MiR-196a Is Upregulated in Gastric Cancer and Promotes Cell Proliferation by Downregulating p27kip1

Ming Sun1, Xiang-hua Liu1, Jin-hai Li2, Jin-song Yang3, Er-bao Zhang1, Dan-dan Yin1, Zhi-li Liu4, Jing Zhou4, Ying Ding1, Su-qin Li5, Zhao-xia Wang1, Xiu-feng Cao3, and Wei De1

Abstract

Aberrant expression of miR-196a has been frequently reported in cancer studies. However, the expression and mechanism of its function in gastric cancer remains unclear. Quantitative real-time PCR was carried out to detect the relative expression of miR-196a in gastric cancer cell lines and tissues. SGC7901 cells were treated with miR-196a inhibitors, mimics, or pcDNA/miR-196a to investigate the role of miR-196a in cell proliferation. Higher expression of miR-196a in gastric cancer tissues was associated with tumor size, a higher clinical stage, and was also correlated with shorter overall survival of patients with gastric cancer. Exogenous down-regulation of miR-196a expression significantly suppressed the in vitro cell-cycle progression, proliferation, and colony formation of gastric cancer cells, and ectopic miR-196a expression significantly enhanced the development of tumors in nude mice. Luciferase assays revealed that miR-196a inhibited p27kip1 expression by targeting one binding site in the 3'-untranslated region (3'-UTR) of p27kip1 mRNA. qPCR and Western blot assays verified that miR-196a reduced p27kip1 expression at both mRNA and protein levels. The p27kip1-mediated repression in cell proliferation was reverted by exogenous miR-196a expression. A reverse correlation between miR-196a and p27kip1 expression was noted in gastric cancer tissues. Our study shows that aberrant overexpression of miR-196a and consequent downregulation of p27kip1 could contribute to gastric carcinogenesis and would be targets for gastric cancer therapies and further developed as potential prognostic factors. Mol Cancer Ther; 11(4); 842–52. ©2012 AACR.

Introduction

Gastric cancer is the second leading cause of cancer death and is the most common gastrointestinal malignancy in East Asia, Eastern Europe, and parts of Central and South America (1). Malignant proliferation has been major reason for the dismal outcome of patients with gastric cancer. Increased cell proliferation is a common feature of gastric malignant proliferation. Gastrectomy remains the mainstay treatment of gastric cancer, but the prognosis for advanced stage patients is still very poor (2). Therefore, better understanding of the pathogenesis and identification of the molecular alterations is essential for the development of diagnostic markers that aid novel effective therapies for gastric cancer (3–5). Because a miRNA is able to regulate hundreds of genes (6), the aberrant expression of miRNA has been frequently reported in gastric cancer, suggesting that miRNA dysregulation is involved in the development and progression of gastric cancer (7).

miRNAs are a class of small noncoding RNAs, which act as posttranscriptional regulators by inhibiting gene expression through either cleavage of the target mRNA or translational repression (8). Generally, one miRNA can regulate multiple target genes and one gene can be repressed by multiple miRNAs, which results in the formation of complex regulatory pathways (9). Previous studies have suggested that miRNA have major functions during carcinogenesis and may function as oncopgenes or tumor suppressors based on their inhibition of tumor suppressor or oncogene target miRNAs, respectively (10–12). For instance, let-7 family has been shown to be underexpressed in various cancers and can impair tumorigenesis through inhibition of RAS family, HMGA2, and other oncogenes (13, 14). The differential expression profile of miRNAs during gastric cancer has been established. However, the genes targeted by most of these miRNAs are still unknown (15–17). Thus, elucidating the biologic consequences of miRNA dysregulation and identifying the targets of miRNAs is critical to understanding miRNA pathways and their underlying molecular mechanisms. This will help us to better understand the pathogenesis...
and development of gastric cancer and to develop miRNA-directed diagnostics and therapeutics against this deadly disease.

In the initial step of our study, we conducted qPCR analysis of miR-196a expression in human gastric cancer tissues and showed that miR-196a was overexpressed in tumor-derived samples and gastric cancer cell lines compared with matched normal samples. miR-196a upregulation was also correlated with tumor size, advanced pathologic stage, and patient survival time. Further functional analyses of miR-196a indicated that the enhanced expression of miR-196a could promote gastric cell-cycle progression and proliferation in vitro and in vivo. Luciferase reporter assay and Western blot confirm that miR-196a may function as an oncogene by directly targeting p27kip1 (also known as CDKN1B). The p27kip1-mediated repression in cell proliferation was reverted by exogenous miR-196a expression. A reverse correlation between miR-196a and p27kip1 expression in gastric cancer tissues was noted by consecutive further analysis.

