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MiR-616-3p promotes angiogenesis and EMT in gastric cancer via the PTEN/AKT/mTOR pathway

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ABSTRACT

Dysregulation of microRNAs has been demonstrated to be involved in a variety of biological events related to cancer, including proliferation, metastasis, angiogenesis and immune escape. MiR-616-3p is located on the chromosome region 12q13.3, however, its potential role and clinical implications in gastric cancer remain poorly understood. The current study aimed to investigate the potential role of miR-616-3p in gastric cancer. The results showed that miR-616-3p was up-regulated in cancer tissues. Higher expression of miR-616-3p in tumor tissues also predicted poor prognosis. Furthermore, loss- and gain-of-function *in vitro* revealed that miR-616-3p promoted angiogenesis and EMT in gastric cancer cells. Mechanistically, further analysis demonstrated that the effects of miR-616-3p on metastasis and angiogenesis occurred through the down-regulation of PTEN, a direct target of miR-616-3p. We propose that the restoration of PTEN expression may block miR-616-3p-induced EMT and angiogenesis. Collectively, our findings suggest that the miR-616-3p-PTEN signaling axis might be a potential therapeutic target for gastric cancer.

1. Introduction

Gastric cancer is one of the most prevalent human malignancies. Worldwide, the incidence of gastric cancer is the fourth highest among malignant tumors, while mortality rate ranks second [1,2]. In recent years, with advances in medical treatment technology, the combined application of chemotherapy, radiotherapy and surgical treatment has contributed profoundly to improve prognosis in gastric cancer patients [3,4]. Nevertheless, the low overall survival rate is still unsatisfactory. Therefore, understanding the pathogenesis and genetic control mechanisms of gastric cancer and characterizing proliferation, apoptosis, invasion, metastasis and other biological behavior-related regulatory molecules are of great importance and will help to develop novel strategies in clinical diagnosis, treatment and prognosis monitoring.

MiRNAs are a class of non-coding small RNAs of approximately 22 nucleotides in length and are widely present in eukaryotes, with highly conserved, temporal and tissue specificities among species [5]. MiRNAs are involved in regulating post-transcriptional expression levels by inhibiting the translation of target mRNAs [6]. MiRNAs often control aberrant expression in human malignancies and function as either oncogenes or tumor suppressors, depending on the biological roles of target genes [7–9]. Dysregulation of miRNAs is closely related to a wide range of biological processes such as tumor incidence, development and drug resistance [10,11]. For example, miR-612 suppresses metastasis in HCC by targeting the kinase AKT2 [12]. The serum expression of miR-21 is associated with chemosensitivity in advanced pancreatic cancer [13].

MiR-616-3p is elevated in several types of tumors, including prostate cancer [14]and lung cancer [15]. A recent study also demonstrated that the up-regulation of miR-616-3p expression was correlated with poor survival in hepatocellular carcinoma patients [16]. However, to date, there are no studies investigating its role in

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gastric cancer. In this study, we explored the expression and clinical significance of miR-616-3p in GC tissues. We also tested whether miR-616-3p modulates tumor angiogenesis and metastasis in gastric cancer cells. Furthermore, mechanistic investigation suggests that miR-616-3p may exert its biological function by targeting PTEN, a direct target gene, via the AKT/mTOR pathway.

2. Materials and methods

2.1. GC tissues

A total of 63 paired gastric cancerous and adjacent noncancerous tissues were obtained from biobank in Fudan University Shanghai Cancer Center (FUSCC). These patients underwent surgery in FUSCC between 2008 and 2009. None of the patients had received chemotherapy or radiotherapy before surgery. The tumors were staged according to the TNM staging system of the American Joint Committee on Cancer (7th edition). The clinicopathology characteristics of the patients were listed in Supplementary Table 1. Written informed consent was obtained from the patients for all the samples. The study methodologies and the experiments were approved by the ethics committee of Fudan University Shanghai Cancer Center (FUSCC).

2.2. Cell lines and culture conditions

The human gastric cancer cell lines MKN-28, AGS, SGC-7901 and MGC-803 were purchased from the cell bank of the Chinese Academy of Science (Shanghai, China). The gastric normal epithelial cell GES-1 and HEK 293 T cells were also obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO2.

