polyvinylidene difluoride membrane (ISEQ00010; Millipore, Billerica, MA). For Western blot analysis, membranes were first incubated with 8% skimmed milk/Tris-buffered saline/Tween 20 at room temperature for 4 hours and then with a primary antibody overnight at 4°C. The following primary antibodies were used: mouse monoclonal anti-β-catenin (sc-59778; Santa Cruz Biotechnology Inc., Dallas, TX), mouse monoclonal anti-vimentin (sc-6260; Santa Cruz Biotechnology Inc.), and rabbit polyclonal anti-β-actin (ab8227; Abcam, Cambridge, UK). The following day, membranes were washed three times with Tris-buffered saline/Tween 20 and incubated for 1 hour with a secondary antibody (1:10,000 dilution for anti-mouse horseradish peroxidase—linked antibody 7074; 1:20,000 dilution for anti-rabbit horseradish peroxidase—linked antibody 7074; Cell Signaling Technology, Danvers, MA) and then detected using an ECL kit (RPN2235; Amersham ECL Select; GE Healthcare Life Sciences, Buckinghamshire, UK). Data were quantified using ImageJ software version 1.48u (NIH, Bethesda, MD; [http://imagej.nih.gov/ij](http://imagej.nih.gov/ij)) and β-actin or glyceraldehyde-3-phosphate dehydrogenase levels.
Wound-Healing Assay

SGC-7901 or BGC-823 cells were seeded at $2 \times 10^6$ cells per well in 6-well plates, grown to 100% confluency, and then kept in serum-free RPMI 1640 medium for 24 hours. Next, a wound was generated across the cell monolayer using a 100-µL plastic pipette tip. Cell migration into the wound area was inspected under an inverted microscope for up to 24 hours after scarification. Quantitative analysis of the wound closure was calculated by measuring the initial width of the wound and the final width of the wound and calculating the distance of wound closure.

Matrigel Invasion Assay

SGC-7901 or BGC-823 cells were seeded at $5 \times 10^4$ cells per well with serum-free medium into the upper Matrigel invasion chamber (Corning Inc., Corning, NY), the filter of which was precoated with Matrigel (Sigma-Aldrich Shanghai Trading Co. Ltd., Shanghai, China) and the lower chamber of which was filled with a medium containing 10% fetal bovine serum as a chemoattractant. The cells were cultured for 20 hours and, at the end of the experiment, the cells remaining in the upper chamber were carefully removed and the cells that had invaded the bottom of the membrane were fixed and stained with hematoxylin. Quantification was performed by counting the stained cells.

Three-Dimensional Cell Culture

Matrigel was dissolved at 4°C overnight and then used to coat 24-well plates (100 µL per well). After a 30-minute incubation at 37°C, BGC-823 or SGC-7901 cells ($2 \times 10^4$) suspended in 2% liquid Matrigel were inoculated into the Matrigel-coated wells. Cells were then grown in a cell culture box for 10 to 14 days, and fresh culture medium was added every 3 to 4 days.

Tube Formation Assay

Cells ($5 \times 10^4$ viable cells per well) were seeded onto a 48-well polystyrene plate coated with Matrigel (120 µL per well), which had been incubated at 37°C and 5% CO₂ for 30 minutes. Six hours after seeding, representative phase-contrast images were obtained at ×4 magnification.

CAM Assay

Fertilized white leghorn chicken eggs were incubated in an incubator at 37°C with 60% humidity. A small window was made in the shell on day 7 of chick embryo development under aseptic conditions. The window was resealed with sterile adhesive tape, and the eggs were returned to the incubator until day 11 of chick embryo development. On day 11, 10-µL cell suspensions of BGC-823 or SGC-7901 cells were placed on top of the chick chorioallantoic membrane (CAM), and the eggs were resealed and returned to the incubator for 72 hours until day 14 ($n = 6$ chicken embryos per cell line). Fixation was performed using formaldehyde and acetone (1:1), and prefixation was performed for 15 minutes through the window. The transplanted tumors and surrounding chorioallantoic membrane were integrally unloaded for observation and imaging.

Gelatin Zymography Assay

For the gelatin zymography assay, proteins were extracted from the cultured supernatant of the BGC-823 or SGC-7901 gastric cancer cells, with or without miRNA transfection. Equal amounts of protein samples (30 µg) were separated by electrophoresis under denaturing conditions (SDS-PAGE). After electrophoresis, the gel was washed four times in electrophoresis buffer and incubated overnight in gelatin (0.1%). After washing twice in electrophoresis buffer, the gel was incubated in substrate buffer (0.1% gelatin, 50 mM Tris, pH 8.8) for 48 hours at 37°C. Next, after the gel was rinsed three times, it was stained using SimplyBlue Safestain (Invitrogen) and incubated for 3 hours at room temperature under gentle agitation. Next, SimplyBlue SafeStain was removed, and the gel was destained according to the manufacturer’s instructions. The gel was scanned with an image analysis system (Quantity One; Bio-Rad, Hercules, CA).

