

MicroRNA-429 inhibits gastric cancer migration and invasion through the downregulation of specificity protein 1

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Received May 27, 2015; Accepted October 5, 2016

DOI: 10.3892/ol.2017.5869

Abstract. microRNAs (miRs) have been reported to have an important role in tumorigenesis and tumor progression. Although miR-429 has been shown to be downregulated in gastric cancer (GC), the function of miR-429 in the metastasis of GC has yet to be investigated. In the present study, GC cells were transfected with miR-429, and reverse transcription-quantitative polymerase chain reaction, cell migration assays, cell invasion assays, western blot analysis and luciferase assays were conducted to investigate the role of miR-429 in GC cells. It was demonstrated that miR-429 expression was markedly increased following transfection of the cells with miR-429. Furthermore, miR-429 was shown to inhibit the migration and invasion of GC cell lines. In addition, this study provided evidence that miR-429 directly targets specificity protein 1 in GC cells. The results of the present study may enhance current knowledge regarding the molecular basis of cancer metastasis and provide a potential therapeutic strategy for GC.

Introduction

Gastric cancer (GC) is a type of malignant digestive tract tumor and is the second most common cause of cancer-associated mortality worldwide (1). It is estimated that there are 100,000 new cases of GC and >700,000 GC-associated mortalities worldwide each year (2). The pathogenesis of GC is multifactorial, and genetic and epigenetic alterations of oncogenes, tumor suppressor genes and growth factors have been implicated in the development of GC (3). Treatment of GC predominantly involves surgery, chemotherapy and radiotherapy. Despite recent advances in the treatment of GC, >50% of all patients

with advanced stage GC succumb to recurrence or metastasis, and eventually death, even after a subtotal gastrectomy (4). However, currently, there exists no effective method to predict and prevent the metastasis of GC. Therefore, understanding the molecular mechanisms underlying tumor metastasis are important to further improve the survival of patients with GC.

microRNAs (miRs), which are a class of small (~22-nucleotide) non-coding RNA molecules, are widely expressed in numerous organisms (5). miRs regulate the expression of their downstream target genes by base pairing with the 3'-untranslated region (UTR) of mRNA, leading to mRNA cleavage or translation repression (6). Previous studies have verified that miRs are aberrantly expressed in various types of human cancers (7-9). Increasingly, evidence has suggested that these dysregulated miRs are critical in the proliferation, apoptosis, migration and invasion of tumor cells (10). To date, a number of dysregulated miRs have been observed in GC, and have been shown to participate in GC cell proliferation, apoptosis, migration and invasion, as well as in sensitivity to chemotherapy and radiotherapy by regulating different tumor-related target genes (11-14). Therefore, further investigation of the function of miRs will provide insight into the mechanisms of GC development and identify therapeutic targets.

miR-429 has been reported to be downregulated in GC; however, whether miR-429 has a role in the metastasis of GC has yet to be investigated. The present study aimed to elucidate the effect of miR-429 on GC motility and to investigate its underlying mechanisms.

Materials and methods

Cell culture. The human GC cell lines, SGC-7901 and AGS, were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an atmosphere of 5% CO₂ at 37°C.

Cell transfection. Mature miR-429 mimics, negative control (NC) miR mimics and the luciferase reporter plasmid were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cell transfection and co-transfection were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

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Key words: gastric cancer, microRNA-429, specificity protein 1, metastasis

