Original Paper

MicroRNA-449a Inhibits Proliferation and Induces Apoptosis by Directly Repressing E2F3 in Gastric Cancer

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Key Words

Gastric cancer • MiR-449a • E2F3 • Proliferation • Apoptosis

Abstract

Background: MicroRNA-449a is a tumor suppressor that is down-regulated in multiple tumors types. However, the role of miR-449a in gastric cancer (GC) remains largely unknown. **Methods:** MiR-449a expression was up-regulated using miR-449a mimics, and the role of miR-449a in GC was assessed using cell viability and apoptosis assays. miR-449a target genes were confirmed using luciferase activity, RT-PCR and western blot assays. **Results:** miR-449a was downregulated in gastric cancer cell lines and gastric cancer tissues. Restoration of miR-449a expression inhibited gastric cancer cell proliferation and colony formation. Significant G0/G1 arrest was observed in gastric cancer cells transfected with miR-449a mimics. Furthermore, combination therapy with miR-449a with cisplatin displayed greater anti-tumor effects than treatment with cisplatin alone. We also identified E2F3 (E2F transcription factor 3), an important transcription factor involved in the proliferation and metastasis of tumor cells, as a direct target gene of miR-449a. Furthermore, silencing E2F3 elicits similar a repressive effect as overexpression of miR-449a in gastric cancer cells, and E2F3 overexpression rescued the repressing effects of miR-449a mimics. **Conclusions:** This study indicates that the miR-449a/E2F3 axis plays an important role in proliferation and apoptosis in gastric cancer. Therefore, miR-449a represents a novel target for gastric cancer therapy.

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Introduction

Gastric cancer (GC) is one of the most common types of malignancies around the world. Over the past 20 years, the incidence and mortality rate of GC have decreased worldwide, but it still ranks as the second leading cause of cancer death and the most common gastrointestinal malignancy in East Asia [1]. Despite recent advances in gastrectomy, chemotherapy, and radiotherapy, more than half of all advanced stage GC patients die of recurrence, even after undergoing curative gastrectomy [2]. Therefore, elucidation of the regulatory mechanisms modulating GC tumorigenesis and the molecular alterations underlying this disease is imperative for developing novel targeted therapies for gastric cancer [3].

MicroRNAs (miRNAs) are a family of small, non-coding RNAs that negatively regulate the expression of target genes by altering mRNA translation or stability [4]. miRNAs play important roles in a wide variety of physiological or pathological processes, including tumorigenesis. An increasing body of evidence indicates that microRNA expression is dysregulated in human cancers, implying an essential role for miRNAs during tumor progression [5]. MiRNAs function as regulatory molecules, acting as oncogenes or tumor suppressor genes in tumorigenesis. Furthermore, abnormal miRNA expression is associated with the development and progression of GC [6]. However, the exact molecular mechanisms by which miRNAs influence GC remain unclear. The role of miRNAs in GC tumorigenesis warrants further investigation.

MicroRNA-449a (miR-449a) is an important miRNA that has been identified in previous cancer studies. MiR-449a acts as tumor suppressor, and it is downregulated in various types of cancer, including gastric cancer [7]. However, little is known about the role of miR-449a in GC tumorigenesis and progression. The aim of the present study is to explore the role of miR-449a in GC cell proliferation and apoptosis.

Materials and Methods

Tissue sample collection and cell culture

Twelve primary gastric cancer tissue samples and non-tumor gastric tissues were collected from Wujiang first people's hospital. Written informed consent was obtained from each participant. This study was approved by the institutional review board of Wujiang first people's hospital. Tissue samples were quickly frozen in liquid nitrogen immediately after surgical removal and stored at -80 °C. Normal human gastric epithelial cells (NGEC) and the gastric cancer cell line, SGC-7901, were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM medium, supplemented with 10% fetal bovine serum (FBS; Hyclone, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated in an atmosphere of 5% CO₂ at 37 °C. Primary normal human gastric epithelial cells (NGEC) were established as described previously [8].

