MicroRNA-221-3p Plays an Oncogenic Role in Gastric Carcinoma by Inhibiting PTEN Expression

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Gastric carcinoma is one of the most common malignancies in men, and microRNA plays a critical role in regulating the signaling networks of gastric carcinoma tumorigenesis and metastasis. We first report the functional characteristics of miR-221-3p in gastric carcinoma. Quantification in gastric carcinoma cell lines and tumor samples reveals significantly increasing miR-221-3p expression. Moreover, a high level of miR-221-3p is correlated with a poor prognosis for gastric carcinoma patients. Ectopic miR-221-3p expression significantly promotes gastric carcinoma cell proliferation, invasion, and sphere formation, while silencing miR-221-3p significantly inhibits these abilities in gastric carcinoma cells. Tests in vivo showed that miR-221-3p significantly promotes tumor growth in xenograft mouse models. In this study, we reveal that miR-221-3p targets PTEN mRNA and downregulates PTEN, which is the possible mechanism of miR-221-3p-induced oncogenic properties. Collectively, we reveal a critical role for miR-221-3p in gastric carcinoma development and progression.

Key words: miR-221-3p; Gastric carcinoma; PTEN; Akt

INTRODUCTION

Gastric carcinoma is one of the most common malignancies and a leading cause of cancer-related death1,2. Recently, there have been significant advances in disease management and treatment, but gastric carcinoma patients still have a very dismal long-term prognosis1. The main challenges in the treatment of gastric carcinoma involve intrahepatic recurrence and metastasis, which simultaneously predict a poor outcome for gastric carcinoma patients3. The identification of critical players that suppress or promote these processes may lead to novel therapeutic targets for improving the prognosis of these patients.

In the past decades, increasing attention has been paid to the important biological and pathological roles of microRNAs (miRNAs), which are small, endogenous, noncoding RNAs composed of 18–23 nucleotides (nt) that posttranscriptionally regulate gene expression by targeting the 3′-untranslated regions (3′-UTRs) of mRNAs4. Many miRNAs are proto-oncogenes or tumor suppressors5, and in recent years, numerous studies have shown that aberrant expression of miRNAs is associated with the development and progression of gastric carcinoma6. A number of miRNAs have been shown to be aberrantly expressed during gastric carcinoma development7. In addition, some of these miRNAs might have prognostic significance7. miRNAs play a critical role in regulating the gastric carcinoma tumorigenesis and metastasis signaling networks8.

Mounting evidence has shown that the poor prognosis of patients with gastric carcinoma and therapeutic failure are associated with a number of abnormally activated signaling pathways, among which phosphoinositide 3-kinase (PI3K)/Akt signaling represents one of the most important regulatory pathways for the malignancy9. Moreover, Akt signaling contributes to oncogenesis by activating multiple downstream effector molecules. Of note, activated Akt phosphorylates tumor suppressor FOXO3a and impairs the transcription of its target genes related to cell growth arrest such as p21, inactivation of which has also been implicated in the promotion of tumor angiogenesis10. In addition, mTOR, another substrate subjected to phosphorylation by Akt, enhances phosphorylation of S6K1 and 4E-BP1 and plays a crucial role in the regulation of ribosomal protein synthesis, for example, production of cyclin D1 and...
Figure 1. miR-221-3p is upregulated in gastric carcinoma tumors and gastric carcinoma cells. (A) Quantification of miR-221-3p in gastric carcinoma cell lines showing higher expression than in normal human stomach epithelial cells (AGS1). (B) Average expression of miR-221-3p in gastric carcinoma cells and control cells. (C) Quantification of miR-221-3p in human gastric carcinoma tumors (T) and normal liver tissue (N). **p<0.01 based on the Student’s t-test. Error bars, SD.
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VEGF-A at both the transcriptional and translational levels. It has been found that, mediated by the above molecular mechanisms, both AKT/FOXO3a and AKT/mTOR pathways underlie lung cancer development and progression. Thus, inhibitors targeting these pathways might represent potential therapeutic agents against gastric carcinoma. In the framework of gene expression regulation, it is widely recognized that miRNA-mediated posttranscriptional repression plays an important role, largely attributed to the capability of an miRNA to inhibit multiple target mRNAs through binding to their 3'-UTRs.