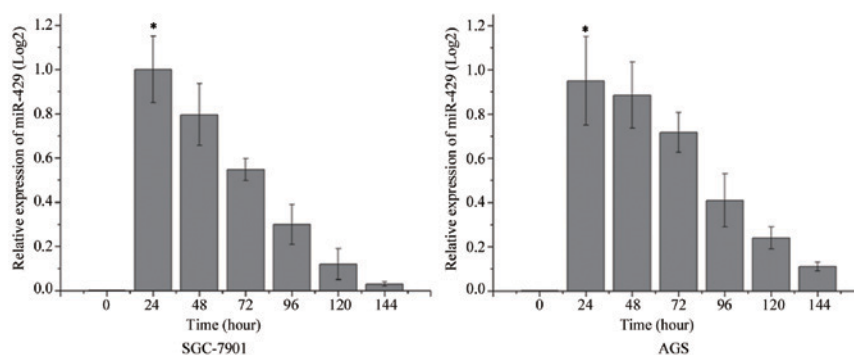


Figure 1. Expression of miR-429 in SGC-7901 and AGS gastric cancer cells prior to and following transfection with miR-429. The basal expression levels of miR-429 (0 h) in SGC-7901 and AGS cells were only at the detectable limit. The expression of miR-429 was markedly increased until 144 h after transfection. * $P < 0.05$. miR-429, microRNA-429.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from GC cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, RNA (500 ng) was reverse transcribed into cDNA using a reverse transcription kit (Tiangen Biotech Co., Ltd., Beijing, China). qPCR was performed on the ABI 7300 thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR PrimeScript miRNA RT-PCR kit. Every sample was analyzed three times. U6 was used as an internal control.

Cell migration and invasion assays. *In vitro* migration and invasion assays were performed using Transwell plates (BD Biosciences, Franklin Lakes, NJ, USA) with 8- μ m pores. After transfection with miR-429 or NC, the cells (1×10^4 cells) in 20 μ l RPMI-1640 medium were added to the upper chamber of the Transwell plates. RPMI-1640 medium containing 20% FBS was added to the upper chamber as a chemoattractant. After 12-h incubation, cells on the upper surface were removed using cotton wool and the cells attached to the bottom were fixed with methanol and stained with 0.5% crystal violet. For the invasion assays, cells (1×10^4 cells) in 20 μ l RPMI-1640 medium were added to the upper chamber pre-coated with Matrigel (BD Biosciences). After 24-h incubation, cells on the upper surface were removed using cotton wool and the cells attached to the bottom were fixed with methanol and stained with 0.5% crystal violet. Images were captured and the cells were counted using a photomicroscope (Olympus Corporation, Tokyo, Japan).

Western blot analysis. Primary antibodies used in this study included rabbit anti-human monoclonal specificity protein 1 (Sp1; dilution, 1:1,000; catalog no., 9389; Cell Signaling Technology, Inc., Beverly, MA, USA) and rabbit anti-human monoclonal β -actin (dilution, 1:1,000; catalog no., 8457; Cell Signaling Technology, Inc.). GC cells were lysed using radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with a protease inhibitor cocktail at 72 h following transfection. Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked with 5% skimmed in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1 h, and then incubated

with the primary antibodies overnight at 4°C. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (dilution, 1:200; catalog no., 7074; Cell Signaling Technology, Inc.) was used to detect the primary antibodies. Finally, the bands were visualized using enhanced chemiluminescence reagents and images were captured using the FluorChem imaging system (ProteinSimple, San Jose, CA, USA). AlphaEase FC software (version 4.0.1; ProteinSimple, San Jose, CA, USA) was used to analyze the western blotting results.

Luciferase assay. Sp1 was identified as a target of miR-429 using TargetScan (http://www.targetscan.org/vert_71/). To determine whether Sp1 is a direct target of miR-429, luciferase assays were performed. Luciferase reporter plasmids, including pGL3 Sp1 3'UTR wild type (WT) and pGL3 Sp1 3'UTR mutant type (Mut), were synthesized and confirmed by GenePharma Co., Ltd. GC cells were seeded into 24-well plates at a density of 40-50% confluence and co-transfected with miR-429 or NC and the luciferase reporter plasmid using Lipofectamine 2000, according to the manufacturer's protocol. After a 48-h incubation, the cells were harvested and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega Corporation, Mannheim, Germany). Each assay was replicated three times.

Statistical analysis. Data are presented as the mean \pm standard deviation, and compared using Stata 10.0 software (StataCorp LP, College Station, TX, USA). Double-tailed P-values < 0.05 were considered to be statistically significant.