Synthesis and transfection of miRNA/siRNA and plasmid DNA

miR-449a mimics, negative controls (NC), and siRNA targeting human E2F3 mRNA (GenBank no. NM. 001949) were designed and synthesized by Shanghai GenePharma Company (Shanghai, China). For transfection, 4 × 10³ cells per well were seeded into 96-well plates in antibiotic-free growth medium. Once the cells reached 80–90% confluence, they were transfected with human miR-449a mimics or negative control (NC). For E2F3 or scrambled siRNA, cells were transfected at 30–40% confluence using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol. The pCDNA3.1-E2F3 plasmid, which encodes the entire E2F3 coding sequence but lacks the normal 3'-untranslated sequence, was purchased from Houzai Biotech Company (Nanjing, China). This plasmid was transfected as described previously [9]. Experiments were performed 72 h after transfection.

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

To quantitate the expression of miR-449a, total RNA, including miRNA, was extracted from the cell lines and tissue samples using TRIzol reagent (Life Technologies, Inc., Carlsbad, CA, USA). RNA was synthesized into cDNA using M-MuLV Reverse Transcriptase (Fisher Scientific, Pittsburgh PA, USA). Real-time PCR was

conducted using SYBR Green mix (Takara, Dalian, China) and a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) under the following conditions: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s and 60 °C for 30 s. The expression of miR-449a was normalized to U6 RNA and calculated using the $2^{-\Delta\Delta Ct}$ method. E2F3 mRNA levels were detected as described previously [10].

Cell proliferation assay

Cell proliferation was determined using the Cell Counting Kit-8 assay kit (Takara, Dalian, China), according to the manufacturer's instructions. Cells were seeded at a density of 3×10^4 per well in 96-well plates, and each well was transfected with miR-449a mimic (50 nM), inhibitor (100 nM) or the proper negative control. Seventy-two hours after transfection, the absorbance was read at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA).

Cell cycle assay

The cell cycle assay was performed as previously described [11]. Seventy-two hours after transfection, cells were harvested and washed twice at 4 °C with PBS containing 0.5% BSA. Cells were collected, re-suspended in 300 μ l PBS, and fixed by adding 700 μ l of 100% ethanol at 4 °C for 24 h. Cells were washed twice in ice cold PBS to remove ethanol and then resuspended in a propidium iodide (PI) solution containing 100 μ g/ml PI and 50 μ g/ml RNase (Sigma, USA) at 37 °C for 30 min in the dark. Cell number in each phase of the cell cycle was determined using Calibur Flow Cytometers and analyzed with CELL Quest software.

Analysis of clonogenicity in vitro

Aliquots of viable SGC-7901 cells (1,000 per well) transfected with miR-449a mimic or NC were placed in six-well plates 24 h after transfection and maintained in DMEM medium supplied with 10% fetal bovine serum for 2 weeks. The number of colonies containing more than 50 cells were stained with crystal violet and counted. Triplicate wells were measured for each group.

Apoptosis analysis by annexin-V FITC/PI double staining

The cells were treated with 5 μ M cisplatin. To identify apoptotic cells, annexin V and PI staining was performed using an Annexin V-FITC Apoptosis Detection kit (Becton, Dickinson and Company, San Jose, CA, USA), according to the manufacturer's instructions. After treatment, 5×10^5 cells were collected by centrifugation at 1500 rpm for 5 min. The cell were re-suspended in 200 μ l of binding buffer and incubated with 5 μ l FITC Annexin V and 1 μ l of propidium iodide solution for 30 min at room temperature. Apoptosis was detected using a Calibur Flow Cytometer (Becton, Dickinson and Company, San Jose, CA, USA). Apoptotic cells were defined as annexin V-positive/propidium-negative.

Hoechst 33258 staining assay

After treatment, apoptotic cells were detected using Hoechst 33258 staining (Beyotime, Haimen, China), according to the manufacturer's protocols. Stained cells were observed under a fluorescent microscope using 350-nm excitation and 460-nm emission.

Luciferase reporter assay

The 3'-untranslated region (3'UTR) of E2F3 mRNA containing the predicted miR-449a binding site was amplified by PCR using the Takara PCR Amplification Kit (Takara, Dalian, China). The corresponding mutant constructs were created using the Quick Mutagenesis Stratagene kit (Stratagene, La Jolla, CA). PCR products were digested and cloned into the psiCHECK-2 reporter vector (Promega, USA). Cells (0.5×10^5) were seeded in 24-well plates and cultured for 24 h. Reporter plasmids (200 ng psiCHECK-2-E2F3-wild or psiCHECK-2-E2F3-mut) and 100 nmol/L miR-449a mimics were cotransfected into SGC7901 cells using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). After 48 h, the cells were lysed and reporter activity was determined using a Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Western blotting