Materials and Methods

Tissue collection

Gastric cancer tissues and normal tissues were obtained from patients who had undergone surgery at Jiangsu province hospital between 2007 and 2008 and were diagnosed with gastric cancer (stage II, III, and IV) based on histopathologic evaluation. No local or systemic treatment was conducted in these patients before the operation. All the tissue samples were collected, immediately snap frozen in liquid nitrogen, and stored at −80°C until RNA extraction. The study was approved by the Research Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, PR China). Informed consents were obtained from all patients.

Cell lines and culture conditions

Human gastric adenocarcinoma cancer cell lines MGC-803, MKN-28, and SGC-7901 and the normal gastric epithelium cell line (GES-1) were obtained from Chinese Academy of Sciences Committee on Type Culture Collection cell bank. All cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 10% FBS (Invitrogen) at 37°C in incubator with 5% CO2.

RNA extraction and qPCR analyses

Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Invitrogen). For qPCR, RNA was reverse transcribed to cDNA from 1 μg of total RNA by using a Reverse Transcription Kit (Takara). Real-time PCR (RT-PCR) analyses were conducted with Power SYBR Green (Takara). All protocols were carried out according to the manufacturer’s instructions. Results were normalized to the expression of U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequence:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5'-AACGCTTCACGAATTTGCCGT-3'</td>
<td>5'-AGAGGCCAGGGATGATGTTCTG-3'</td>
</tr>
<tr>
<td>U6</td>
<td>5'-CTCGCTTCGGCCAGCACCA-3'</td>
<td>5'-GACTCATGACCCAGTCCATGC-3'</td>
</tr>
</tbody>
</table>

qPCR and data collection were carried out on ABI 7500.

miRNA mimic and inhibitor transfection

Gastric cancer cells were transfected with miR-196a mimic, miR-196a inhibitor (Applied Biosystems), or scrambled sequence (GenePharma) with a final concentration 100 pm by using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. At 48 hours after transfection, cells were harvested for Western blotting or qPCR analyses.

Cell proliferation assays

A cell proliferation assay was conducted with MTT kit (Sigma; ref. 18) according to the manufacturer’s instruction. For the colony formation assay, 500 cells were placed into each well of 6-well plate and maintained in media containing 10% FBS for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma) in PBS for 15 minutes. Colony formation was determined by counting the number of stained colonies in 3 randomly selected fields with an inverter microscope. Triplicate wells were measured in each treatment group.

Flow cytometry

SGC-7901 cells transiently transfected with miR-NC, miR-196a mimics, anti-miR-NC, miR-196a inhibitors, pEGFP-NC, and stably transfected with pEGFP-p27kip1 were harvested 48 hours after transfection by trypsinization. After the double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide was done by the FITC Annexin V Apoptosis Detection Kit (BD

miR-196a:

Reverse-transcribed primer

5'-GTCAGAAGGAATGTACAGCCACACAAACA-3';
Forward: 5'-CGTCAGAAAGGAATGTCACAG-3';
Reverse: 5'-ACCTGCGTATGTTCTCATGT-3';

p27kip1:

Forward: 5'-TGCAACCCAGCATTCTCTACTCA-3';
Reverse: 5'-CAACGAGTGATGTATCTGATAAAACAAGG-3';

U6:

Reverse transcribed Primer:

5'-AACGCTTCAGAATTTGCCGT-3';
Forward: 5'-CTCGCTTCGGCCAGCACCA-3';
Reverse: 5'-AACGCTTCAGAATTTGCCGT-3';

GAPDH:

Forward: 5'-AGAGGCCAGGGATGATGTTCTG-3';
Reverse: 5'-GACTCATGACCCAGTCCATGC-3';

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Biosciences) according to the manufacturer’s recommendations, the cells were analyzed with a flow cytometry (FACScan; BD Biosciences) equipped with a CellQuest software (BD Biosciences; ref. 19). Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells, and then the relative ratio of early apoptotic cells were compared with control transfectant from each experiment. Cells for cell-cycle analysis were stained with propidium oxide by the CycleTEST PLUS DNA Reagent Kit (BD Biosciences) following the protocol and analyzed by FACScan. The percentage of the cells in G0–G1, S, and G2–M phase were counted and compared (20).