In the current study, we identify that miR-221-3p is highly expressed in multiple subtypes of gastric carcinoma tissues and causes simultaneous downregulation of PTEN, leading to activation of both Akt pathways, consequently leading to accelerated proliferation and enhanced angiogenesis in gastric carcinoma.

MATERIALS AND METHODS

Clinical Specimens and Cell Culture

A total of 50 gastric carcinoma specimens and 5 adjacent gastric carcinoma tissue samples, frozen in liquid nitrogen, were obtained from Xinhua Hospital affiliated to Shanghai Jiao tong University. No patients had received any antitumor treatments before biopsy. The human gastric carcinoma cell lines (AGS, SNU-16, KATO-III, SNU-1, SNU-5, Hs 746T, and NCI-N87) and normal gastric cell line (GES-1) were cultured in the indicated media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Rockville, MD, USA) and 1% streptomycin/penicillin at 37°C with 5% CO2 according to the ATCC (Manassas, VA, USA).

RNA Extraction, Reverse Transcription, and RT-PCR

Total RNA was extracted from paraffin-embedded samples by the acid phenol–chloroform method and from freshly frozen samples or cells with TRIzol reagent (Invitrogen). Total RNA was reverse transcribed with a First-Strand cDNA Synthesis Kit (Invitrogen). Real-time polymerase chain reactions (RT-PCRs) were conducted using Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen) on the PRISM 7900HT system (Applied Biosystems, Foster City, CA, USA). All reactions were done in triplicate, and reactions without reverse transcriptase were used as negative controls. The U6 or GAPDH was used as the endogenous control, and the $2^{-\Delta\Delta C_{T}}$ equation was used to calculate the relative expression levels.

Oligonucleotide Transfection and Generation of Stably Transfected Cell Lines

Cells were seeded into six-well plates, transfected with miR-221-3p mimics or miR controls (50 nM; GenePharma, Shanghai, P.R. China) using Lipofectamine™ RNAiMAX (Invitrogen) and transfected with siMIF (100 nM; Invitrogen) or siRNA controls using Lipofectamine 2000 reagent (Invitrogen), and then harvested for assays 48 h later. The lentiviral plasmid pEZX-MR03 (GeneCopoeia, Rockville, MD, USA) expressing miR-221-3p (Cat. HmiR0274-MR03) or scrambled miRNA (Cat. CmiR0001-MR03) and Lenti-Pac HIV Expression Packaging mix (GeneCopoeia) were cotransfected into gastric cancer cells using EndoFectin Lentivirus transfection reagent (GeneCopoeia). After transfection for 48 h, lentiviral particles were harvested and then transduced into the gastric cancer cells, and the stably transfected cells were selected using puromycin (2 ng/ml) and validated by RT-PCR.

Figure 2. miR-221-3p is upregulated in gastric carcinoma and inversely correlates with patient survival. (A) Quantification of miR-221-3p in normal liver, stages I–II gastric carcinoma, and stages III–IV gastric carcinoma. (B) Correlation analysis of expression data and patient survival data from TCGA showing that miR-221-3p levels are a risk indicator for survival. **$p<0.01$, ###$p<0.01$ based on the Student’s $t$-test. (A) #Student’s $t$-test to stages I–II. Error bars, SD.
Figure 3. miR-221-3p accelerates gastric carcinoma cell proliferation in vitro. (A) Quantification of miR-221-3p in SNU-16 and SNU-1 cell lines with overexpressing miR-221-3p. (B) Quantification of miR-221-3p in AGS and NCI-N87 cell lines with silencing miR-221-3p. (C) MTT assay reveals cell growth curves of miR-221-3p overexpressing cells SNU-16 and SNU-1. (D) MTT assay reveals cell growth curves of miR-221-3p silencing cells AGS and NCI-N87. **p<0.01 based on the Student’s t-test. Error bars, SD.
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**MTT Assay and Colony Formation Assay**

Gastric carcinoma cells were seeded at 1,500 cells per well in 96-well plates after transfection. An MTT assay was performed to test cell proliferation at 1, 2, 3, and 4 days, and the absorbance was measured at 490 nm with a spectrophotometric plate reader. For colony formation assay, cells were plated at 500 cells per well in six-well plates after transfection and cultured for 14 days. Colonies were fixed with methanol, stained with 0.5% crystal violet, and counted under an inverted microscope.