Western blotting was performed according to standard methods, as described previously [12]. Membranes were probed with polyclonal rabbit antibodies against anti-E2F3 (1:500; Abcam, Cambridge, MA,

USA). The membranes were stripped and re-probed with an anti-GAPDH rabbit polyclonal antibody (Abcam, Cambridge, MA, USA) as a loading control. The blot was developed using enhanced chemiluminescence solution (Beyotime, Haimen, China) and photographed using the FluorChem imaging system (Alpha Innotech Corp., San Leandro, CA, USA). The intensity of each spot was analyzed with AlphaEaseFC software.

Statistical analysis

All data are represented as the mean $\pm$ standard deviation of at least three independent experiments. The results were analyzed using a two-tailed Student's *t* test. Results were considered statistically significant at $p < 0.05$.

Results

MiR-449a expression is down-regulated in gastric cancer

To evaluate the role of miR-449a in the development of gastric cancer, we detected the expression levels of miR-449a in the human gastric cancer cell line SGC-7901 and normal human gastric epithelial cells (NGEC) using quantitative RT-PCR. As shown in Fig. 1A, miR-449a expression was markedly down-regulated in SGC7901 cells compared with normal human gastric epithelial cells, as determined by real-time PCR analysis. To analyze the clinical relevance of this result, we extended our analysis to assess the expression of miR-449a in a series of 12 human primary gastric cancer tissues and paired normal adjacent gastric tissues. Similar to gastric cancer cell lines, all tumor samples had lower levels of miR-449a expression compared with paired normal tissues (Fig. 1B). These data confirm that miR-449a is down-regulated in gastric cancer and indicate that miR-449a may be a potential tumor suppressor.

MiR-449a suppresses growth and promotes apoptosis in SGC-7901 cells

To investigate the biological effect of miR-449a on gastric cancer, we evaluated the effect of miR-449a on the growth of SGC-7901 cells. As shown in Fig. 2A, CCK-8 assays revealed that ectopic expression of miR-449a significantly suppressed gastric cancer cell growth. Similar results were observed using colony formation assays (Fig. 2B and 2C). To explore the mechanism by which miR-449a inhibits cell proliferation, we analyzed the cell-cycle distribution in transfected cells using flow cytometry. miR-449a mimics induced significant G0/G1 arrest in miR-449a-overexpressing SGC-7901 cells compared with control cells (Fig. 2D and 2E). Furthermore, Annexin V and Hoechst 33258 staining (Fig. 2F, 2G and

Fig. 1. MiR-449a is down-regulated in human gastric cancer cell lines and tissues. A: Real-time PCR analysis of miR-449a expression in NGEC and gastric cancer SGC-7901 cell lines. B: MiR-449a expression in primary gastric cancer tissues (T) compared to paired adjacent normal tissues (ANT) from 12 patients. Experiments were repeated at least three times. Bars denote the mean of three independent experiments. * $P < 0.05$.

Fig. 2. MiR-449a suppresses growth and promotes apoptosis in SGC-7901 cells. A: Cell proliferation was measured using CCK-8 assays. Overexpression of miR-449a inhibited SGC-7901 cell proliferation. B, C: Colony formation ability was determined using colony formation assays. Overexpression of miR-449a inhibited the colony formation ability of SGC-7901 cells. D, E: Cell-cycle distribution was detected using flow cytometry. F: Apoptosis was detected using Hoechst 33258 staining assays. G, H: Apoptosis was detected by annexin-V FITC/PI double-staining. Overexpression of miR-449a enhanced apoptosis induced by 5 μ M cisplatin in SGC-7901 cells. * $P < 0.05$.

2H) demonstrated that miR-449a overexpression promoted apoptosis in response to 5 μ M cisplatin. These results suggest that miR-449a is a tumor suppressor in gastric cancer cells *in vitro*.