**Hoechst staining assay**

SGC-7901 cells were cultured in 6-well cell culture plates, and Hoechst 33342 (Sigma) was added to the culture medium; changes in nuclear morphology were detected by fluorescence microscopy using a filter for Hoechst 33342 (365 nm). For quantification of Hoechst staining assay a n d compared (20).

**Plasmid constructs**

To ectopically express p27kip1, the synthetic p27kip1 sequence was subcloned into pEGFP-N3 vector. To construct a luciferase reporter vector, p27kip1 3′-untranslated region (3′-UTR) fragment containing putative binding sites for miR-196a was amplified by PCR using the following primers:

**Forward:** 5′-CACAATCGAGTTCAAAAATA

**Reverse:** 5′-AAAGATCCAAATATTATCTACTTGCTGT

AAATC-3′;

and cloned downstream of the luciferase gene in the pLuc Luciferase vector (Ambion Inc.) and named p27kip1 3′-UTR-WT. Site-directed mutagenesis of the miR-196a target site in the p27kip1 3′-UTR was carried out by the QuickChange mutagenesis kit (Stratagene) and named p27kip1 3′-UTR-Mut, in which 3′-UTR-WT was used as a template. For the mutated construct, the miR-196a target site 5′-CATGTGACTACT-3′ was substituted with a 5′-GTTTCAATGATGA-3′ fragment.

**Luciferase assay**

After placed into 48-well plates, HEK 293T cells were cotransfected with pCDNA-miR-196a or pCDNA (200 ng), pRL-TK (2 ng; Promega), luciferase reporter plasmids (50 ng) containing wild-type (WT) or mutant type (Mut) of p27kip1 3′-UTR using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, firefly and Renilla luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega; ref. 18).

**Tumor formation assay in a nude mouse model**

Five-week-old female athymic BALB/c nude mice were maintained under specific pathogen-free conditions and manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. Tumor cells transiently transfected with miR-196a inhibitor or stably transfected with pCDNA/miR-196a, and vector control were harvested from 6-well cell culture plates, washed with PBS, and resuspended at a concentration of 2 × 10⁷ cells per mL. Of the suspending cells, 0.1 mL was subcutaneously injected into either side of the posterior flank of the same nude mouse. Tumor volumes were examined once a week when the implantations were starting to grow bigger. Five weeks after injection, these mice were sacrificed and examined the growth of subcutaneous tumors.

**Western blot assay and antibodies**

Cells were lysed using mammalian protein extraction reagent RIPA (Beyotime) supplemented with protease inhibitors cocktail (Roche) and phenylmethylsulfonyl-fluoride (Roche). Fifty micrograms of protein extractions were separated by 10% SDS-PAGE, transferred to 0.22 μm nitrocellulose (NC) membranes (Sigma) and incubated with specific antibodies. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). GAPDH antibody was used as control; goat anti-p27kip1 (1:1,000) was purchased from Sigma; rabbit anti-Bcl2, Bax, and caspase-3 (1:1,000) were from Cell Signaling Technology.

**Immunohistochemistry**

Gastric tumor and nontumor tissue samples were immunostained for p27kip1. The signal was amplified and visualized with 3,3′-diaminobenzidine chromogen, followed by counterstaining with hematoxylin. Expression was considered to be positive when 50% or more cancer cells were stained. Anti-p27kip1 (1:50) were purchased from BioWorld.

**Statistical analysis**

The Students t test (2 tailed), one-way ANOVA, and Mann–Whitney U test were conducted to analyze the in vitro and in vivo data by SPSS 16.0 software. P values less than 0.05 were considered significant.