Luciferase Assay

TargetScan (http://www.targetscan.org, last accessed May 20, 2015) was used to predict whether miR-506 targeted the...
3′-UTRs of ETS1. Mutations in the miR-506 target sites were generated. For the luciferase assay, cells (2 × 10^5) were seeded in triplicate in 6-well plates and allowed to settle for 24 hours. Different concentrations of miR-506 mutant, inhibitor, mimics, or the control-luciferase plasmid were synthesized at Kunming Medical University and RiboBio Co Ltd. Plasmids were transfected into gastric cancer cells using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s recommendations. Luciferase signals were measured 48 hours after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI), according to the manufacturer’s protocol. Three independent experiments were performed, and the data are presented as the means ± SD.

Immunohistochemistry

An EnVision detection kit (GK500705; Dako, Glostrup, Denmark) was used for immunohistochemical analysis of ETS1 and matrix metalloproteinase (MMP)-9 proteins in gastric cancer tissues, according to the manufacturer’s protocol. Briefly, primary antibodies were diluted in phosphate-buffered saline, according to the manufacturers’ recommendations: 1:50 for ETS1 (NCL-ETS-1; Novocastra, Newcastle upon Tyne, UK) and 1:400 for MMP-9 (sc-21733; Santa Cruz Biotechnology Inc.). A total volume of 50 μL diluted antibody solution was added to each section and incubated overnight at 4°C. The next day, after washing with phosphate-buffered saline, sections were incubated with Dako REAL EnVision/HRP (Dako Corp., Carpinteria, CA) for 3 minutes, after which slides were rinsed in water, counterstained with hematoxylin, and mounted with Eukitt (O. Kindler GmbH & Co, Freiburg, Germany). ETS1 and MMP-9 protein expression in gastric cancer tissue specimens, as detected by antibodies, was reviewed and scored under a light microscope by two independent pathologists.

**Table 1** Association of miR-506 Expression with Clinicopathological Data from Gastric Cancer Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of samples</th>
<th>miR-506 high expression tumors, No. (%)</th>
<th>P value</th>
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<tr>
<td>Female</td>
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<td>&lt;50</td>
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<tr>
<td>≥50</td>
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<tr>
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<td>Metastasis (lymph node and distant)</td>
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<td>Lymph node metastasis, N stage</td>
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<td>Distant metastasis, M stage</td>
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<td>I</td>
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<tr>
<td>IV</td>
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<td>2 (25.00)</td>
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*P < 0.05.

Table 1: Association of miR-506 Expression with Clinicopathological Data from Gastric Cancer Patients

![Figure 2](image-url) Regulation of epithelial-to-mesenchymal transition by miR-506 overexpression. A: Matrigel invasion: SGC-7901 cells (1 × 10^5) were transfected with miR-506 mimics or negative control (NC), and the number of cells that invaded the basal side of the Matrigel-coated transwell inserts was counted after 24 hours. B: Wound-healing assay: cells from the same transfection as in A were seeded into 6-well plates, and a wound was generated at 24 hours after transfection. C: The pseudopodia growth of SGC-7901 cells from the same transfection as in A was assessed through three-dimensional cell culture. The cells were grown in a cell culture box for 10 to 14 days. D: Western blot analysis of the epithelial adherent markers E-cadherin and α-catenin, and mesenchymal markers N-cadherin and vimentin, in the same SGC-7901—transfected cells as in A. Both blots were reprobed with β-actin to control for protein loading. Error bars represent means ± SD (A); n = 3 to 7 (A); n = 3 (C). *P < 0.05.
Statistical Analysis

All statistical analyses were performed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL). The t-test was used to evaluate whether there was a significant difference between the two groups of data in all of the pertinent experiments. \( P < 0.05 \) (using a two-tailed paired t-test) was considered statistically significant.