E2F3 is a direct target of miR-449a in gastric cancer cells

Because E2F3 is associated with the development and progression of cancer, it is essential to elucidate regulatory mechanisms that converge on this molecule. Analysis using

Fig. 3. E2F3 is a direct target of miR-449a in gastric cancer cells. **A:** TargetScan predicted a miR-449a target sequence in the 3'-UTR of E2F3 mRNA. **B:** E2F3-3'UTR-wild or E2F3-3'UTR-mut plasmids were transfected into miR-449a-treated SGC-7901 cells and assayed for luciferase activity. **C:** The mRNA levels of E2F3 were detected by RT-PCR in SGC-7901 cells transfected with miR-449a mimics or NC. **D, E:** E2F3 protein levels were detected by western blotting in SGC-7901 cells transfected with miR-449a mimics or NC. **F:** E2F3 expression was detected by immunohistochemistry in gastric cancer tissue and non-tumor gastric tissues. * $P < 0.05$.

TargetScan Release 6.2 (<http://www.targetscan.org/>) indicated that E2F3 is a potential target of miR-449a (Fig. 3A), a miRNA that was previously reported to be down-regulated in various tumors. To validate E2F3 as a miR-124 target, we conducted luciferase reporter assays using E2F3-3'UTR-wild or E2F3-3'UTR-mut in miR-449a transfected SGC7901 cells (Fig. 3B). The luciferase activity of the E2F3-3'UTR-wild plasmid decreased (52%) following treatment with miR-449a mimics, whereas the inhibitory effect of the miR-449a mimics was not observed with the mutated construct (Fig. 3B). These results indicate that miR-449a suppresses gene expression through binding the 3' UTR of E2F3 mRNA. Moreover, qRT-PCR and western blot analyses demonstrated that the expression of E2F3 mRNA and protein were decreased after treatment with miR-449a mimics in SGC-7901 cells (Fig. 3C, 3D and 3E).

Fig. 4. MiR-449a mimics and siE2F3 elicit similar repressive effects. A: E2F3 mRNA levels were detected by RT-PCR in SGC-7901 cells transfected with siE2F3 or scrambled siRNA. B, C: E2F3 protein levels were detected by western blotting in SGC-7901 cells transfected with siE2F3 or scrambled siRNA. D: Cell proliferation was measured using CCK-8 assays in SGC-7901 cells transfected with siE2F3 or scrambled siRNA. E, F: Cell-cycle distribution was detected by flow cytometry in SGC-7901 cells transfected with siE2F3 or scrambled siRNA. G, H: Apoptosis was detected using Hoechst 33258 staining assays in SGC-7901 cells transfected with siE2F3 or scrambled siRNA. G, H: Apoptosis was detected using annexin-V FITC/PI double-staining in SGC-7901 cells transfected with siE2F3 or scrambled siRNA. * $P < 0.05$.

In human tissue samples, E2F3 expression was up-regulated compared with paired normal tissues (Fig. 3F). Taken together, these results demonstrate that E2F3 is a direct target of miR-449a.

MiR-449a mimics and siE2F3 elicit similar repressive effects

To determine whether E2F3 plays an important role in gastric cancer cells, we transfected E2F3-specific siRNA (si-E2F3) into cells to knockdown E2F3 expression. As shown in Fig. 4A, 4B and 4C, transfection with siE2F3 effectively reduced E2F3 at the mRNA and protein levels, as determined by RT-PCR and western blotting, respectively. Next, we treated the gastric cancer cells with scrambled or siE2F3 and evaluated cell viability. Treatment with the siE2F3 decreased SGC-7901 cells growth relative to scrambled siRNA (Fig. 4D). siE2F3-transfected cells also displayed a similar growth arrest phenotype to that observed with miR-449a mimic treatment (Fig. 4E and 4F). Treatment with the siE2F3 promoted SGC-7901 cell apoptosis relative to scrambled siRNA (Fig. 4G, 4H and 4I). These data indicate that miR-449a mimics and siE2F3 elicit similar repressive effects on SGC-7901 cells.

Overexpression of E2F3 reverses the repressive effects of miR-449a mimics

Next, we examined whether overexpression of E2F3 was able to reverse the repressive effects of miR-449a mimics. SGC-7901 cells were co-transfected with miR-449a mimics and pCDNA3.1-E2F3, which encodes the entire E2F3 coding sequence but lacks its normal 3'-untranslated sequence. CCK-8 assays revealed that E2F3 overexpression rescued the

Fig. 5. Overexpression of E2F3 reverses the repressive effects caused by miR-449a mimics. A: Cell proliferation was measured using CCK-8 assays in SGC-7901 cells transfected with miR-449a mimics alone or co-transfected with miR-449a mimics and pCDNA3.1-E2F3. B: Apoptosis was detected by annexin-V FITC/PI double-staining in SGC-7901 cells transfected with miR-449a mimics alone or co-transfected with miR-449a mimics and pCDNA3.1-E2F3. * $P < 0.05$.

growth-repressing effects of miR-449a mimics (Fig. 5A). Next, we examined the effect of E2F3 overexpression on SGC-7901 cells apoptosis. As shown in Fig. 5B, fewer apoptotic cells were detected after cotransfection with pCDNA3.1-E2F3 and miR-449a mimics compared to miR-449a alone. These data suggest that the repressive effects of miR-449a are in part facilitated by the down-regulation of E2F3.

