Targeting MicroRNA-21 Suppresses Gastric Cancer Cell Proliferation and Migration via PTEN/Akt Signaling Axis

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Abstract
MicroRNA plays a pivotal role in various human cancers, especially in human gastric cancer. In the present study, we evaluated the effect of microRNA-21 (miR-21) on the gastric cancer cell proliferation, migration, apoptosis and the related signaling cascades. Here, we showed that down-regulation of miR-21 markedly reduced gastric cancer cell proliferation (AGS and NCI-N87 cells) in a time dependent manner. Moreover, our findings revealed that silencing miR-21 dramatically blocked gastric cancer cell migration and movement, which might be related to down-regulation of vimentin expression. We also found that down-regulation of miR-21 promoted cell apoptosis and repressed cell cycle progression. Further investigation showed that down-regulation of miR-21 significantly increased phosphatase and tensin homolog (PTEN) protein expression level, but not transcription level (mRNA level), which in turn decreased Akt phosphorylation at Thr308 and Ser473. Collectively, our results uncover that miR-21 targets PTEN/Akt signaling pathway and regulates cell proliferation, migration and apoptosis in human gastric cancer cells. Our findings may provide a therapeutic target for treatment of human gastric cancer.

Keywords
micro RNA-21, PTEN, Akt, gastric cancer, proliferation, migration

Introduction
Gastric cancer (GC) is one of the most prevailing malignant tumors in human, which is characterized by rapid progression, poor prognosis and low five-year survival. So far, the major therapeutic approaches have not been discovered in GC because the molecular mechanisms of GC progression are still completely unknown¹. It is well documented that abnormal miRNA expression has been shown to be closely related to the occurrence and progression of GC². In recent studies, the dysfunction of miRNA regulation has been closely linked to the gastric carcinogenesis through disturbing tumor suppressors and facilitating the expression of oncogenes. Furthermore, dysfunction of miRNA also leads to changes in down-stream phenotypes, including cell proliferation, apoptosis, motility and invasion³. MiRNAs results in degradation and attenuation of translation of the targets mRNA through complete or incomplete binding to the target genes⁴. Specifically, miroRNA-21 (miR-21) is well recognized and closely involved in translational regulation. In addition to gastric cancer⁵, previous studies have reported that miR-21 was also overexpressed and involved in many human cancers, such as colon cancer⁶, lung cancer⁷, and breast cancer⁸. These findings suggest that the functionality of miR-21 is closely related to development of human cancer and promotes cancer cell infiltration and metastasis with the role of oncogene. Nevertheless, the current gap is that the innate interaction between the miR-21 and the downstream signaling axis is completely unknown.

Based on recent studies, the role of miR-21 in GC and the related molecular mechanisms are investigated. We found that down-regulation of miR-21 markedly reduced gastric cancer cell proliferation (AGS and NCI-N87 cells) in a time...
dependent manner. Moreover, our findings revealed that silencing miR-21 dramatically blocked gastric cancer cell migration and movement, which might be related to down-regulation of vimentin expression. We also found that down-regulation of miR-21 promoted cell apoptosis and repressed cell cycle progression. Further investigation showed that down-regulation of miR-21 significantly increased phosphatase and tensin homolog (PTEN) protein expression level, but not transcription level (mRNA level), which in turn decreased Akt phosphorylation at Thr308 and Ser473. We propose that miR-21 regulates cell proliferation and invasion in gastric cancer cells via targeting PTEN/Akt signaling cascades.

**Materials and Methods**

**Cell Culture**

The human gastric cancer cell line AGS cells were obtained from Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Grand Island, NY, USA), 100 IU/ml penicillin and 100μg/ml streptomycin (Sigma, St. Louis, MO, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. Trypsin (0.25%, Invitrogen, Carlsbad, CA, USA) solution was used to subculture the cells from the culture flask.

**Lentivirus Preparation and Infection**

Lentivirus encoding short hairpin RNA (shRNA) targeting miR-21 and its negative control (shRNA-NC) were constructed by Hanyin Biotechnology Company (Shanghai, China). AGC cells were seeded into 6-well plates with a density of 1×10⁶ cells per well and maintained for 12 h at 37°C. Then, the medium was replaced by McCoy’s 5A medium (Gibco Laboratories, Grand Island, NY, USA). With a multiplicity of infection (MOI) of one, 2 mL of lentivirus containing indicated shRNA was added into each well. Following incubation for 12 h at 37°C, the medium was replaced by fresh McCoy’s 5A medium again. Infection efficiency was evaluated by GFP expression via flowcytometry. The cells were harvested for real time-PCR analysis to evaluate the knockdown efficiency after being cultured for 96 h at 37°C.

