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Original Article

Negative correlation and opposite bio-functions of miR-29a and miR-425 in human gastric cancer

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Abstract: Increasing evidence support that microRNAs (miRNAs) are key regulators involved in the progression of gastric cancer as tumor suppressors or oncogenes. MiR-425 and miR-29a are both functionally vital miRNAs in gastric cancer, however, their correlation and underlining mechanisms remain unknown. Expression of miR-425 and miR-29a in human gastric cancer samples and cell lines were examined by quantitative real-time PCR (qRT-PCR). Correlation between the two miRNA expression, and correlation of their expression levels with clinicopathologic features were analyzed. After transfected with miR-29a or miR-425 mimics, the cell proliferation and migration ability were assessed by CCK-8 assay and transwell assay, respectively. MiR-425 was significantly upregulated whereas miR-29a was markedly downregulated in gastric cancer samples compared to the paired normal samples. Negative correlation was found in these two miRNAs expression. Expression of miR-425 and -29a were both associated with differentiation and lymph node metastasis. Cellular functional assays confirmed that miR-425 promoted cell proliferation and migration. However, miR-29a exerted the opposite. MiR-425 and miR-29a may act as oncogenic or tumor suppressing miRNAs, respectively in gastric cancer. Understanding the internal mechanisms may provide more insights into their correlation and facilitate the future therapy for gastric cancer.

Keywords: Gastric cancer, miR-425, miR-29, cell proliferation and migration, opposite function

Introduction

Gastric cancer is the fourth and fifth most frequent cancer among males and females, respectively all around the world, and becomes a global killer [1]. Mainly due to the difficulty in early diagnosis of this disease in clinical practice, its incidence remains high and it is a major cause for cancer-related mortality in China [2]. It is now well recognized that Helicobacter pylori (H pylori) is the most important acquired aetiological agent for this cancer, which initiates chronic inflammation and leads to malignancy transformation [3, 4]. In addition, the prognosis fact for gastric cancer patients at advanced stages remains very poor, left a low survival rate [5, 6].

Beside protein-coding gene, studies have revealed that multiple non-coding gene expression alterations are involved in the development and progression of gastric cancer. As a class of small, single-stranded endogenous non-coding RNA molecules, microRNAs (miRNAs) function as post-transcriptional regulators of gene expression, impacting on over one-third of the human genes, thus participates in a broad range of biological events [7-9]. Notably, miRNAs play key roles in human cancers: they act either as oncogenes or as tumor suppressors, as well as cancer biomarkers for diagnosis or prognosis [10-12].

MiR-425, also known as miR-425-5p, which mapped to human chromosome 3, has been verified to have an abnormally high expression in all gastric cancer cell lines compared with the GES-1 cell line. And, *in vitro* and *in vivo* experiments had demonstrated that reducing miR-425-5p expression could inhibit gastric cancer cell proliferation, invasion and migration [13]. MiR-29a is a member of the conserved miR-29s family. Decreased expression of miR-29a has been reported in multiple cancers, including in gastric cancer [14]. Previous studies revealed that miR-29a was significantly decreased in gastric cancer and was linked with cell growth, migration, and invasion [15-