**Results**

**miR-196a expression is upregulated in human gastric cancer tissues**

To validate levels of miR-196a expression, we conducted qRT-PCR analysis to measure the miR-196a expression in 36 gastric cancer tissues and 3 gastric cancer cell lines compared with normal counterparts. The results showed that expression of miR-196a was significantly upregulated in gastric cancer tissues and cells (Fig. 1A and B). The clinical pathology findings of 31 patients with gastric cancer with an average age of 61 years (age ranged from 27–77) are shown in Supplementary Table S1. Next, we examined the correlation of miR-196a expression with clinical pathology features. miR-196a upregulation was correlated with tumor size (P = 0.031; Fig. 1C) and...
advanced pathologic stage ($P = 0.006$; Fig. 1D). However, miR-196a expression was not correlated with patients’ age, gender, tumor position, or tumor cell differentiation (Supplementary Table S1). Kaplan–Meier survival analysis and log-rank tests using patient postoperative survival was conducted to further evaluate the correlation between miR-196a expression and prognosis of patients with gastric cancer. According to the median ratio of relative miR-196a expression (40.9) in tumor tissues, the 31 patients with gastric cancer were classified into 2 groups: High miR-196a group ($n = 13$): miR-196a expression ratio ≥ median ratio; low miR-196a group ($n = 18$): miR-196a expression ratio ≤ mean ratio. From the Kaplan–Meier survival curve, we observed that patients with high levels of miR-196a expression ($n = 13$) had significantly shorter survival times than those with low levels of miR-196a expression ($n = 18$, $P < 0.001$, log-rank test; Fig. 1E). Thus, it was concluded that overexpression of miR-196a might have important roles in gastric cancer progression and development.

**Exogenous downregulation or overexpression of miR-196a in gastric cancer cells**

To upregulate or downregulate the expression of miR-196a, miR-196a mimics or miR-196a inhibitors were transiently transfected into SGC-7901 cells, respectively. In addition, to stably sustain the expression of miR-196a in

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**Figure 1.** The level of miR-196a expression in gastric cancer tissues, cells, and its clinical significance. A and B, miR-196a was detected in 36 pairs of gastric cancer tissues by qPCR. Data are presented as fold change in tumor tissues relative to normal tissues. C, miR-196a expression was significantly higher in patients with big tumors than in patients with small tumors. D, miR-196a expression was significantly higher in patients with a higher pathologic stage than in patients with a lower pathologic stage. E, patients with high levels of miR-196a expression showed reduced survival times compared with patients with low levels of miR-196a expression ($P < 0.001$, log-rank test). **, $P < 0.05$; **, $P < 0.01$. 

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SGC-7901 cells, pCDNA/miR-196a was transfected into SGC-7901 cells. The cells were collected 48 hours after transfection for detection of miR-196a expression. qRT-PCR assays revealed that miR-196a expression was upregulated by 7.9-fold after transfection of miR-196a mimics and downregulated by 0.52-fold after transfection of miR-196a inhibitors, whereas the expression of miR-196a was upregulated by 2.16-fold in stably transfected SGC-7901/miR-196a cells, as compared with control cells (Fig. 2A).

Effect of miR-196a on cell proliferation and apoptosis in vitro

The significant increase of miR-196a expression in gastric cancer samples prompted us to explore the possible biologic significance of miR-196a in tumorigenesis. To determine the impact of miR-196a on gastric cancer cell proliferation, MTT assay was conducted to detect cell viability. According to the results of the MTT assay, we found that cells transiently transfected with anti-miR-196a inhibitors had significantly reduced growth, and proliferation of cells transfected with miR-196a mimics was increased compared with that of cells transfected with miR-NC controls (Fig. 2B). To further examine whether the effect of miR-196a on proliferation of SGC-7901 cells reflected cell-cycle arrest, cell-cycle progression was analyzed by flow cytometric analysis. The results revealed that SGC-7901 cells transfected with miR-196a inhibitors had an obvious cell-cycle arrest at the G1–G0 phase and had a decreased G2–S-phase, whereas cells transfected with the miR-196a mimic displayed a decreased G1–C0 and an increased G2–S-phase (Fig. 2C and D).

To determine whether SGC-7901 cell proliferation was influenced by apoptosis we conducted the Hoechst staining assay. Quantification of the number of cells with condensed and fragmented nuclei indicated that miR-196a inhibitor–treated SGC-7901 cells displayed no obvious change in apoptosis compared with control (Fig. 2E). Consistent with our finding from the Hoechst staining assay, flow cytometry showed that the fraction of early apoptotic cells was not significantly different in miR-196a inhibitor–treated SGC-7901 cells compared with anti-miR-NC–treated cells (Fig. 2F). Furthermore, Western blot analysis indicated that the expression level of cleaved caspase-3 displayed no significant change in miR-196a inhibitor–transfected SGC-7901 cells (Fig. 2G). These results indicated that enhanced miR-196a expression promoted gastric cancer cells proliferation and did not influence cell apoptosis.
miR-196a directly targets p27kip1 by interacting with its 3'-UTR