**RNA Isolation and Real Time-PCR**

Total-RNA from cells in different groups was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s instructions. Real-time PCR was conducted using SYBR-Premix Ex Taq™ (TaKaRa, Shiga, Japan) according to the manufacturer’s protocol.

**Cell Proliferation Assay**

AGS cells were seeded into 96-well plate at 4000 cells per well the day before infection. The cells were infected with miR-21 negative control (NC) and miR-21 shRNA. Without any treatment was served as control group. Cell viability and proliferation were assessed by cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD, USA) and 5-bromo-2-deoxyuridine (BrdU) incorporation ELISA kit (Roche, Indianapolis, IN, USA) respectively, according to the manufacturer’s protocol. Cell viability and proliferation detection was measured by absorbance at 450 and 492 nm, respectively, using an Infinite 200 plate reader (TECAN, Mönndedorf, Switzerland).

**Alive Measurement of Cell Bio-Behaviors**

The cell bio-behaviors including total cell number, cell differentiation and cell movement were measured by the real-time cell monitoring system using a Cell-IQ Cell-IQ Alive Image Monitoring System (Chip-Man Technologies, Tampere, Finland), equipped with a phase-contrast microscope (Nikon CFI Achromat phase contrast objective with 10× magnification) and a camera. The equipment was controlled by Imagen software (Chip-Man Technologies, Tampere, Finland). Images were captured at 5 min intervals for 72 h. Analysis was carried out with a freely distributed Image software (McMaster Biophotonics Facility, Hamilton, ON, Canada), using the Manual Tracking plug in created by Fabrice Cordelières (Institut Curie, Orsay, France). Cell-IQ system uses machine vision technology for monitor and record time-lapse data, and it can also analyze and quantify cell functions and morphological parameters. Here, we used this system to discriminate cell stage (dividing/stable stage) and calculate cell numbers of each stage during proliferation. Also, Cell-IQ was programmed to quantify the movement of each individual cell in the image field. The distance of total cell movement indicated the high migratory intention of cells. AGS cells were cultured in Cell-IQ system with 24-well plates (8×10³ cells/well) for 24 h and then cells were translated with and without miR-21 shRNA for another 72 h. Cell-IQ system automatically discriminated cell stage and calculated total cell number, cell differentiation and cell movement. Each group contained 6–12 replicate image sites.

**Immunofluorescence**

AGS cells were cultured on glass cover slips and incubated overnight before infection. The cells were infected with miR-21 negative control (NC) and miR-21 shRNA. Cells without any treatment were served as control group. After 72 h, the cells were fixed with 4% paraformaldehyde for 20 min at 37°C, followed by permeabilization in methanol for 15 min at –20°C. The cells were incubated in blocking buffer (5% bovine serum albumin in PBS) for 1 h at room temperature followed by incubation with ki67 antibody (Abcam, cat# ab15580, Cambridge, MA, USA; diluted 1:200 in blocking
buffer) at 4°C overnight. Cells were washed 3 times for 10 min in PBS, and then incubated for 2 hr with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody for ki67 (diluted 1:200 in blocking buffer; Invitrogen, USA) at room temperature. Nuclei were stained with DAPI for 1 hr at room temperature before observation. Images were acquired using a LSM510 META confocal microscope (Zeiss, Jena, Germany) through a 40× oil objective (NA = 1.3) and the data were analyzed with Zeiss Rel 3.2 image processing software (Zeiss, Germany).

**Scratch Wound-Healing Motility Assay**

When AGS cells were seeded and grown to confluency, a scratch was set with a pipette tip running though the dish and cultured under standard conditions for 0 hr, 48 hr and 72 hr. Plates were washed twice with fresh medium to remove nonadherent cells and then photographed. The cell migration was evaluated by counting cells that migrated from the wound edge.

**Apoptosis Assay**

For apoptosis assays, AGS cells were harvested 24 or 48 hr after infection, and then washed with PBS. A FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, CA, USA) was added to the cells. As per the manufacturer’s instructions, the cells were stained and analyzed by flow cytometer (BD Biosciences, USA) within 30 mins after staining. The results were analyzed using FlowJo 10.0.7 software (Treestar Inc., USA).

**Cell Cycle Assay**

For cell cycle analysis, AGS cells were harvested 24 or 48 hr after infection, and then washed with PBS. A FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, CA, USA) was added to the cells. As per the manufacturer’s instructions, the cells were stained and analyzed by flow cytometer (BD Biosciences, USA) within 30 mins after staining. The results were analyzed using FlowJo 10.0.7 software (Treestar Inc., USA).