**Western Blot Analysis**

Western blot analysis was conducted using anti-phospho-AKT (ser473), anti-AKT, anti-4E-BP1 (Epitomics, Burlingame, CA, USA), anti-phospho-FOXO3a (ser253), and phospho-4E-BP1 (Ser65; Cell Signaling Technology, Danvers, MA, USA), anti-p21, anti-cyclin

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**Figure 4.** miR-221-3p accelerates gastric carcinoma cell colony formation. (A) Representative micrographs of crystal violet-stained cell colonies overexpressing miR-221-3p in SNU-16 and SNU-1 cell lines analyzed by colony formation assay for 14 days and quantification of number of clones. (B) Representative micrographs of crystal violet-stained cell colonies silencing miR-221-3p AGS and NCI-N87 cell lines analyzed by colony formation assay for 14 days and quantification of number of clones. **p<0.01 based on the Student’s t-test. Error bars, SD.
D1, and anti-PTEN (BD Pharmingen, San Diego, CA, USA) antibodies.

Cell Invasion Assays

The effects of miR-221-3p or PTEN expression on cell migration and invasion were assessed using Matrigel assays as previously described

In Vivo Tumor Growth Model

Male BALB/c nude mice aged 4 to 6 weeks were purchased from the Hunan Slac Jingda Laboratory Animal Co., Ltd. (Shanghai, P.R. China). For the tumor growth assay, SNU-1 cells stably overexpressing miR-221-3p or scramble miRNA were resuspended in PBS and 1 × 10⁶ cells (200 μl) and subcutaneously injected in the dorsal flank of nude mice. Tumor size was measured every 3 days, and tumor volumes were calculated with the following formula: volume = (L × W²)/2, in which L is the longest diameter, and W is the shortest diameter. Six weeks later, mice were sacrificed, and tumors were dissected and weighed. Animal handling and research protocols were approved by the Animal Care and Use Ethics Committee.

Luciferase Reporter Assay

The 3'-UTR sequence of PTEN that was predicted to interact with miR-221-3p, or a mutated sequence within the predicted target site, was synthesized and inserted into the XbaI and FseI sites of the pGL3 control luciferase reporter vector (Promega, Madison, WI, USA). The luciferase reporter assay was performed as previously described

Statistical Analysis

Statistical analysis data were described as the mean ± SD. DFS was estimated using the Kaplan–Meier method. The relationship between survival period and each of the variables was analyzed using the log-rank test for categorical variables. Comparisons between different groups were undertaken using the Student’s two-tailed t-test. The criterion of statistical significance was \( p < 0.05 \). Statistical analysis was done with the SPSS/Win11.0 software (SPSS Inc., Chicago, IL, USA).

Figure 5. miR-221-3p promotes gastric carcinoma cell tumor growth in vivo. (A) Cells stably overexpressing miR-221-3p in SNU-1 were subcutaneously injected into nude mice. Six weeks later, SNU-1 cells stably overexpressing miR-221-3p had larger tumors than controls. (B) Representative picture of tumors formed. (C) The growth curves of tumor volumes. (D) Tumor weight. **\( p < 0.01 \) based on the Student’s t-test. Error bars, SD.
RESULTS

Aberrant Expression of miR-221-3p in Human Gastric Carcinoma Was Correlated With Poor Prognosis

In order to confirm whether miR-221-3p is related to gastric carcinoma, miR-221-3p levels were measured in gastric carcinoma specimens (n=50) and normal gastric tissues (n=6), as well as in gastric carcinoma cell lines (AGS, SNU-16, KATO-III, SNU-1, SNU-5, Hs 746T, and NCI-N87) and normal gastric epithelial cell line (GES-1). miR-221-3p was significantly higher in gastric carcinoma cell lines (Fig. 1A and B) than in normal gastric cells. In order to further confirm the role of miR-221-3p in gastric carcinoma, we measured the expression levels of miR-221-3p in gastric carcinoma samples. miR-221-3p was expressed higher in gastric carcinoma samples than in adjacent gastric carcinoma tissues (Fig. 1C). Moreover, in tumors, miR-221-3p expression was highest in stages III–IV tumors and higher in stages I–II tumors than in normal gastric tissues (Fig. 2A). Of note, higher miR-221-3p stage was often closely related with poorer prognosis. So we studied whether miR-221-3p was related to patient survival. More patients with lower miR-221-3p could survive after treatment than those with higher miR-221-3p (Fig. 2B). Altogether, these data demonstrate that miR-221-3p is upregulated in gastric carcinoma, and higher miR-221-3p expression possibly predicts poor patient survival.