2.3. RNA isolation and quantitative real-time PCR analysis

The total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA samples (500 ng each) were then reverse-transcribed into cDNA using the PrimeScriptTM RT reagent kit (Takara, Dalian, China). For the miR-616-3p reverse-transcription, the 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCT GCTCAA-3′ sequence was added into the RT reaction system as the miR-616-3p reverse-transcription primer. For the quantification, real-time PCR was performed with SYBR Premix Ex Taq (Takara) with the Applied Biosystems Prism 7900 system (Applied Biosystems, Life technologies, USA). The levels of mature miRNA-616-3p expression were normalized to U6. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method. The primers used in the qRT-PCR were as follows: miR-616-3p-Forward: 5′-ACACTCCAGCTGGGAGT-CATTGGAGGGTTT-3′, miR-616-3p-Reverse: 5′-TGGTGTCGTGGA GTCG-3’; U6-Forward: 5′-CTCGCTTCGGCAGCACA-3′, U6-Reverse: 5′-AACGCTTCACGAATTTGCGT-3’.

2.4. Transwell assay and wound healing

For the migration assays, 4×10^4 transfected MKN-28 and AGS cells in 200 μL serum-free medium were plated in the top chamber with the non-coated membrane (24-well insert; pore size, 8 μm; BD Falcon™; Becton Dickinson, Franklin Lakes, NJ, USA), and 600 μL containing 10% FBS medium was added into the lower chamber. For the invasion assays, Matrigel (1:10) (BD biosciences) was polymerized in transwell inserts for 45 min at 37 °C and then 1×10^5 cells were seeded into the top chamber. After 24 h of incubation for migration and 48 h for the invasion assay, the cells that invaded the lower side of the chamber were fixed with 4% paraformaldehyde, stained with 1% crystal violet, and counted.

For the wound healing assay, the cells were seeded in a six-well plate at a confluence of 90%. Then, a micropipette tip was used to create scratch wounds, and the medium was replaced with fresh serum-free medium. Pictures of wound healing were taken at 0 and 24 h.

2.5. Western blot

Western blot was performed as described previously [17,18]. Briefly, the cells were lysed by RIPA buffer (Beyotime, Shanghai, China) with protease inhibitors and phosphatase inhibitors (Bimake, Shanghai, China). Then, the protein concentrations of the cell lysates were quantified using the BCA protein assay kit (Beyotime, Shanghai, China). Finally, a total of 20 μg protein was loaded onto a 10% SDS–PAGE gel and then transferred into polyvinylidene fluoride (PVDF) membranes. After blocking with 10% skim milk for 1 h at room temperature, the membranes were probed with the primary antibodies overnight at 4 °C. The next day, the membranes were washed with TBST and then incubated with horseradish peroxidase (HRP)–conjugated secondary antibody for 1 h at room temperature. Protein was detected by a luminescent image analyzer (ImageQuant LAS4000 mini). β-actin was used as an endogenous control. Primary antibodies against E-cadherin (1:1000), vimentin (1:1000), Snail (1:1000), slug (1:1000), mTOR (1:1000), *p*-mTOR (Ser2448) (1:1000), AKT (1:1000), *p*-AKT (Ser473) (1:1000), VEGFR2 (1:1000) and β-actin (1:2000) were purchased from Cell Signaling Technology (Cambridge, MA, USA). VEGFA (1:1000) was obtained from Abcam Company (Cambridge, MA, USA). The secondary antibodies against rabbit and mouse (1:10000) were from Jackson ImmunoResearch.

2.6. Dual-luciferase reporter assay

By using a web-based miRNA program Target Scan (http://www.targetscan.org/) and miRBase (http://www.mirbase.org/), we predicted that hsa-miR-616-3p can bind with the 3′-UTR region of PTEN. The wild-type (WT) and mutant 3′-UTR of PTEN were cloned into a luciferase vector containing the Renilla luciferase gene. For the luciferase assay, HEK 293 T cells were co-transfected with miR-616-3p mimics or inhibitors or NC and reporter plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Then, 48 h post transfection, firefly luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corporation, Fitchburg, WI, USA), according to the manufacturer's instructions.

2.7. Transfection and lentivirus infection

PTEN-overexpression plasmid was kindly gifted from Dr. Qin Y (Pancreatic Cancer Institute of Fudan University, Shanghai, China). The miR-616-3p mimics and inhibitors and their corresponding NC (Negative Control) were purchased from Ribobio (Guangzhou, China). For transient transfection, 3 μg PTEN-overexpression plasmid and 100 nM mimics or inhibitors were transfected into the cells using Lipofectamine 2000 according to manufacturer's instructions.

The miR-616-3p overexpression plasmid and lentiviral package were completed by Hanheng biotechnology Company (Shanghai, China). The miR-616-3p knockdown plasmid and lentiviral package were prepared by Hanyin Company (Shanghai, China). The MKN-28 cells were infected with miR-616-3p overexpression lentivirus, and the AGS cells were infected with knockdown lentivirus. The targeted cells were selected by GFP or puromycin.

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