MiR-196a Is Up-regulated in Gastric cancer and Promotes Cell proliferation by Down-regulating p27kip1

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Abstract:
Aberrant expression of miR-196a has been frequently reported in cancer studies. However, the expression and mechanism of its function in gastric cancer (GC) remains unclear. Real-time quantitative PCR was performed to detect the relative expression of miR-196a in GC 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 GC tissues was associated with tumor size, a higher clinical stage, and was also correlated with shorter overall survival of GC patients. Exogenous downregulation of miR-196a expression significantly suppressed the in vitro cell cycle progression, proliferation and colony formation of GC 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 GC tissues. Our study demonstrates that aberrant over-expression of miR-196a and consequent down-regulation of p27kip1 could contribute to gastric carcinogenesis, and would be targets for gastric cancer therapies and further developed as potential prognostic factors.

Introduction
Gastric cancer (GC) 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 GC patients. 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 GC\(^3-5\). Since a miRNA is able to regulate hundreds of genes\(^6\), the aberrant expression of miRNA has been frequently reported in GC, suggesting that miRNA dysregulation is involved in the development, and progression of GC\(^7\).

MicroRNAs (miRNAs) are a class of small non-coding RNAs, which act as post-transcriptional 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 oncogenes or tumor suppressors based on their inhibition of tumor suppressor or oncogene target
mRNAs, 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 GC has been established. However, the genes targeted by most of these miRNAs are still unknown(15-17). Thus, elucidating the biological 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 performed qPCR analysis of miR-196a expression in human GC tissues and showed that miR-196a was over-expressed in tumor-derived samples and GC cell lines compared with matched normal samples. MiR-196a upregulation was also correlated with tumor size, advanced pathological 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 GC tissues was noted by consecutive further analysis.

Materials and Methods

Tissue collection

GC tissues and normal tissues were obtained from patients who had underwent surgery at Jiangsu province hospital between 2007 and 2008 and were diagnosed with gastric cancer (stage I,III,IV ) based on histopathological 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, 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 medium (invitrogen, Carlsbad, Calif) supplemented with 10% fetal bovine serum (invitrogen, Carlsbad, Calif) at 37°C in incubator with 5% CO2.
RNA extraction and qPCR analyses

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

miR-196a:
Reverse transcribed Primer
5' GTCAGAAGGAATGATGCACAGCCAACAACA 3';
Forward 5' CGTCAGAAGGAATGATGCACAG 3';
Reverse 5' ACCTGCCTAGGTAGTTTCATGT 3';
p27kip1:
Forward 5' TGCAACCGACGATTCTTCTACTCAA 3';
Reverse 5'CAAGCAGTGATGTATCTGATAAACAAGG3';
U6:
Reverse transcribed Primer: 5' AACGCTTCACGAATTTGCGT 3';
Forward: 5' CTCGCTTCGCACAGACACA 3';
Reverse: 5' AACGCTTCACGAATTTGCGT 3';
GAPDH:
Forward: 5’ AGAGGCAGGGATGATGTTCTG 3’;
Reverse: 5’ GACTCATGACCACAGTCCATGC 3’;
qPCR and data collection were performed on ABI 7500.

**miRNA mimic and inhibitor transfection**

Gastric cancer cells were transfected with miR-196a mimic, miR-196a inhibitor (Applied Biosystems, Carlsbad, Calif), or scrambled sequence (Genefarme, Shanghai, China) with a final concentration 100pm by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. At 48 hr after transfection, cells were harvested for Western blot or qPCR analyses.

**Cell proliferation assays**

A cell proliferation assay was performed with MTT kit (Sigma, St. Louis, Mo) (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, St. Louis, Mo) in PBS for 15 minutes. Colony formation was determined by counting the number of stained colonies in 3 randomly selected fields using 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-p27 kip1 were harvested 48 hr after transfection by trypsinization. After the double staining with FITC-Annexin V and Propidium iodide (PI) was done using the FITC Annexin V Apoptosis Detection Kit (BD 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)(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 to control transfectant from each experiment. Cells for cell cycle analysis were stained with PI using 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 six-well cell culture plates, and Hoechst 33342 (Sigma, St Louis, MO, USA) 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 33342 staining, the percentage of Hoechst-positive nuclei per optical field (at least 50 fields) was counted.
Plasmid constructs

To ectopically express p27 kip1, the synthetic p27 kip1 sequence was subcloned into pEGFP-N3 vector. To construct a luciferase reporter vector, p27 kip13′-UTR fragment containing putative binding sites for miR-196a was amplified by PCR using the following primers:

Forward: 5′-CACAAACTCGAGTTTAAAAATACATATCGCTGACTTC-3′;
Reverse: 5′-AAGGATCCAATATTTATCTACTTGCTGTAAATC-3′;

and cloned downstream of the luciferase gene in the pLUC Luciferase vector (Ambion, Inc., Austin, TX, USA) and named p27kip1-3′-UTR-WT. Site directed mutagenesis of the miR-196a target site in the p27kip1-3′-UTR was performed using the Quick-change mutagenesis kit (Stratagene, Heidelberg, Germany) 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′-CATTGTACTACCT-3′ was substituted with a 5′-GTTTCATGATGGA-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, Madison, WI, USA), luciferase reporter plasmids (50 ng) containing wild-type (WT) or mutant-type (MT) of p27 kip1-3′UTR using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif). 48hr after transfection, firefly and Renilla luciferase activities
were measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA)(18).