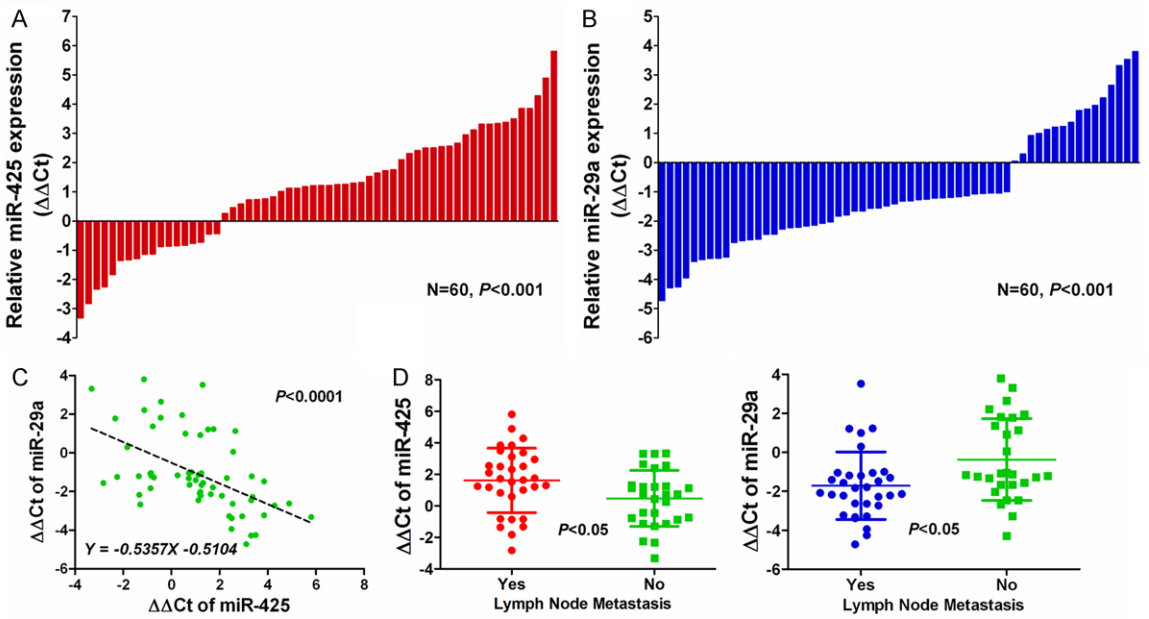

Figure 1. QRT-PCR analysis of miR-425 and miR-29a in paired GC tissues and their correlation with lymph node metastasis. A. The miR-425 level was upregulated in 58.3% (35 of 60) of GC tumor tissues, relative to paired normal tissues (N=60, *P*<0.001). B. The miR-29a level was downregulated in 70.0% (42 of 60) of GC tumor tissues, relative to paired normal tissues (N=60, *P*<0.001). C. Negative correlation of miR-425 and miR-29a expression in paired GC tissues (N=60, *P*<0.0001). D. Average relative expression of miR-425 or miR-29a levels in lymph node metastatic and non-lymph node metastatic paired gastric tissues (N=60, *P*<0.05).

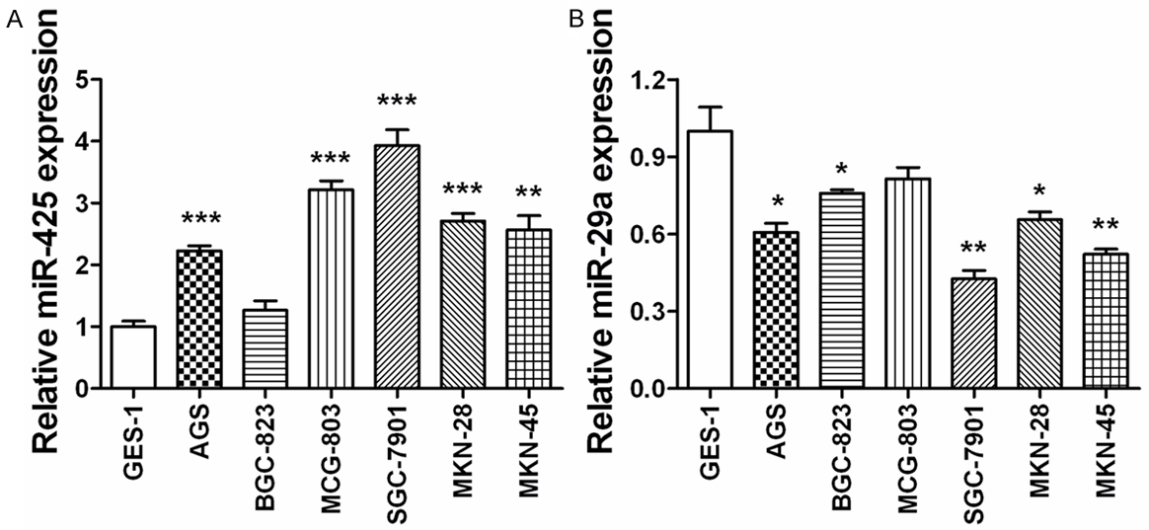

Figure 2. QRT-PCR analysis of miR-425 and miR-29a in GC cell lines. A. Average relative expression of miR-425 in six gastric cancer cell lines AGS, BGC-823, MCG-803, SGC-7901, MKN-28 and MKN-45 and one human normal gastric epithelial cell line GES-1. B. Average relative expression of miR-29a in six gastric cancer cell lines AGS, BGC-823, MCG-803, SGC-7901, MKN-28 and MKN-45 and one human normal gastric epithelial cell line GES-1. Data are means ± SD of three independent experiments. *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.