miR-221-3p Predicted Oncogenic Properties in Gastric Carcinoma Cells

miR-221-3p was confirmed to be expressed aberrantly in gastric carcinoma cells, so we next assessed the

Figure 6. miR-221-3p accelerates invasion of gastric carcinoma cells. (A) Representative results of invasive ability of SNU-1 and SNU-16 cells transfected with miR-221-3p mimics or miR control. (B) Representative results of invasive ability of AGS and NCI-N87 cells transfected with anti-miR-221-3p mimics or anti-miR control. **p<0.01 based on the Student’s t-test. Error bars, SD.
functional role of miR-221-3p in gastric carcinoma cells. We established cell lines overexpressing miR-221-3p or silencing miR-221-3p. The expression levels of miR-221-3p were quantified by qRT-PCR (Fig. 3A and B). In the MTT assay, overexpression of miR-221-3p in SNU-16 and SNU-1 resulted in a higher growth rate in gastric carcinoma cells compared with controls (Fig. 3C), whereas silencing miR-221-3p in AGS and NCI-N87 cells significantly inhibited gastric carcinoma cell growth (Fig. 3D). In addition, the colony formation assay revealed that miR-221-3p promoted viability of gastric carcinoma cells compared with controls (Fig. 4A), whereas silencing miR-221-3p inhibited this ability (Fig. 4B). These tests in vitro revealed that miR-221-3p could promote gastric carcinoma proliferation.

To confirm whether the biologic effect of miR-1905 observed in cultured cells is relevant to gastric carcinoma growth in vivo, SNU-1-miR-221-3p and control cells, respectively, were subcutaneously inoculated into BALB/c athymic mice. Tumors formed by cells overexpressing miR-221-3p grew more rapidly than control (Fig. 5A and B), and the difference in average tumor volume between experimental and control animals continued to increase threefold at the experimental endpoint (6 weeks) (Fig. 5C). In parallel, increases in sizes and weights of tumors excised from animals in the miR-221-3p overexpression group were also observed compared with those of the control group (Fig. 5D). In vivo and in vitro tests therefore revealed that miR-221-3p promoted cell proliferation and tumor growth.

Of note, invasive ability is another characteristic of tumor cells. We next assessed the effects of miR-221-3p on gastric carcinoma cell invasion. In the results, overexpression of miR-221-3p significantly increased the number of invaded gastric carcinoma cells in the Matrigel assay (Fig. 6A) while silencing miR-221-3p restrained this progress (Fig. 6B), indicating that miR-221-3p promoted cell invasion in the Matrigel assay. Altogether, miR-221-3p acts as a cancer inducer in gastric carcinoma cells, and it could promote proliferation, tumor growth, and invasion of gastric carcinoma cells.

miR-221-3p Directly Targets PTEN, and PTEN Levels Are Inversely Correlated With miR-221-3p Levels

How does miR-221-3p play an essential role in tumor growth and invasion? We focused on the PTEN pathway. First, PTEN expression was inversely proportional to miR-221-3p expression by linear analysis (Fig. 7A). Moreover, overexpression of miR-221-3p significantly inhibited PTEN expression in gastric carcinoma cells (Fig. 7B), while silencing miR-221-3p increased PTEN expression (Fig. 7C). To verify whether PTEN was a direct target of miR-221-3p, the luciferase reporter containing the complimentary seed sequence of miR-221-3p at the 3'UTR region of PTEN mRNA was constructed (Fig. 8A). In the luciferase assay, the luciferase activity was significantly decreased in gastric carcinoma cells with overexpression of miR-221-3p cotransfected with 3'UTR-PTENwt FLuci vector and miR-221-3p mimic compared with those cotransfected with 3'UTR-PTENmut FLuci vector and miR-221-3p mimic (Fig. 8B and C). Meanwhile increasing activity was found in the gastric carcinoma cells that silenced miR-221-3p, suggesting that the fragment at the 3'-UTR of the PTEN mRNA was the complementary site for the miR-221-3p seed region (Fig. 8D and E). Thus, PTEN was a direct target of miR-221-3p. Altogether, miR-221-3p directly affects PTEN expression.
miR-221-3p Played its Oncogenic Properties by Activating Both PI3K/AKT/4E-BP1 Signaling Pathways