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

5-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/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. 5 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 PMSF (Roche). 50 ug protein extractions were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to 0.22μm 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 (1:1000) were purchased from sigma, rabbit anti-Bcl2, Bax, and Caspase3 (1:1000) were from Cell Signaling Technology.

**Immunohistochemistry**

Gastric tumor and non-tumor tissue samples were immunostained for p27 kip1. The signal was amplified and visualized with 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 bioword.

**Statistical Analysis**

Student’s t-test (two-tailed), One-way ANOVA and Mann-Whitney test were performed to analyze the in vitro and in vivo data using SPSS 16.0 software. P values less than 0.05 were considered significantly.

**RESULTS**

**MiR-196a expression is up-regulated in human gastric cancer tissues**

To validate levels of miR-196a expression, we performed qRT-PCR analysis to measure the miR-196a expression in 36 GC tissues and 3 GC cell lines compared with normal counterparts. The results showed that expression of miR-196a was significantly up-regulated in GC tissues and cells (Fig 1A,B).
The clinical pathology findings of 31 GC patients with an average age of 61 years (age ranged from 27 to 77) are shown in Supplementary Table 1. 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 pathological stage (P=0.006) (Fig 1D). However, miR-196a expression was not correlated with patients' age, gender, tumor position or tumor cell differentiation (Tab 1). Kaplan–Meier survival analysis and log-rank tests using patient postoperative survival was performed to further evaluate the correlation between miR-196a expression and prognosis of gastric cancer patients. According to the median ratio of relative miR-196a expression (40.9) in tumor tissues, the 31 gastric cancer patients were classified into two 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 over-expression of miR-196a might have important roles in gastric cancer progression and development.

**Exogenous down-regulation or over-expression of miR-196a in GC cells**

To up or down-regulate 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 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 up-regulated by 7.9-fold after transfection of miR-196a mimics and down-regulated by 0.52-fold after transfection of miR-196a inhibitors, while the expression of miR-196a was up-regulated 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 GC samples prompted us to explore the possible biological significance of miR-196a in tumorigenesis. To determine the impact of miR-196a on GC cell proliferation, MTT assay was performed 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 cytometry analysis. The results revealed that SGC-7901 cells transfected with miR-196a inhibitors had an obvious cell cycle arrest.
cycle arrest at the G1/G0 phase and had a decreased G2/S phase, while cells transfected with the miR-196a mimic displayed a decreased G1/G0 and an increased G2/S phase (Fig 2C,D).

To determine whether SGC-7901 cell proliferation was influenced by apoptosis we performed 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 GC 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 GC proliferation, we performed a bioinformatics screen to identify potential downstream target genes that normally have a tumor suppressive effect. Based on this rationale two genes (p27kip1 and IGFBP3) were selected. We cloned the wild-type 3' UTRs of these two genes, and inserted them into the
region immediately downstream of a luciferase reporter gene (Fig 3A). Subsequently, pCDNA/miR-196a vectors were co-transfected 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. However, luciferase activity did not drop sharply in the UTRs with mutant binding sites, when compared to 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 SGC7901 cells transfected with miR-196a inhibitor or mimics was up-regulated or down-regulated compared to cells transfected with control (Fig 3C). Western blot analysis showed that the expression of p27kip1 protein in SGC7901 cells transfected with miR-196a inhibitor was up-regulated compared to cells transfected with negative control (Fig 4D). These data demonstrated that miR-196a could regulate p27kip1 at both mRNA and protein levels.

**MiR-196a promotes tumorigenesis of GC cells in vitro and vivo**

To explore whether the level of miR-196a expression affects tumorigenesis, colony formation assays were performed 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, while 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 two 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 ± 0.105 g) compared to the control and miR-196a inhibitor groups (0.462 ±0.104 g and 0.468 ± 0.115 g, respectively ( Fig 4C ) . qRT-PCR analysis of miR-196a expression was then performed 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 vivo.

*Over-expression of p27kip1 also elicits cell cycle arrest*
To validate whether p27kip1 could also elicit cell cycle arrest and decreased GC 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 performed 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, D). Next, flow cytometry 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 to cells transfected with pEGFP-NC (Fig 5C). These data indicated that over-expression of p27kip1 could arrest cell cycle progression and decrease proliferation of SGC-7901 cells, which was consistent with results of down-regulated 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 GC cell proliferation, we performed rescue experiments. After
transfection with pEGFP-p27kip1, SGC-7901 cells were co-transfected with pCDNA/miR-196a1. We found that co-transfection 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 co-transfection could partially rescue miR-196a-promoted proliferation in SGC-7901 cells (Fig 5F). Meanwhile, flow cytometry analysis indicated that co-transfection could partially rescue the decreased G1 phase and the decrease in S and G2/M phases induced by miR-196a over-expression (Fig 5G). These data indicated that miR-196a promotes SGC-7901 cell proliferation through down-regulation of p27kip1 expression.