17]. Although separate expression pattern and function of miR-425 and miR-29a had been examined, the internal correlation between the two miRNAs remained largely unknown. In the present study, we investigated their expression, clinical significance and biological roles in gastric cancer, and attempt to find out the correlation between the two miRNAs.

Table 1. Correlation of the expression of miR-425 with clinicopathologic feature

Clinical Characteristics	miR-425 Non-increased (ΔΔCt≤1) n=25	miR-425 Increased (ΔΔCt>1) n=35
Age		
<60	11	16
≥60	14	19
		P=0.8953
Gender		
Male	16	21
Female	9	14
		P=0.7534
Tumor Location		
Cardia	5	11
Body	16	17
Pylorus	4	7
		P=0.4789
Differentiation		
Well & Moderately	17	14
Poorly	8	21
		P=0.0324
Tumor Grade		
I+II	13	15
III+IV	12	20
		P=0.4840
Lymph Node Metastasis		
Yes	9	23
No	16	12
		P=0.0229

Materials and methods

Patients and tissues

Altogether 60 gastric cancer patients hospitalized in Huaihe Hospital of Henan University from August 2015 to August 2016 were enrolled in our study. The stage of these patients was evaluated according to TNM Classification of Malignant Tumors. The gastric cancer tissue and the adjacent normal tissue of each patient were collected during surgery, frozen in liquid nitrogen immediately and stored at -80°C for RNA extraction. Informed consents were obtained from all the patients conformed to the guideline of the ethics committee of Huaihe Hospital of Henan University.

Cell culture

The human gastric epithelial cell line GES-1 or gastric cancer cell lines AGS, BGC-823, MCG-803, SGC-7901, MKN-28 and MKN-45 was from American Type Culture Collection (ATCC). Cells were cultured in DMEM or 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO_2.

MicroRNA mimics and transfections

MiR-425 and miR-29a mimic with corresponding controls were from GenePharma (Shanghai, China). MKN-45 or SGC-7901 cells were seeded in six-well or other plates and transfected using lipofectamine 2000 according to the manufacturer's instructions.

RNA extraction and qRT-PCR

Total RNA from tissues or cells was extracted by TRIzol reagent (Invitrogen, USA) following the instructions. Reverse transcription was performed using M-MLV system (Promega) by specific primers from RiboBio (Guangzhou, China). Quantitative real-time PCR (qRT-PCR) was performed by using SYBR mix (TOYOBO, Japan) on ABI 7500 system. U6 was used for as internal controls. The relative expression of microRNA was computed by $2^{-\Delta\Delta CT}$ method.

CCK-8 assay

Cell proliferation rate was calculated by CCK-8 assay. After transfection with microRNA mimics or controls, 2×10^3 cells were seeded in 96-well plates. At day1, day2 and day3, the absorbance at 450 nm was measured by using CCK-8 Kit (Dojindo). The experiments were repeated in triplicate.

Cell migration assay

The migration ability was measured by transwell assay. 18 h after transfection, cells were seeded into the upper chamber of the transwell with 200 μl serum-free medium (10^5 cells per well). Complete medium with 10% FBS was added to the lower chamber. The 24-well plate was incubated at 37°C. 24 h later, nonmigratory cells on the upper surface of upper chamber were removed slightly by cotton swabs. The migrated cells on the bottom of the membrane was fixed, stained, counted under light microscope and photographed at a ×200 magnifica-

Table 2. Correlation of the expression of miR-29a with clinicopathologic feature

Clinical Characteristics	miR-29a Non-decreased (ΔΔCt≥-1) n=18	miR-29a Decreased (ΔΔCt<-1) n=42
Age		
<60	8	19
≥60	10	23
		P=0.9548
Gender		
Male	11	26
Female	7	16
		P=0.9538
Tumor Location		
Cardia	3	13
Body	11	22
Pylorus	4	7
		P=0.5089
Differentiation		
Well & Moderately	13	18
Poorly	5	24
		P=0.0370
Tumor Grade		
I+II	9	19
III+IV	9	23
		P=0.7347
Lymph Node Metastasis		
Yes	6	26
No	12	16
		P=0.0421

tion for five randomly selected fields. The experiments were performed in triplicate.

Statistics analysis

Statistics Analysis was performed by SAS v9.2 software. All experiments were performed at least triplicate. Two-tailed student's t-test, linear regression and the κ^2 test were performed, and statistically significant level was set at P<0.05. Mean ± SD is displayed in the figures.