Results

High Expression of miR-506 Is Associated with Longer Survival Times of Gastric Cancer Patients

To observe whether miR-506 expression levels affected the clinical outcomes of gastric cancer patients, we used quantitative real-time PCR in a blinded manner to detect miR-506 expression levels in a cohort of human gastric cancer samples. Strikingly, miR-506 expression levels robustly stratified advanced clinical stage and early-stage patients (Figure 1A and Table 1). Patients whose primary gastric cancer lesions expressed high miR-506 levels (miR-506 levels greater than the mean) exhibited significantly...
miR-506 Inhibits the EMT of Gastric Cancer Cells

Because epithelial-to-mesenchymal transition (EMT) is an important process that enables cancer cells to invade their surroundings and metastasize, we examined whether miR-506 overexpression correlated with EMT in human gastric cancer cell lines. We observed, as expected, that miR-506 overexpression reduced the ability of SGC-7901 cells to invade through Matrigel (Figure 2A). In addition, wound-healing assays revealed that the migration ability of SGC-7901 cells was diminished (Figure 2B). These results indicated that miR-506 could be a potential suppressor of gastric cancer metastasis. Furthermore, the growth of gastric cancer cell pseudopodia is considered a prometastatic phenotype, and this was robustly decreased by miR-506 overexpression (Figure 2C). Consistent with this, the expression of E-cadherin and α-catenin was elevated, and the expression of N-cadherin and vimentin was decreased by miR-506 overexpression (Figure 2D), suggesting that miR-506 acts as an epithelial transition promoter in gastric cancer cells.

Consistent with the miR-506 overexpression results, miR-506 inhibitors significantly increased the invasive (Figure 3A) and motive (Figure 3B) ability of BGC-823 cells. Furthermore, miR-506 suppression increased the growth of gastric cancer cell pseudopodia (Figure 3C).

Similarly, Western blot analysis showed that miR-506 inhibition caused decreased expression of E-cadherin and α-catenin, as well as increased expression of N-cadherin and vimentin (Figure 3D). Collectively, these findings indicate that miR-506 plays a role in EMT suppression in gastric cancer cells.

miR-506 Suppresses Angiogenesis in Gastric Cancer

Because angiogenesis is an important factor in cancer metastasis, we examined the angiogenic effects of miR-506 expression in gastric cancer cells. A Matrigel-based tube formation assay using SGC-7901 cells showed that miR-506 overexpression suppressed vascular tubule development (Figure 4A). Accordingly, as quantified by histological morphometric evaluation of CAM sections, vessel density was significantly reduced in SGC-7901 cells with miR-506 overexpression (Figure 4B). Consistent with this, gelatin zymography assays showed that the expression levels of MMP-9 and MMP-2 were decreased by miR-506 overexpression (Figure 4C). This suggests that miR-506 contributes to extracellular matrix maintenance in the gastric cancer microenvironment. Conversely, miR-506 inhibition in BGC-823 cells promoted the formation of a tubular polygonal network (Figure 4D) and induced vessel growth on the CAM (Figure 4E). Furthermore, gelatin zymography assays showed that miR-506 expression inhibition was associated with the expression of MMP-9 and MMP-2 (Figure 4F). These findings indicate that miR-506 is necessary and sufficient for angiogenesis suppression during gastric cancer progression.

ETS1 Is a Direct Target of miR-506 Regulation

We next investigated the direct molecular targets of miR-506. The miR-506 target genes were predicted and selected using TargetScan software. On the basis of our analysis, we found that miR-506 targets the 3′-UTRs of ETS1 (Figure 5A). The expression levels of ETS1 and MMP-9 were lower in miR-506–transfected SGC-7901 cells; however, their expression was augmented after miR-506 inhibition in BGC-823 cells (Figure 5B). To confirm miR-506

Figure 5 Identification of ETS1 as a direct target gene of miR-506. A: miR-506 targets the 3′-untranslated region (UTR) of ETS1 of multiple species, such as Homo sapiens, Pan troglodytes, Macaca nemestrina, and Mus musculus. B: miR-506 was overexpressed or inhibited in BGC-823 and SGC-7901 gastric cancer cell lines, respectively, to monitor the expression of ETS1 and matrix metalloproteinase (MMP)-9 by Western blot analysis. C: Luciferase activity of ETS1 3′-UTR in SGC-7901 and BGC-823 cells transfected with control miRNA [negative control (NC)], miR-506 mimics, or miR-506 inhibitors. Error bars represent means ± SD (C). n = 3 (C). *p < 0.05.
interaction with the 3′-UTR of ETS1, wild-type or mutant miR-506–binding sequences from the 3′-UTR of the ETS1 gene were cloned into the 3′-UTR of the luciferase reporter gene for luciferase assays. Overexpression of miR-506 with the wild-type ETS1 3′-UTR in SGC-7901 and BGC-823 cells resulted in a significant decrease in luciferase activity; however, luciferase activity was not decreased by overexpression of miR-506 with mutant 3′-UTR miR-506–binding sequences (Figure 5C). Collectively, these results indicate that ETS1 may be a potential target mediating miR-506–dependent regulation in gastric cancer cells.