Results

Expression of miR-429 in GC cell lines prior to and following transfection with miR-429. RT-qPCR was conducted to assess the transfection efficiency following transfection of SGC-7901 and AGS cells with miR-429. As shown in Fig. 1, the basal expression of miR-429 in GC cells was at the detection limit. Following transfection of GC cells with miR-429, the expression level of miR-429 was markedly increased until ~144 h later, as the level of miR-429 declined gradually over time.

miR-429 suppresses GC cell migration and invasion. Transwell assays were used to assess the effects of miR-429 on GC

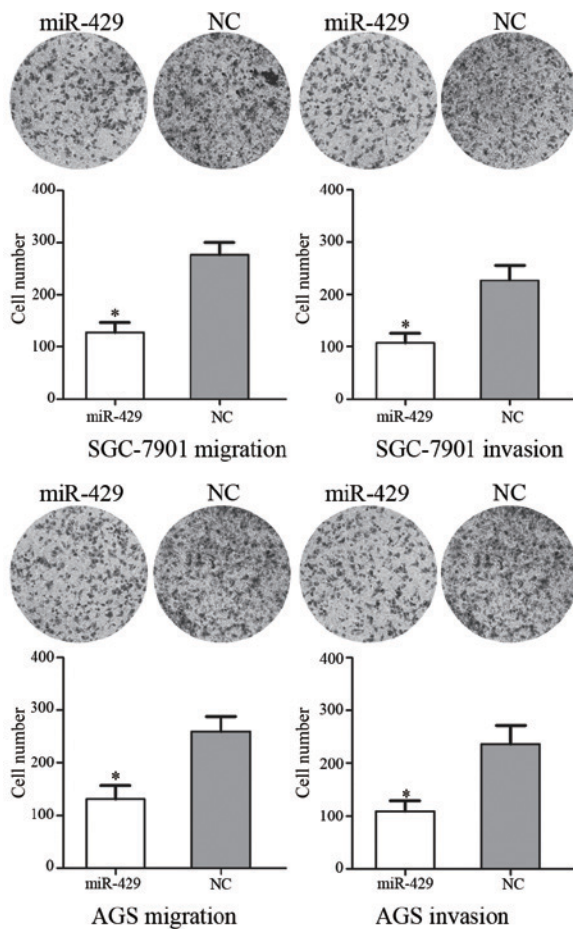


Figure 2. miR-429 inhibits gastric cancer cell migration and invasion. After a 12-h incubation, the number of SGC-7901 and AGS cells that had transversed the Transwell membrane was decreased for the cells transfected with miR-429, as compared with the NC. Similarly, after 24-h of incubation, the number of SGC-7901 and AGS cells that had transversed the Transwell membrane pre-coated with Matrigel was decreased for the cells transfected with miR-429, as compared with the NC. * $P<0.05$. miR-429, microRNA-429; NC, normal control.

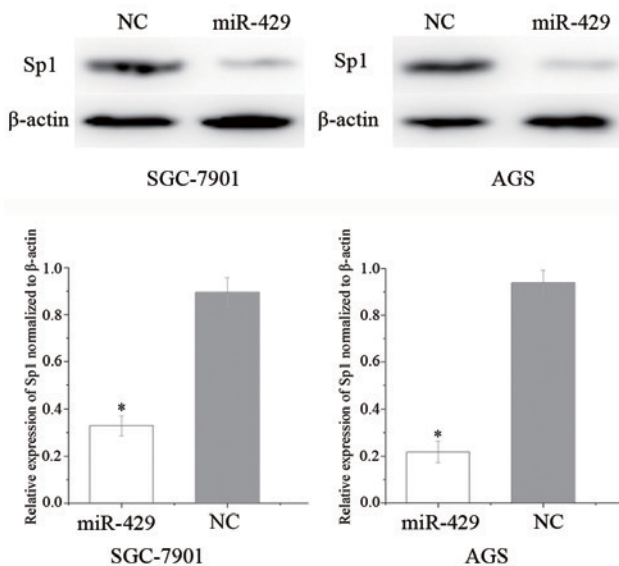


Figure 3. Western blotting and densitometric analyses demonstrated that Sp1 expression was significantly reduced in SGC-7901 and AGS gastric cancer cells following transfection of the cells with miR-429. * $P<0.05$. Sp1, specificity protein 1; NC, normal control; miR-429, microRNA-429.