To further explore the mechanism by which miR-196a promotes gastric cancer proliferation, we conducted a bioinformatics screen to identify potential downstream target genes that normally have a tumor suppressive effect. On the basis of this rationale, 2 genes [p27kip1 and insulin-like growth factor–binding protein 3 (IGFBP3)] were selected. We cloned the wild-type 3'-UTR of these 2 genes and inserted them into the region immediately downstream of a luciferase reporter gene (Fig. 3A). Subsequently, pcDNA/miR-196a vectors were cotransfected with different luciferase 3'-UTR constructs into HEK293T cells. We found that miR-196a decreased the relative activity of the luciferase reporter containing the wild-type 3'-UTR of p27kip1 mRNA. To test whether p27kip1 mRNA is a direct target for miR-196a, we mutated the predicted binding site of miR-196a in the 3'-UTR. Luciferase activity did not drop sharply in the UTRs with mutant-binding sites, when compared with Mut-type counterparts (Fig. 3B).

We next determined whether miR-196a could regulate p27kip1 at both mRNA and protein levels. qPCR analysis showed that the expression of p27kip1 mRNA in SGC-7901 cells transfected with miR-196a inhibitor or mimics was upregulated or downregulated compared with cells transfected with negative control (Fig. 4D). Western blot analysis showed that the expression of p27kip1 protein in SGC-7901 cells transfected with miR-196a inhibitor was upregulated compared with cells transfected with negative control (Fig. 4D). These data showed that miR-196a could regulate p27kip1 at both mRNA and protein levels.

miR-196a promotes tumorigenesis of gastric cancer cells in vitro and vivo

To explore whether the level of miR-196a expression affects tumorigenesis, colony formation assays were conducted to detect cell growth viability in pcDNA/miR-196a stably transfected SGC-7901 cells, miR-196a inhibitor transiently transfected SGC-7901 cells, and SGC-7901 cells. According to the results of growth curves, we found that pcDNA/miR-196a stably transfected SGC-7901 cells had significantly increased growth viability, whereas miR-196a inhibitor–transfected SGC-7901 cells had reduced growth viability (Fig. 4A). Furthermore, pcDNA/miR-196a stably transfected SGC-7901 cells, miR-196a inhibitor transiently transfected SGC-7901 cells and SGC-7901 cells were inoculated into female nude mice. Five weeks after injection, the pcDNA/miR-196a group had a high mortality rate, and the tumors formed in this group were substantially bigger than those in the other 2 groups (Fig. 4B). However, the tumor volume between the miR-196a inhibitor group and the control group was not significantly different, possibly because of transient transfection. Moreover, the mean tumor weight at the end of the experiment was markedly higher in the pcDNA/miR-196a group (0.925 ± 0.108 g) than in the control and miR-196a inhibitor groups (0.462 ± 0.104 and 0.468 ± 0.115 g, respectively; Fig. 4C). qRT-PCR analysis of miR-196a expression was then conducted in selected tumor tissues. The results showed that the levels of miR-196a expression in tumor tissues formed from pcDNA/miR-196a cells were higher than those of tumors formed in control and anti-miR-196a inhibitor groups (Fig. 4D). These results indicated that higher miR-196a expression could promote tumor growth in vitro and in vivo.

Overexpression of p27kip1 also elicits cell-cycle arrest

To validate whether p27kip1 could also elicit cell-cycle arrest and decreased gastric cancer cell proliferation, we constructed pEGFP-p27kip1 and transfected the vector into SGC-7901 cells. We then selected stably transfected SGC-7901/pEGFP-p27kip1 cells by G418 treatment. The expression levels of p27kip1 mRNA and protein in SGC-7901/pEGFP-p27kip1 cells were significantly increased compared with pEGFP-NC-transfected cells (Fig. 5A). MTT and colony formation assays were then conducted to detect the cell viability. The results of the MTT assay and growth curves revealed that the cells transfected with pEGFP-p27kip1 had a significant growth inhibition when compared with cells transfected with pEGFP-NC (Fig. 5B and D). Next, flow cytometric analysis indicated that the cell-cycle progression of SGC-7901/pEGFP-p27kip1 cells was stalled at the G1–G0 phase with a significant decrease in S and G2–M phases compared with cells transfected with pEGFP-NC (Fig. 5C). These data indicated that overexpression of p27kip1 could arrest cell-cycle progression and decrease proliferation of SGC-7901 cells, which was consistent with results of downregulated miR-196a in SGC-7901 cells.