To verify the mechanism of miR-221-3p, we examined the role of miR-221-3p-mediated inhibition of PTEN in gastric carcinoma. Overexpressing miR-221-3p in SNU-1 and SNU-16 cells remarkably increased the effective proteins (Fig. 9A) participating in the PTEN signaling pathway containing phosphorylated Akt, phosphorylated P13K, and phosphorylated 4E-BP1. Meanwhile, silencing miR-221-3p in AGS and NCI-N87 cells

Figure 8. PTEN is a direct target of miR-221-3p. (A) Wild-type and mutant-type miR-221-3p target sequences of PTEN 3’-UTR. (B, C) Relative luciferase activity of PTEN in cells after cotransfection with wild-type (WT) or mutant (mut) PTEN 3’-UTR reporter genes and miR-221-3p mimics or control in SNU-1 and SNU-16 cells. (D, E) Relative luciferase activity of PTEN in cells after cotransfection with wild-type (WT) or mutant (mut) PTEN 3’-UTR reporter genes and anti-miR-221-3p mimics or control in AGS and NCI-N87 cells. **p<0.01 based on the Student’s t-test. Error bars, SD.
robustly suppressed these proteins (Fig. 9B). These results indicated that miR-221-3p indeed activated the Akt pathways, p21 and cyclin D1 are the targets of Akt, so we detected the expressions of p21 and cyclin D1. Decreased p21 and increased cyclin D1 expression could be caused by miR-221-3p overexpression (Fig. 9C), whereas it has the opposite effect on the regulation of p21 and cyclin D1 in gastric carcinoma cells silencing miR-221-3p (Fig. 9D). The above results revealed that miR-221-3p activated both PI3K/AKT/4E-BP1 signaling pathways. In order to further confirm the activation of the PI3K/AKT/4E-BP1 signaling pathways, we used pathway inhibitors. We found that the P13K inhibitor LY294002 significantly decreased phosphorylation of P13K and Akt (Fig. 10A), the Akt inhibitor decreased phosphorylation of Akt but not P13K (Fig. 10C), the mTOR inhibitor rapamycin decreased phosphorylation of 4E-BP1 (Fig. 10E), and these three inhibitors suppressed the proliferation of miR-221-3p overexpressing SNU-1 cells (Fig. 10B, D, and F). These data suggested that miR-221-3p promoted the proliferation of gastric carcinoma cells by simultaneously activating both PI3K/AKT/4E-BP1 pathways.

Repression of PTEN in Gastric Carcinoma Cells Restrains miR-221-3p-Induced Proliferation

In order to confirm the essential role of PTEN in miR-221-3p-induced oncogenic properties, we restored PTEN expression in SNU-1-miR-221-3p cells (Fig. 11A), and we found that PTEN restoration significantly decreased phosphorylation of Akt, P13K, and 4E-BP1 in SNU-1-miR-221-3p cells (Fig. 11B). Moreover, PTEN silencing in AGS-anti-miR-221-3p cells (Fig. 11C) increased phosphorylation of Akt, P13K, and 4E-BP1 in AGS-anti-miR-221-3p cells (Fig. 11D). To further confirm the role of PTEN, we examined the proliferation of indicated cells.

Figure 9. miR-221-3p activates both AKT/FOXO3a and AKT/mTOR signaling pathways. (A, B) Western blot analysis of phospho-P13K (p-P13K), total P13K (T-P13K), phospho-AKT (p-AKT), total AKT (T-AKT), phospho-4E-BP1 (p-4E-BP1), and total 4E-BP1 in indicated cells. (C, D) Western blot analysis of protein expression of p21 and cyclin D1 in indicated cells.
Figure 10. AKT/FOXO3a or AKT/mTOR signaling pathway inhibitors restrained the proliferation of miR-221-3p-overexpressing cells. Western blot analysis shows the effect of LY294002 (A), AKT inhibitor III (C), or rapamycin (E). MTT shows the effect of LY294002 (B), AKT inhibitor III (D), or rapamycin (F) on miR-221-3p-overexpressing cells. **p<0.01 based on the Student’s t-test. Error bars, SD.
PTEN restoration significantly decreased the proliferation of SNU-1-miR-221-3p cells (Fig. 11E). Meanwhile, silencing PTEN in AGS-anti-miR-221-3p cells showed the opposite function (Fig. 11F). These data suggest that PTEN is essential for miR-221-3p-induced proliferation.