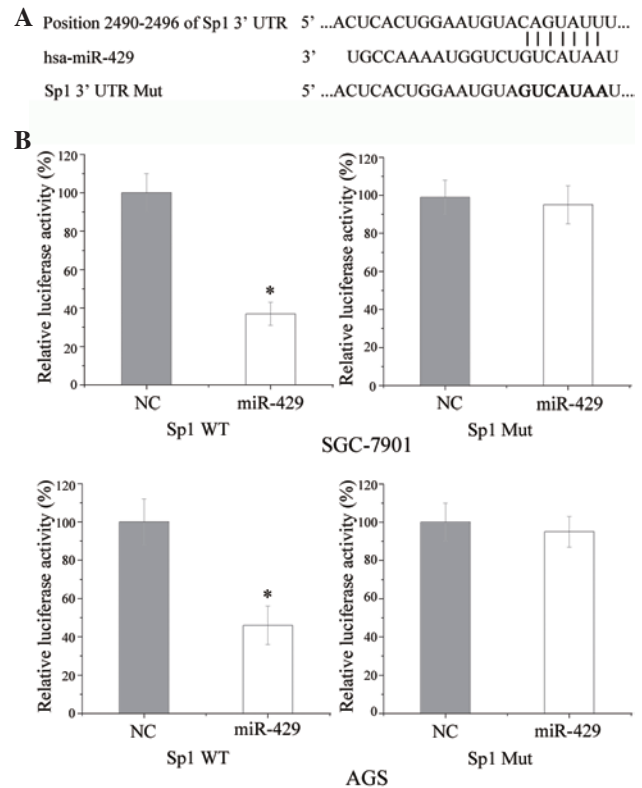


Figure 4. Sp1 is a target of miR-429. (A) TargetScan was used to demonstrate that Sp1 mRNA contained a miR-429 7-nucleotide seed match at position 2,490-2,496 of the Sp1 3'-UTR. (B) Luciferase activity assay verified direct binding of miR-429 to the sites of Sp1 predicted by TargetScan. It showed that overexpression of miR-429 significantly inhibited the WT but not the Mut luciferase activity of Sp1 in gastric cancer SGC-7901 and AGS cell lines. * $P<0.05$. Sp1, specificity protein 1; miR-429, microRNA-429; 3'-UTR, 3'-untranslated region; WT, wild-type; Mut, mutant; NC, normal control.

SGC-7901 and AGS cell migration and invasion (Fig. 2). In the migration assay, it was demonstrated that miR-429 transfection resulted in a $53.69\pm5.42\%$ decrease in SGC-7901 cells and a $48.27\pm6.23\%$ decrease in AGS cells. In the invasion assay, it was observed that miR-429 transfection led to a $56.54\pm5.68\%$ decrease in SGC-7901 cells and a $52.81\pm7.39\%$ decrease in AGS cells. These results suggest that miR-429 inhibits the motility of GC SGC-7901 and AGS cells.

Sp1 is downregulated following transfection of GC cells with miR-429. Western blot analysis was performed to investigate the effect of increased expression levels of miR-429 on Sp1 expression in GC cells. As shown in Fig. 3, Sp1 was significantly downregulated in GC SGC-7901 ($P=0.021$) and AGS ($P=0.014$) cell lines following overexpression of miR-429. These results suggest that miR-429 reduces the protein expression level of Sp1 in GC cells.

Sp1 is a direct target of miR-429 in GC. Sp1 was identified as a target of miR-429 using TargetScan. As shown in Fig. 4A, Sp1 mRNA contained a miR-429 7-nucleotide seed match at position 2,490-2,496 of the Sp1 3'-UTR. To verify whether miR-429 directly targets Sp1, a luciferase activity assay was performed. As shown in Fig. 4B, miR-429 significantly inhibited the luciferase activity of the vector carrying the wild-type but not the mutant Sp1 3'-UTR in GC SGC-7901 and AGS

cells. These results suggest that Sp1 may be a direct target of miR-429 *in vitro*.