Inhibition of p27kip1 is potentially involved in the oncogene function of miR-196a

To investigate whether p27kip1 was involved in the miR-196a–induced increase in gastric cancer cell proliferation, we carried out rescue experiments. After transfection with pEGFP-p27kip1, SGC-7901 cells were cotransfected with pcDNA/miR-196a1. We found that cotransfection of pEGFP-p27kip1 and pcDNA/miR-196a could rescue the decreased expression of p27kip1 protein induced by ectopic expression of miR-196a (Fig. 5E). Moreover, MTT assay indicated that the cotransfection could partially rescue miR-196a–promoted proliferation in SGC-7901 cells (Fig. 5F). Meanwhile, flow cytometric analysis indicated that cotransfection could partially rescue the decreased G1 phase and the decrease in S and G2–M phases induced by miR-196a overexpression (Fig. 5G). These data indicated that miR-196a promotes SGC-7901 cell proliferation through downregulation of p27kip1 expression.

Inverse relationship between the expression of p27kip1 and miR-196a

To assess the relationship between p27kip1 and miR-196a expression in gastric cancer, we examined p27kip1 by
qPCR and immunohistochemistry in 20 pairs of gastric cancer tissues and in 4 gastric cancer cell lines. The results showed that the mRNA levels of p27kip1 was generally lower in gastric cancer tissues and cells, when compared with matched normal tissues and cells, respectively (Fig. 6A and B). We also found that 85% normal gastric tissues showed a p27kip1-positive signal, whereas most tumor-derived tissues exhibited low levels of p27kip1 protein compared with the paired normal tissues (Fig. 6C and Supplementary Table S2). Further analysis revealed that expression of miR-196a is inversely correlated with p27kip1 protein level in gastric cancer (Fig. 6D). These data indicated that the p27kip1 level was mostly opposite to levels of miR-196a expression in gastric cancer.

Figure 3. miR-196a directly targets the p27kip1 gene. A, a human p27kip1 and IGFBP3 3′-UTR fragment containing wild-type (WT) or mutant (Mut) miR-196a-binding sequence was cloned downstream of the luciferase reporter gene. B, the luciferase reporter plasmid containing wild-type or mutant p27kip1 3′-UTR was cotransfected into HEK-293T cells with pCDNA-miR-196a or pCDNA-miR-NC. Luciferase activity was determined by the dual luciferase assay and shown as the relative firefly activity normalized to Renilla activity. C, the level of p27kip1 mRNA was determined by qPCR. D, the expression of p27kip1 protein was analyzed by Western blotting. GAPDH was used as control. *, P < 0.05; **, P < 0.01; NS, not significant. CMV, cytomegalovirus.
Discussion

The aberrant expression of overexpressed miR-196a in non–small cell lung carcinoma, colorectal cancer, and glioblastoma has been found (21–23). Similarly, Luthra and colleagues have revealed that the significantly high expression of miR-196a promoted esophageal cell proliferation, anchorage-independent growth and suppressed apoptosis by directly targeting Annexin A1 (ANXA1; ref. 24). However, reduced expression of miR-196a in melanoma could enhance cells migratory potential (25). As miR-196a expression in multiple tumors has been found to be significantly higher than controls, it might be useful as a candidate marker for diagnosis of cancers.

In our study, we identified that miR-196a was dramatically upregulated in gastric cancer tissues and cells, suggesting that high expression of miR-196a was involved in gastric carcinogenesis. Specifically, miR-196a expression was found to be significantly higher at later stages of tumor development or had undergone more extensive metastasis compared with counterparts. Moreover, the overall survival time of patients who lacked miR-196a expression was significantly longer than that of patients with moderate or strong expression of miR-196a. This suggests that the expression of miR-196a could be used to develop as a new independent prognosis or progression marker of gastric cancer. Further studies showed that overexpression of miR-196a has the effect of promoting gastric cancer cell G1–S transition and proliferation.