**DISCUSSION**

In the past few years, miRNAs were reported to play an important role in cancer initiation and tumor progression. miR-221-3p is a new member of the miRNA family. In this study, we investigated the expression, function, and mechanisms of the action of miR-221-3p in gastric carcinoma. We found that miR-221-3p is a tumor-promoting factor in gastric carcinoma, where it acts as an oncogene by regulating PTEN expression. In gastric carcinoma cells, miR-221-3p overexpression robustly promoted cell proliferation and invasion in vitro. In contrast, silencing endogenous miR-221-3p remarkably abrogated the proliferation and invasion of gastric carcinoma cells. At the molecular level, PI3K/AKT/4E-BP1 pathways

Figure 11. Restoration of PTEN inverses miR-221-3p-induced proliferation. (A, C) Western blotting confirmation of PTEN in indicated cells. (B, D) Western blot analysis of indicated phosphorylated proteins in indicated cells. MTT shows the effect on indicated cells after restoration (E) or depletion (F) of PTEN. \(^*p<0.01\) based on the Student’s \(t\)-test. Error bars, SD.
contributed to an miR-221-3p-mediated malignant phenotype of gastric carcinoma cells, likely mediated by suppressing PTEN expression. Of note, the close correlation between high miR-221-3p expression and low expression of PTEN, as well as with the malignant properties of gastric tumors, was also confirmed in implanted tumors and in clinical gastric carcinoma samples, suggesting a possible role for miR-221-3p in the development and progression of gastric carcinoma.

Gastric carcinoma is one of the most common malignancies and one of the leading causes of cancer-related deaths worldwide. Despite recent advances in disease management and treatment, gastric carcinoma patients still have a very dismal long-term prognosis. For advanced-stage gastric carcinoma patients, the overall 5-year survival rate is less than 19%. The main challenges in the treatment of gastric carcinoma involve intrahepatic recurrence and metastasis, which simultaneously predict a poor outcome for gastric carcinoma patients. The identification of critical players that suppress these processes may lead to novel therapeutic targets for improving the prognosis of these patients. Various previous studies have identified some key signaling transduction cascades that are implicated in the progression, invasion, and metastasis of gastric carcinoma, such as the EGFR/PI3K pathway, the RhoGTPase/Rho effector pathway, the MAPK pathway, and the SNAK/JNK pathway. However, the underlying molecular mechanisms of gastric carcinoma metastasis are far from being fully understood.

Hundreds of genes were found to be the target of miRNA that harbor a target sequence in their 3′-UTR complementary to the seed region of the miRNA. Several studies have reported that certain miRNAs can directly target PTEN. PTEN is located at 10q23.3 and encodes a dual-specificity phosphatase with lipid and protein phosphatase activities. PTEN suppressed migration and genetic deletion of the Pten tumor suppressor gene that promotes cell motility, and overexpression or reconstitution of PTEN inhibited cell motility in a variety of cell types. Mechanistically, PTEN repressed cell motility through a variety of pathways, and PI3K/AKT is one important target of PTEN. In this study, we found that overexpression of miR-221-3p significantly decreased PTEN in gastric carcinoma cells to inhibit phosphorylated PI3K and AKT, resulting in an increase in proliferation, migration, and invasion. PTEN overexpression could restrain the increase in proliferation and invasion in miR-221-3p-overexpressed gastric carcinoma cells.

In summary, miR-221-3p was highly expressed in gastric carcinoma and promoted proliferation and invasion of gastric carcinoma cells. Moreover, miR-221-3p was correlated with a poor prognosis for gastric carcinoma patients, and miR-221-3p may be a novel therapeutic target of gastric carcinoma.

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