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

To assess the relationship between p27kip1 and miR-196a expression in GC, we examined p27kip1 by qPCR and immunohistochemistry in 20 pairs of GC tissues and in 4 GC cell lines. The results showed that the mRNA levels of p27kip1 was generally lower in GC tissues and cells, when compared with matched normal tissues and cells, respectively (Fig 6A,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 Table2). 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 GC.

Discussion

The aberrant expression of over-expressed miR-196a in non small cell lung cancer, colorectal cancer, and glioblastoma has been found (21-23). Similarly, R Luthra et al. 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)(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 GC. Further studies demonstrated 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 performed luciferase reporter assay and western blot to confirm that p27kip1 is a target of miR-196a in GC cells. The G1-S transition of the cell cycle in mammalian cells is controlled by cyclins, cyclin-dependent kinases (CDKs) 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 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 post-transcriptional regulation may play an important role in the reduction of p27kip1.

Our results showed that the expression levels of p27kip1 are down-regulated in human GC tissues and cell lines, and inversely correlated
with the expression levels of miR-196a. Moreover, we established p27kip1 over-expression 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 over-expression of miR-196a. Thus, it was concluded that down-regulation of p27kip1 might be a mechanism by which miR-196a exerts its oncogene functions.

Although p27kip1 was negatively regulated directly by miR-196a in GC 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 GC 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, down-regulation of miR-196a has the effect of suppressing GC cell proliferation both in vitro and 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 GC and help with GC treatment.
Acknowledgments

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Reference


Figure legends

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

**Figure 2.** Effect of miR-196a on cell proliferation and apoptosis. (A) The relative expression level of miR-196a in SGC-7901 cells, transfected with miR-196a mimics (or miR-NC) or inhibitors (or Anti-miR-NC), or pCDNA/miR-196a, was tested by qPCR. (B) 48 hr after transfection, MTT assays were performed to determine the proliferation of SGC7901 cells. (CD) The bar chart represented the percentage of cells in G0/G1, S, or G2/M phase, as indicated. (E) Hoechst staining assay of cell apoptosis, the percentage of Hoechst-positive nuclei per optical field (at least 50 fields) was counted. (F) The apoptotic rates of cells were detected by flow cytometry. (G) Western blot assays of cleaved caspase-3. *P<0.05 and **P<0.01.

**Figure 3.** MiR-196a directly targets the p27 kip1 gene. (A) A human p27 kip1 and IGFBP3 3’ UTR fragment containing wild-type or mutant miR-196a binding
sequence was cloned downstream of the luciferase reporter gene. (B) The luciferase reporter plasmid containing wild-type or mutant p27 kip1 3’ UTR was co-transfected into HEK-293T cells with pCDNA-miR-196a or pCDNA-miR-NC. Luciferase activity was determined using the dual luciferase assay and shown as the relative firefly activity normalized to renilla activity. (C) The level of p27 kip1 mRNA was determined by qPCR. (D) The expression of p27 kip1 protein was analyzed by western blot. GAPDH was used as control. *P<0.05, **P<0.01 and N.S. not significant.

**Figure 4.** Effect of miR-196a on tumor growth *in vitro* and *vivo*. (A) Colony-forming growth assays were performed 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 s.d. (C) Tumor weights are represented as means of tumor weights ± s.d. (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 performed three times with three technical replicates. *P<0.05, **P<0.01 and N.S. not significant.

**Figure 5.** Over-expression of miR-196a could partially block the oncogene
function of p27kip1 over-expression. SGC-7901 cells were transfected with pEGFP-p27kip1 or pEGFP-NC. (A) The expression of p27 kip1 mRNA and protein were analyzed by qPCR and western blot. GAPDH was used as control. (B) MTT analysis of SGC-7901 cell proliferation. (C) Cell cycle analysis of SGC-7901 cells was shown. The bar chart represents the percentage of cells in G0/G1, S, or G2/M phase as indicated. (D) Colony-forming growth assay was performed to determine the proliferation of SGC-7901 cells. The colonies were counted and captured. (E) The expression of p27 kip1 protein was analyzed by western blot. (F) MTT analysis of SGC-7901 cell proliferation was shown. (G) Cell cycle analysis of SGC-7901 cells was shown. *P<0.05, **P<0.01 and N.S. not significant.

Figure 6. The level of p27 kip1 expression in GC tissues and cell lines. (AB) The level of p27 kip1 mRNA in GC tissues and cell lines was analyzed by qPCR. (C) The level of p27 kip1 protein in GC tissues was analyzed by immunohistochemistry. (D) Analysis of the relationship between miR-196a expression and p27 kip1 protein level. *P<0.05, **P<0.01 and N.S. not significant.
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