As very few studies had ever addressed the mechanism of miR-196a function effects, we conducted luciferase reporter assay and Western blotting to confirm that p27kip1 is a target of miR-196a in gastric cancer cells. The G1–S transition of the cell cycle in mammalian cells is controlled by cyclins, cyclin-dependent kinases (CDK), and their inhibitors, and deregulation of CDK inhibitors is a common feature in tumor cells (26). p27kip1, a member of the Kip/Cip family of CDK inhibitors and was first identified during TGF-β-induced G1 arrest (27). p27kip1 was first known to prevent cell-cycle progression by inhibiting cyclin E/CDK2 activity in the nucleus through binding of its N-terminal domain with cyclin E/CDK2

Figure 4. Effect of miR-196a on tumor growth in vitro and vivo. A, colony-forming growth assays were conducted to determine the proliferation of SGC-7901 cells. The colonies were counted and captured. B, the tumor volume was calculated once a week after injection of SGC-7901 cells stably transfected with pcDNA/miR-196a, pcDNA/miR-NC, or transiently transfected with anti-miR-196a inhibitor. Points, mean (n = 3); bars indicate SD. C, tumor weights are represented as means of tumor weights ± SD. D, qPCR analysis of miR-196a expression in tumor tissues formed from SGC-7901/miR-196a, SGC-7901/anti-miR-196a, or SGC-7901/miR-NC. All experiments were carried out 3 times with 3 technical replicates. *, P < 0.05; **, P < 0.01; N.S., not significant.
complexes, thus blocking ATP loading, and physically occluding the catalytic cleft of the CDK. Moreover, cytoplasmic p27kip1 could also induce cell apoptosis (28–32). As a tumor suppressor, the absence or reduction of p27kip1 expression has been implicated in the genesis, progression, and decreased patient survival of many human malignancies, including breast, gastric, prostate, and lung carcinomas and is strongly associated with poor clinical outcomes in the majority of studies (33–36). However, no homozygous deletions and only rare point
mutations have been found in the human p27kip1 gene (27), which indicates that miRNA-mediated posttranscriptional regulation may play an important role in the reduction of p27kip1.

Our results showed that the expression levels of p27kip1 are downregulated in human gastric cancer tissues and cell lines and inversely correlated with the expression levels of miR-196a. Moreover, we established p27kip1 overexpression in the SGC-7901 cell line by stable transfection of pEGFP-p27kip1 and selecting with the antibiotic, G418. In addition, further experiments indicated that the increased p27kip1 level in the nucleus could induce cell-cycle arrest and inhibit cell proliferation, consistent with miR-196a inhibitor transfection, which could be rescued by concomitant overexpression of miR-196a. Thus, it was concluded that downregulation of p27kip1 might be a mechanism by which miR-196a exerts its oncogene functions.

In our study, we found that p27kip1 was negatively regulated by miR-196a in gastric cancer cells. However, many studies have clearly indicated that one miRNA could regulate multiple genes, whereas one gene could also be controlled by multiple miRNAs (37). Thus, miR-196a might regulate other target genes that may be involved in cell proliferation and metastasis in gastric cancer cells, and further studies will be needed to clarify this point.

In summary, our study showed that miR-196a is dramatically upregulated in gastric cancer tissues and cell lines and that the high expression of miR-196a is significantly associated with tumor size, poor pT stage, pN stage, and patients' survival time. Moreover, downregulation of miR-196a has the effect of suppressing gastric cancer cell proliferation both in vitro and in vivo by targeting p27kip1. Further insights into the functional and clinical implications of miR-196a and its target p27kip1 may contribute to the early diagnosis of gastric cancer and help with gastric cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: M. Sun, Z.X. Wang, X. Cao, W. De
Development of methodology: E.B. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.H. Li, J.S. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Sun, Z.L. Liu, J. Zhou
Writing, review, and/or revision of the manuscript: M. Sun, W. De
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Sun, X. Liu, D.D. Yin, S. Li
Study supervision: M. Sun

Figure 6. The level of p27kip1 expression in gastric cancer tissues and cell lines. A and B, the level of p27kip1 mRNA in gastric cancer tissues and cell lines was analyzed by qPCR. C, the level of p27kip1 protein in gastric cancer tissues was analyzed by immunohistochemistry. D, analysis of the relationship between miR-196a expression and p27kip1 protein level. *: P < 0.01.
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Ming Sun, Xiang-hua Liu, Jin-hai Li, et al.


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