Discussion

The miR-200 family, which is located on chromosome 1 and has been implicated in numerous cancers, can be divided into two clusters: The first cluster consists of miR-200a, miR-200b and miR-429, and the second cluster comprises miR-200c and miR-141 (15). Previous studies have demonstrated that miR-429 is dysregulated in various cancers, including non-small cell lung cancer (16), Ehrlich ascites tumor cells (17), renal cell carcinoma (18), colorectal carcinoma, nasopharyngeal carcinoma (19) and endometrial endometrioid carcinomas (20). In addition, the expression level of miR-429 was upregulated in bladder cancer and ovarian carcinoma (21,22), and its upregulation in patients with serous ovarian carcinoma was correlated with a poor prognosis (22). Therefore, miR-429 is likely involved in carcinogenesis and cancer progression, and may have different roles depending on the type of cancer.

In osteosarcoma, overexpression of miR-429 inhibited cell proliferation and enhanced cell apoptosis. Furthermore, miR-429 was shown to exert tumor-suppressing effects in osteosarcoma by binding to the 3'-UTR of zinc finger E-box binding homeobox 1 (ZEB1) (23). In esophageal carcinoma, miR-429 was shown to be downregulated and its expression was significantly associated with the occurrence of lymph node metastases. Upregulation of miR-429 inhibited cell migration and enhanced cell apoptosis in esophageal carcinoma cell lines by directly targeting B-cell lymphoma-2 and Sp1 (24). In breast cancer, downregulation of miR-429 was shown to prevent the bone metastasis of cancer cells. Conversely, ectopic expression of miR-429 markedly suppressed breast cancer cell invasion through downregulation of ZEB1 and V-Crk avian sarcoma virus CT10 oncogene homolog-like (25). Together, these findings suggested that miR-429 may be used for the development of novel therapeutic strategies for cancer.

miR-429 was downregulated in human GC tissues and the level of miR-429 was associated with lymph node metastasis. Upregulation of miR-429 inhibited the proliferation of tumor cells and their attachment to fibronectin and laminin in a dose-dependent manner (26). However, no previous study has investigated the functional role of miR-429 in GC cell migration and invasion. In the present study, miR-429 was shown to inhibit cell migration and invasion by directly targeting Sp1. These findings suggested that Sp1 should be investigated as a target therapy to block GC from becoming invasive.

Sp1 is a sequence-specific DNA-binding protein that was the first transcription factor to be cloned from mammalian cells in 1983 (27). The expression level of Sp1 has been shown to be upregulated in numerous human cancers, including GC (28), breast cancer (29), hepatocellular carcinomas (30), thyroid cancer (31), colorectal cancer (32), pancreatic cancer (33) and lung cancer (34). Increasingly, evidence has suggested that Sp1 may be involved in a variety of cellular processes, including cell growth, survival, differentiation, tumor development and tumor progression (35-38). miRs, which regulate the expression of their target genes by base pairing with seed sequences in the 3'-UTR of mRNAs, have been shown to regulate the expression of Sp1 (39,40). In a previous study, miR-22 was reported

to be downregulated in GC and inhibited cell proliferation, migration and invasion by targeting Sp1 (41). The results of the present study suggested that miR-429 suppresses GC cell migration and invasion by directly targeting Sp1. Therefore, miR-429 could be investigated for its value in the early detection of tumor recurrence and as a potential target of therapy to prevent GC from becoming invasive.

The metastasis of GC is a key step in tumor progression and is an indicator of a poor prognosis (42). Metastasis involves a series of sequential events, including detachment, migration, invasion, extravasation, angiogenesis, survival in the circulatory system and extravasation (43,44). Several miRs have been reported to have an important role in tumor metastasis (45-47). Furthermore, since miRs regulate multiple target genes simultaneously, miR-based therapy is expected to be more efficient than the traditional single target therapy (48,49). In the present study, miR-429 was shown to be an important regulator in tumor cell migration and invasion, which emphasized an essential role of miR-429 in regulating GC metastasis.

In conclusion, the present study demonstrated that upregulation of miR-429 in GC cell lines inhibited GC cell metastasis *in vitro*. In addition, Sp1 was shown to be negatively regulated by miR-429 and to contain a binding site for miR-429 in the 3'-UTR of its mRNA. This newly identified association between miR-429 and Sp1 may potentially provide a novel therapeutic strategy for GC.

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