Results

Upregulation of miR-425 and downregulation of miR-29a in gastric cancer samples and cell lines

To reveal the expression correlation of miR-425 and miR-29a in gastric cancer, a total of 60 gastric cancer samples and the corresponding normal tissues collected during surgery from August 2015 to August 2016 were included for qRT-PCR detection. As shown in **Figure 1A** and **1B**, compared to the paired non-tumor tissues, 58.3% (35/60) of gastric cancer samples showed increased expression of miR-425 (defined as greater than a two-fold increase) and 70.0% (42/60) of these samples showed decreased expression of miR-29a (more than a two-fold decrease). The average fold change of miR-425 was significantly higher and miR-29a was significantly lower in tumor samples than that in non-tumor tissues (*P*<0.001). Pearson's test unveiled that the miR-425 expression levels negatively correlated with the miR-29a levels (r^2=0.2806, *P*<0.0001, **Figure 1C**). Among the tumor samples, we further found that increased miR-425 and decreased miR-29a expression were both correlated with lymph node metastasis status: significantly higher miR-425 and lower miR-29a were observed in gastric cancer with lymph node metastasis, compared with that from gastric cancer without lymph node metastasis (**Figure 1D**). Furthermore, expression of miR-425 and miR-29a were also examined in six gastric cancer cell lines (AGS, BGC-823, MCG-803, SGC-7901, MKN-28, MKN-45) and one gastric epithelial cell line GES-1. Consistent with the previous results, we found that miR-425 was upregulated and miR-29a was downregulated in the above six gastric cancer cell lines compared with that in GES-1 (*P*<0.05, **Figure 2A** and **2B**).

Both miR-425 and miR-29a correlated with tumor differentiation and lymph node metastasis status

We next investigated the clinical significance of miR-425 and miR-29a in gastric cancer, as measured by the correlation between miR-425/-29a expression and clinicopathologic features. As shown in **Tables 1** and **2**, expression of both miR-425 and miR-29a was correlated with differentiation and lymph node metastasis in gastric cancer (*P*<0.05 for both). Specifically, gastric tumors with increased miR-425 tended to be poorly differentiated and with positive lymphatic metastasis, however, decreased miR-29a tumors were poorly differentiated and with positive lymphatic metastasis. This line of results also supported the opposite roles

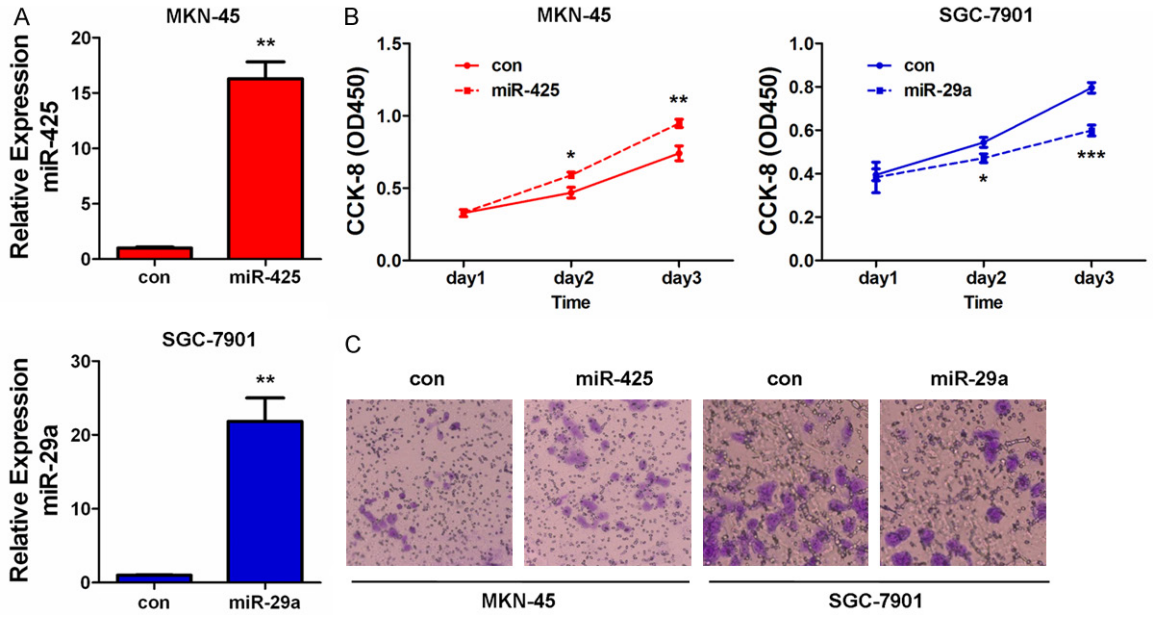

Figure 3. MiR-425 and miR-29a regulated gastric cancer cell proliferation and migration oppositely. MKN-45 or SGC-7901 cells are transfected with miR-425 or miR-29a mimics, and controls. A. QRT-PCT was performed at 48 h post-transfection, U6 was used as an internal control. B. CCK-8 assay was performed at 0 h, 24 h and 48 h post-transfection. C. Migration assay using transwells was performed at 48 h post-transfection. Data are means ± SD of three independent experiments. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

of the two miRNA in predicting pathology and prognosis.