The Expression of ETS1 Is Associated with MMP-9 in Gastric Cancer Tissues

Because MMP-9 is known to participate in gastric cancer metastasis and ETS1 was reported to be an MMP9 transcription factor, we examined the expression of both proteins in gastric cancer tissues. Protein expression levels were examined in a cohort of human gastric cancer samples in a blinded manner. Nuclear accumulation of ETS1 was observed in 71.10% (123/173) of the gastric cancer tissues examined (Figure 6, A–C), whereas 75.14% (130/173) of the tissue samples examined stained positively for MMP-9 (Figure 6, D–F). ETS1 expression was associated with MMP-9 expression (r = 0.459, P ≤ 0.0001) (Table 2), indicating that ETS1 and MMP-9 synergistically regulate gastric cancer metastasis.

Discussion

EMT and angiogenesis are two essential processes in cancer progression.16,17 In this study, we report a significant role for miR-506 in gastric cancer EMT and angiogenesis suppression. In addition, we demonstrated that higher endogenous miR-506 expression was associated with longer survival times in gastric cancer patients. miR-506 is located at Xq27.3, a chromosomal region closely associated with fragile X syndrome.18 Down-regulation of miR-506 has been detected in several solid tumors, including serous ovarian cancer and breast cancer,19,20 suggesting that miR-506 plays an important role in tumor suppression. Our study presents evidence that miR-506 is a potent inhibitor of EMT and that miR-506 overexpression is associated with decreased vimentin and increased E-cadherin expression in gastric cancer. We also explored the potential role of miR-506 in angiogenesis and found that miR-506 both suppresses angiogenesis in gastric cancer and is associated with decreased MMP-9 expression. These findings suggest that miR-506 acts as a suppressor of angiogenesis and metastasis in gastric cancer.

We also demonstrated that ETS1 is a direct target of miR-506. The necessity of ETS1 for endothelial cells to adopt an angiogenic, blood vessel–forming phenotype has been well documented.21–23 Accordingly, ETS1 is abundant in regenerating adult tissues and in areas of the developing embryo that require the formation of new blood vessels.24,25

<table>
<thead>
<tr>
<th>MMP-9</th>
<th>ETS1, No. (%)</th>
<th>P value</th>
<th>R</th>
</tr>
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<tr>
<td>Positive (n = 43)</td>
<td>28 (65.12)</td>
<td>15 (34.88)</td>
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</tr>
<tr>
<td>Positive (n = 130)</td>
<td>22 (16.92)</td>
<td>108 (83.08)</td>
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MMP, matrix metalloproteinase.
Because the acquisition of invasive behavior is a part of the endothelial activation program, ETS1 may be responsible for stimulating proteases necessary for these processes.26,27 Likewise, ETS1 may help cancer cells get nutrients and oxygen by inducing tumor vascularization, and may promote tumor metastasis and invasion by activating ECM-degrading proteases in tumor or mesenchymal cells.28 Thus, high ETS1 levels in tumors often correlate with a poorer prognosis.22

ETS1 was reported to be an MMP-9 transcription factor.29 MMP-9 plays a critical role in promoting tumor progression by degrading the extracellular matrix and altering cell adhesion.30 In this study, immunohistochemistry of clinical gastric cancer samples showed that ETS1 expression was associated with MMP-9 expression, indicating that ETS1 and MMP-9 synergistically regulate gastric cancer metastasis. Thus, it is possible that a miR-506–ETS1 axis plays an important role in the regulation of gastric cancer angiogenesis.

In summary, cancer is a complex disease and controlling cancer development and progression requires system-level and integrative approaches. Our study revealed the functional relevance of miR-506 with respect to angiogenesis and metastasis, suggesting that miR-506 acts as a tumor suppressor in gastric cancer. Additional studies will be needed to explore the potential clinical utility of miR-506 as a potential biomarker for gastric cancer prognosis and as a new potential therapeutic target.

Acknowledgments

Z.L. performed cellular functional studies and drafted the manuscript. Z.L. and S.D. performed cellular studies and statistical analysis and wrote the manuscript. J.Z. and J.T. collected the clinical tissue samples. J.Y. communicated and followed up with patients. Y.W. and M.L. performed immunohistochemistry. C.G., R.L., and Y.X. performed cell culture and protein extraction. W.W., X.X., and T.G. collected the clinical tissue samples. J.Y. communicated and followed up with patients. Y.W. and M.L. performed immunohistochemistry. C.G., R.L., and Y.X. performed cell culture and protein extraction. W.W., X.X., and T.G. analyzed clinical samples. H.D. performed real-time PCR. K.Y. designed the study. X.S. conceived, designed, and coordinated the study. All authors read and approved the final manuscript.

References