Discussion

An abundance of *in vivo* and *in vitro* studies have demonstrated that microRNAs (miRNAs) regulate cancer development and progression in carcinogenesis as either tumor suppressors or oncogenes [13]. Despite increasing evidence pointing to a role for miR-449a as a tumor suppressor, the role of miR-449a in human gastric cancer remains unclear [14-16]. In the present study, miR-449a expression was downregulated in gastric cancer tissues and cell lines. We also found that elevated miR-449a levels inhibited gastric cancer cell growth, which was associated with the induction of apoptosis and cell cycle arrest. Luciferase assays confirmed that miR-449a directly binds the 3'-UTR of E2F3 mRNA and inhibits E2F3 translation in gastric cancer cells.

The human miR-449 cluster is located on chromosome 5 in a highly conserved region within the second intron of the CDC20B gene, a homolog of CDC20 [17]. This cluster, which encodes the highly conserved miR-449a and miR-449b, contains similar sequences and secondary structures as the miR-34 family, so they were classified as one family of microRNAs [17]. Under normal conditions, high levels of miR-449a are present in lung, testis and trachea tissues [18]. Growing evidence indicates that deregulation of miRNAs plays an important role in human carcinogenesis [19, 20]. miR-449a is downregulated and displays tumor suppressive effects in several malignant tumors, including lung [21], liver [22] and gastric [23] cancer; breast [24] and bladder carcinoma [7], prostatic carcinoma [25, 26], and oophoroma [27]. Kheir et al. confirmed that miR-449a expression is low or absent in 8 out of 10 primary gastric cancers [23]. Hu et al. demonstrated that miR-449a could modulate the cell cycle and apoptosis by regulating cyclin D1 and Bcl-2 expression in SGC7901 cells [16]. In the present study, we found that miR-449a is downregulated in gastric cancer cell lines and tumor tissues. Furthermore, miR-449a suppressed growth and promoted apoptosis in gastric cancer cells. These data indicate that miR-449a plays a tumor suppressor role in gastric cancer cells.

E2F-family transcription factors play important roles in cell cycle progression and cancer [28]. They are 8 members of the E2F-family: E2F1-E2F3 act as transcriptional activators and

E2F4-E2F8 are considered transcriptional repressors [28]. E2F activators regulate genes necessary for G₁- to S-phase transition and DNA replication. E2F3 acts as a transcriptional activator and increases cellular proliferation through the G1/S transition [29, 30]. Recent studies indicate that E2F3 modulates the expression of several genes, including Cdk1, Aurora-A, and Survivin [28, 31, 32]. In this study, E2F3 was confirmed to be a direct target gene of miR-449a using luciferase activity assays. Our data suggest that the down-regulation of E2F3 by miR-449a causes cell cycle arrest and apoptosis. In contrast, the suppressive effect of miR-449a on cell growth is largely due to the down-regulation of E2F3 expression, because the *E2F3* silencing showed a smaller inhibitory effect on cell growth than ectopic miR-449a expression. Thus, down-regulation of E2F3 represents a molecular mechanism by which miR-449a exerts its tumor suppressor function.

In conclusion, our study revealed that miR-449a is frequently down-regulated in GC. miR-449a inhibits GC cell growth and induces GC cell apoptosis. The tumor suppressor functions of miR-449a in GC involve the inhibition of cell proliferation, induction of G0/G1 arrest and apoptosis by directly targeting E2F3. There are some limitations to our research study. For example, there are no clinical data and no comparison to adjacent normal tissue in this study. Further studies should be carried out to concern the effects of miR-449a in gastric cancer patients with different stages of cancer. Additionally, the lack of use of other commonly used chemotherapeutic drug such as 5-FU, is also limitation of this study.

Disclosure Statement

None.

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