**Western Blot Analysis**

Western blot analysis was performed according to standard procedures. Total protein was isolated from AGS cells. Protein concentrations were determined by BCA Protein Assay kit (Beyotime Biotechnology, China). The membranes containing proteins were incubated with a primary antibody against PTEN, p-Akt-Thr308, p-Akt-Ser473, total Akt activated caspase-3, vimentin, E-cadherin, MMP-9 (Cell Signaling Technology, Danvers, MA, USA), and β-tubulin (Abcam, Cambridge, USA). Signals were detected by indicated secondary antibodies labeled with HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the signal intensity was determined by Quantity One software.

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism (https://www.graphpad.com/). All experiments were performed at least in triplicate under identical conditions and data were represented as means ± standard error of the mean (SEM). Differences between two groups were analyzed by unpaired two-tailed Student’s t test. Difference with *p* < 0.05 was considered statistically significant.

**Results**

**Silencing miR-21 Reduced Human Gastric Cancer Cell Proliferation**

AGS cells were infected with miR-21 shRNA or NC shRNA. The infection efficiency was evaluated by flow cytometry. As shown in Fig. 1A, the infection efficiency reached 99%. Next, the mRNA expression of miR-21 was measured by qRT-PCR. As shown in Fig. 1B, the mRNA level of miR-21 was significantly blocked compared with NC group and normal AGS cells, indicating that miR-21 was a successful knockdown. To investigate the effect of miR-21 on AGS cell proliferation, CCK-8 and BrdU assay were employed. As shown in Fig. 1C and D, blockage of miR-21 remarkably suppressed cell proliferation compared with NC group and normal AGS cells. Next, the same experiments were carried out in NCI-N87 cells and the similar results were obtained (Fig. 1E and F). Taken together, these results suggest that targeting miR-21 can prevent human gastric cancer cell proliferation.

**Down-Regulation of miR-21 Blocked AGS Cell Growth**

The proliferation of AGS and NCI-N87 cells was markedly decreased by miR-21 shRNA, causing significant inhibition of cell proliferation compared with normal cells and cells infected with miR-21 shRNA-NC (Fig. 1). At the same time, AGS cells were infected with or without miR-21 shRNA and the dynamic cell growth was monitored by Cell-IQ Alive Image Monitoring System at indicated time point. As shown in Fig. 2A, the knockdown of miR-21 markedly prevented cell growth compared with NC group and normal AGS cells. Subsequently, the cell growth was monitored by Ki-67 staining after infection of miR-21 shRNA. As shown in Fig. 2B and C, silencing miR-21 greatly diminished Ki-67 expression in AGS cells compared with NC and normal AGS cells. Altogether, these data characterize the
functionality of miR-21 in regulating human gastric cancer cell growth.

**Knockdown of miR-21 Decreased AGS Cell Movement**

To investigate the effect of miR-21 on AGS cell movement, the cells were infected with miR-21 shRNA or NC shRNA. The cell movement was monitored and analyzed. As shown in Fig. 3A, silencing miR-21 dramatically compromised cell movement. Subsequently, the expression level of vimentin, a biological marker which involved in the cell migration, was detected by Western blotting. As shown in Fig. 3B and C, silencing miR-21 dramatically decreases the expression of vimentin. The same experiments were carried out in
NCI-N87 cells and the similar results were obtained (Fig. 3D and E). We also confirmed these results by a wound-healing motility assay. Obviously, in Fig. 4A and B, silencing miR-21 significantly reduced cell movement. Taken together, these results support the idea that miR-21 regulates cell movement and migration in human gastric cancer.

**Silencing miR-21 Promoted Cell Apoptosis and Blocked Cell Cycle Progression in AGS Cells**

The apoptosis of AGS cells was detected through flow cytometry (Fig. 5A and B). Infection of miR-21 shRNA accelerated cell apoptosis of AGS cells, while caspases-3 was...
dramatically activated. As shown in Fig. 5C and D, activated caspase-3 was significantly negatively correlated with miR-21 level in cells. To further explore whether silencing miR-21 alter cell cycle progression, AGS cells were infected with miR-21 shRNA or NC shRNA. As shown in Fig. 6A and B, flow cytometry data showed that silencing miR-21 dramatically propagated in G0/G1 phase of cells and fall back in S-phase of AGS cells.