MiR-425 and miR-29a oppositely affect gastric cancer cell proliferation and migration

Based on these results from **Figure 2A** and **2B**, MKN-45 and SGC-7901 cells were selected for further study. The above results indicated that miR-425 and miR-29a might act oppositely. To test this hypothesis, we assessed the roles of miR-425 and miR-29a by gain-of-function experiments. Either miR-425 or miR-29a mimics were transfected into the MKN-45 or SGC-7901 cells. QRT-PCR assay confirmed that exogenously transducing miRNA was successful (**Figure 3A**). According to the data of the cell proliferation CCK-8 assay, we plotted the absorbency curves at the wavelength of 450 nm at different time points post transfection. We found that miR-425 mimics significantly promoted cell proliferation, whereas miR-29a inhibited cell proliferation (**Figure 3B**). Moreover, transwell migration assays were performed to evaluate the effect of the two miRNAs in gastric cancer migration. As shown in **Figure 3C**, when compared with the control group, the result demonstrated that miR-425 transduction significantly facilitated the migration of gastric cancer cells, and miR-29a had the opposite effects. These data indicated that miR-425 promoted cell proliferation and migration capacity of gastric cancer cells, while miR-29a inhibited the above cellular processes in vitro.

Discussion

Gastric cancer remains one of the most prevalent tumors and it is well known that gastric carcinogenesis is a multistep process including genetic and epigenetic modulations involving various oncogenes and tumor suppressor genes [18]. MiRNAs are now popularly studied in gastric cancer tumorigenesis. Actually, a large number of miRNAs with different biological functions have been identified to be aberrantly expressed and are in correlation with indicated clinicopathological features in gastric cancer, all denoting their possible use as diagnostic, therapeutic and prognostic biomarkers [19]. However, the internal relationship between these important miRNAs seems largely unclear.

MiR-425 and miR-29a have both been reported to be implicated tumorigenesis in a few cancer types. For instance, for miR-425, Sun et al. had found that miR-425 is upregulated in cervical cancer and serum miR-425 may serve as a potential prognostic biomarker for cervical can-

cer [20]. In esophageal squamous cell carcinoma (ESCC), miR-425 functions as an oncogene by targeting the 3'-UTR of SMAD2 and investigators also indicate the potential utility of plasma miR-425 as a novel biomarker for ESCC diagnosis [21]. Importantly, experiments had demonstrated that miR-425-5p could promote gastric cancer cell proliferation, invasion and migration [13]. MiR-29a is another well studied miRNA in multiple cancers. In gastric cancer, former reports revealed that miR-29a was significantly decreased and was negatively linked with cell growth, migration, and invasion [14-17]. Is there some association with the two seemly unrelated miRNAs? We took this question in mind and did the present study.

The answer came out to be interested. At the expression level, we found that the two miRNAs exhibited negative correlation in both tissue samples and cell lines. Also, when analyzing the clinical significance of the two miRNAs, we found that increased miR-425 and/or decreased miR-29a tumors tended to be poorly differentiated and with positive lymphatic metastasis. Importantly, cellular functional assays directly proved that the two miRNAs exerted opposite functions in two gastric cancer cell lines. Although this research is limited by a small sample size, the underlined mechanism responsible for the negative correlation requires to be further studied. Confronting the research fact, the strategy of using miRNAs for targeted therapy in the near future is probably over-optimistic. However, learning more about the internal relationship between different miRNAs will provide more details about the gene regulation network in cancer cells. With the increasing discoveries over years, treating strategy using miRNAs or other non-coding RNAs is surely extremely promising.

In summary, our study concluded that miR-425 and miR-29a are two antagonistic miRNAs with opposite expression pattern as well as opposite bio-functions in gastric cancer. Looking inside the mechanism that governs their negative correlation will improve our understanding of the two miRNAs in gastric cancer in the future.

Disclosure of conflict of interest

None.

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