Silencing miR-21 up-Regulated PTEN/Akt Signaling Pathway

To study the function of miR-21 on PTEN expression in human gastric cancer cells, AGS and NCI-N87 cells were infected with miR-21 shRNA and NC shRNA. Interestingly, as shown in Fig. 7A and F, PTEN mRNA expression was no change compare with NC and control groups. Subsequently, we measured the PTEN protein expression. As shown in Fig. 7B and G, we found that PTEN protein expression was significantly triggered in AGS and NCI-N87 cells, indicating that miR-21 regulated PTEN expression level through post-translation level instead of transcriptional level (Fig. 7C and 7H). Furthermore, we also measured the key downstream targets of PTEN. Our results showed that Akt phosphorylation (Thr308 and Ser473) was markedly restrained, but total Akt was no change (Fig. 7D and I). In addition, MMP-9 expression was significantly decreased as well (Fig. 7E and J). Altogether, these results support our hypothesis that miR-21 up-regulates human gastric cancer cell proliferation, movement and apoptosis by targeting PTEN/Akt signaling pathway.

Discussion

In recent years, accumulated evidence has shown that miR-21 was highly expressed in all types of gastric cancer
Fig. 4. Down-regulation of miR-21 reduced migration ability of AGS cells. (A) AGS cells were infected with or without miR-21 shRNA and the cell migration was detected by scratch wound-healing motility assay. (B) The AGS cell migration was quantified. Data represent mean ± SEM, n = 3; *p < 0.05 vs. NC and control group.
Fig. 5. Down-regulation of miR-21 promoted AGS cells apoptosis. (A) AGS cells were infected with or without miR-21 shRNA and cell apoptosis was detected by flow cytometry. (B) Quantitative analysis of cell death after infection of miR-21 shRNA in AGS cells. (C) Western blot analysis of activated caspase-3 with or without infection of miR-21 shRNA in AGS cells. (D) Quantitative analysis of activated caspase-3 after infection of miR-21 shRNA in AGS cells. Data represent mean ± SEM (n = 3; *p < 0.05 vs. NC and control group).
cells\(^5\), such as AGS, SGC7901 and NCI-N87. While many target genes of miR-21 have also been identified, such as RECK, PDCD4, Serpini1, and FASLG\(^10\)–\(^13\). Further investigations have reported that miR-21 could mediate the drug resistance of chemotherapeutic drugs such as trastuzumab and cisplatin. In addition, Zhang et al.\(^14\) found that up-regulation of miR-21 promoted cell proliferation of AGS cells. In contrast, down-regulation of miR-21 prevented cell proliferation and promoted cell apoptosis. Zhang et al.\(^14\) also reported that the level of miR-21 regulated the healing of BGC-823 gastric cancer cells, whereas treatment of miR-21 inhibitor markedly reduced the healing and repair of gastric cancer cells. At the same time, the migration ability of gastric cancer cells was significantly inhibited by the delivery of miR-21 inhibitor. The result in the current study showed that when AGS and NCI-N87 cells were transfected with miR-21 shRNA, the cell proliferation was blocked (Figs 1 and 2). Furthermore, the cell migration and movement was significantly prevented when miR-21 was down-regulated, which may be related to decrease of the vimentin expression (Figs 3 and 4). These results were similar as above, and it could be confirmed that miR-21 played an important role in the regulation of cellular migration in gastric cancer.

Vanas et al.\(^15\) reported that after inhibition of miR-21, the expression of tumor suppressor gene PTEN was significantly up-regulated. Their results showed that PTEN as the anti-cancer protein was deactived by miR-21 in the development and progression of gastric cancer. Conversely, overexpression of miR-21 resulted in a decreased apoptosis rate of gastric cancer cells. Furthermore, Ziyan et al.\(^16\) reported that miR-21 served as a significant modulator of the anti-tumor effect of CDDP by regulating the expression of bcl-2 in osteosarcoma cells. Eto et al.\(^17\) transfected NCI-N87 cells with miR-21 mimetic, and the results showed that miR-21 down-regulated PTEN and the Akt phosphorylation levels were dramatically increased. Further investigation showed that miR-21 played a key role in the regulation of gastric cancer cell tumor suppressor gene expression, such as PTEN and PDCD4\(^18\). Two different gastric cancer cells (SGC7901 and MKN45), were transfected with miR-21 mimetic, and the results demonstrated that the overexpression of miR-21 could promote the invasion and migration of gastric cancer cells. In contrast, miR-21 inhibitors significantly reduced
Fig. 7. MiR-21 regulated PTEN expression at the post-transcriptional level and decreased Akt activity. (A) AGS cells were infected with or without miR-21 shRNA and the PTEN mRNA expression was measured by qRT-PCR. Data represent mean ± SEM (n = 3). (B) The protein expression levels of PTEN, MMP-9, p-Akt-Thr308, p-Akt-Ser473 and total Akt were detected by Western blotting in AGS cells and the representative pictures were shown. (C–E) Quantitative analysis of the changes of PTEN, MMP-9, p-Akt-Thr308 and p-Akt-Ser473 in AGS cells. Data represent mean ± SEM (n = 3; *p < 0.05 vs. NC and control group). (F) NCI-N87 cells were infected with or without miR-21 shRNA and the PTEN mRNA expression was measured by qRT-PCR. Data represent mean ± SEM (n = 3). (G) The protein expression levels of PTEN, MMP-9, p-Akt-Thr308, p-Akt-Ser473 and total Akt were detected by Western blotting in NCI-N87 cells and the representative pictures were shown. (H–J) Quantitative analysis of the changes of PTEN, MMP-9, p-Akt-Thr308 and p-Akt-Ser473 in NCI-N87 cells. Data represent mean ± SEM (n = 3; *p < 0.05 vs. NC and control group).
cell proliferation, migration and invasion. In the current study, our results uncover that miR-21 shRNA inhibited the proliferation of gastric cancer cells (Fig. 2). In addition, analysis of cell cycle progression showed that down-regulation of miR-21 shRNA blocked cell cycle progression in the stage of G0/G1 (Fig. 6). This data suggest that miR-21 may act as a strong anti-apoptotic factor and regulates cell cycle progression in human gastric cancer.

PI3K/Akt signaling cascades orchestrate in the development and progression of various human cancers. The gain and loss of functions caused by related genes and molecular abnormalities in this pathway could lead to the abnormalities in tumor cell proliferation, apoptosis and invasion. Recent studies have shown that PI3K/Akt signaling axis was involved in the development and progression of multiple human tumors. Once PI3 K was activated, the plasma membrane will produce a large number of second messenger 3,4,5-triphosphate phosphatidylinositol PIP3, PIP3 and intracellular Akt and phosphoinositide dependent kinase (PDK1) bind together. The structural confirmation of Akt was changed. The complex will be delivered to the cell surface. This pathway is a positive-loop and signaling will undergo cascade-amplification in the activation of Akt. Activated Akt rapidly regulates downstream substrates such as Bad, caspase9, NF-κB, GSK3β, etc. and regulates cell proliferation, differentiation, apoptosis and migration by phosphorylation. PTEN is a tumor suppressor gene, while it is a membrane-bound lipid phosphatase that regulates the expression of PIP3 as a tumor suppressor in a variety of systems, and plays important function in the regulation of embryonic development, cell growth, differentiation, apoptosis and migration. Yang et al. found that the expression of PTEN was highly rich in the gastric cancer tissue, and the expression of PTEN is up-regulated after the inhibition of miR-21, suggesting that PTEN could be used as a target to control the development of gastric cancer. PTEN has capacity to dephosphorylate excess PIP3 to PIP2, against the activation of Akt and prevent downstream signaling events dependent on Akt. Akt is a major protein in the anti-apoptotic pathway. The activation of Akt could prevent apoptosis through PTEN. Wu et al. found that PTEN/PI3K/Akt signaling pathway could promote the proliferation, migration, invasion and apoptosis of human esophageal cancer cells. The results in the current study showed that down-regulation of miR-21 shRNA markedly reduced cell movement and promoted cell apoptosis via PTEN/Akt signaling axis in gastric cancer cells (Figs 3–7). Furthermore, our findings also revealed that miR-21 shRNA significantly increased PTEN protein expression, but not PTEN mRNA (Fig. 7). These findings showed that miR-21 inhibited PTEN protein level, rather than transcriptional level, which further suggested that miR-21 regulated PTEN at the post-translation level in gastric cancer. Finally, our results not only further confirmed the miR-21 regulation of PTEN/Akt signaling axis, but also uncovered that miR-21 plays a central role in the regulation of human gastric cell proliferation, migration and apoptosis.

In summary, miR-21 regulates human gastric cancer cell proliferation, motility and apoptosis through control of PTEN/Akt signaling cascades, which may be served as a therapeutic target for treatment of human gastric cancer. Further study will discover the precise functions of miR-21 in human gastric carcinogenesis and development.

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Ethical Approval
The study was approved by the Ethics Committee of Fudan University.

Statement of Human and Animal Rights
Human gastric cancer cell line AGS cells were obtained from Chinese Academy of Sciences (Shanghai, China) and were preserved at the Biomedical Research Center of Zhongshan Hospital (Fudan University, Shanghai, China).

Statement of Informed Consent
There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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