

MiR-425-5p promotes tumor progression via modulation of CYLD in gastric cancer

Y.-F. YAN, F.-M. GONG, B.-S. WANG, W. ZHENG

General Surgery Department II, Chinese PLA General Hospital, Beijing, China

Yong-feng Yan and Fang-ming Gong contributed equally to this work

Abstract. – OBJECTIVE: MicroRNAs (miRNAs) have emerged as important gene regulators and are recognized as key players in carcinogenesis. The present study investigated the role of miR-425-5p in the development and progression of gastric cancer (GC).

PATIENTS AND METHODS: The miR-425-5p level in GC tissues and cells was assayed by qRT-PCR. Then, the effects of miR-425-5p expression on the biological behavior of GC cells were investigated. Analysis of target protein expression was determined by Western blotting. Bioinformatic prediction and luciferase assays were employed to identify the predicted miRNA which regulates CYLD.

RESULTS: miR-425-5p was found to be up-regulated in GC tissues and cell lines. Knockdown of miR-425-5p in GC cells attenuated migration and invasion of GC cells, whereas overexpression of miR-425-5p promoted cell migration and invasion. The luciferase assay demonstrated that CYLD was a direct target of miR-425-5p. Furthermore, the miR-425-5p level was inversely correlated with levels of CYLD in Western blotting assay.

CONCLUSIONS: Our findings indicate that miR-425-5p may contribute to the progression of GC through a mechanism involving CYLD, suggesting that miR-425-5p may have the potential to be a novel important alternative therapeutic target for GC.

Key Words:

miR-425-5p, Gastric cancer, CYLD, Invasion, Migration.

Introduction

Gastric cancer (GC) is the second most common cause of cancer-related death and the fourth most common cancer worldwide¹⁻³. In addition, The Chinese Cancer Registry Annual Report reported that GC is the third commonest malignancy and the second leading cause of cancer death in China⁴. Despite improvements in the

diagnosis and treatment of GC, the current treatments available to patients with advanced GC are very limited because of the distant metastasis. Thus, understanding the precise molecular mechanisms underlying the tumorigenesis and progression of gastric cancer is urgently needed and can provide the basis for the development of novel therapeutic strategies.

MicroRNAs (miRNAs) are endogenous non-coding small RNAs, roughly 18-25 nucleotides in length that can down-regulate gene expression by targeting the 3'-UTR region of specific mRNA sequences⁵⁻⁷. Besides, it is closely related to the development and progression of tumor and plays an important role in the proliferation, apoptosis, metastasis of tumor^{8,9}. miRNAs can function as either oncogenes or tumor suppressors according to the roles of their target genes. For instance, Zhao et al¹⁰ showed that miR-874 was significantly down-regulated in colorectal cancer tissues, and overexpression of miR-874 suppressed cell growth and induced apoptosis in colorectal cancer cells by targeting STAT3. Zhang et al¹¹ reported that miR-198 inhibited tumorous behaviors of human osteosarcoma through directly targeting ROCK1. Zhao et al¹² found that up-regulated expression of miR-494 could inhibit GC cells proliferation, migration, and invasion by targeting IGF-1R. These findings suggested that miRNAs potentially serve as therapeutic targets for novel treatment strategies against tumors, including GC.

In the current work, we detected the expression of miR-425-5p in GC tissues and cell lines. We further investigated the role of miR-425-5p in the metastasis of GC cells. Furthermore, we investigated the exact roles of the miR-425-5p and its underlying molecular mechanisms in GC.

Patients and Methods

Cell Culture and Tissue Samples

Fifteen paired GC and matched normal non-tumor tissues were obtained from our department. All the tissues were immediately stored in liquid nitrogen until use. Written informed consent was obtained from all the patients. All specimens were handled and made anonymous according to the ethical and legal standards. The Ethical Committee of our Hospital approved the investigation.

An immortal gastric epithelial cell line, GES-1, and four GC cell lines, BGC-823, AGS, HGC-27, and MKN-45, were commercially obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The above cells were propagated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS at 37°C in 5% CO₂ cell culture incubator.

Cell Transfection

The hsa-miR-425-5p mimics, hsa-miR-425-5p inhibitor, and negative control miRNA (NC) were chemically synthesized by Shanghai GenePharma Co., Ltd. (Songjiang, Shanghai, China). Lipofectamine 2000 (Invitrogen, Eugene, OR, USA) was used for transfection according to the manufacturer's protocol.

Quantitative Reverse Transcription-polymerase Chain Reaction

Total RNA extraction and reverse transcription were performed in strict accordance with the manufacturer's instructions. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the PrimeScript RT Reagent Kit and SYBR Premix Ex Taq kit (TaKaRa Bio, Inc., Otsu, Shiga, Japan). PCR parameters for cycling were as follows: 95°C for 20 seconds, 40 cycles of PCR at 95°C for 3 seconds, and 60°C for 30 seconds. All reactions were done in a 10-mL reaction volume in triplicate. The result compared with U6 and GAPDH was calculated by the 2-dCt method. Primers used for Real-time PCR were designed by Primer Express 3.0 and synthesized in Invitrogen.

Western Blotting

A total of 20 mg of protein was used for Western blotting. After gels electrophoresis, samples were transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% skimmed milk in TBST and incubated

with the antibody against CYLD (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight 4°C. Second antibodies conjugated with horseradish peroxidase (anti-mouse IgG and anti-rabbit IgG) were used to detect primary antibodies. For HRP detection, an ECL chemiluminescence kit (CW BIO) was used. Protein quantity was detected by β -catenin as a loading control.

Wound Healing Assay

The BGC-823 cells transfected with RNA were plated in 6-well plates. When the cells grew to full confluence, a line was scratched using a pipette tip. The cells were then washed with serum-free medium and incubated with serum-free DMEM.

Cell Migration and Invasion Assays

We used a transwell cell migration and Matrigel invasion assay to measure the migration and invasion ability of BGC-823 cell lines. For migration assay, 5×10⁴ transfected cells were placed in the upper chamber of each insert. For invasion assay, 5×10⁴ transfected cells were placed in the upper chamber of each insert coated with 150 mg Matrigel. The lower chamber was filled with 500 μ L Roswell Park Memorial Institute-1640 (RPMI-1640) medium with 10% FBS to attract cells. After cells had been incubated for 36 hours at 37°C and 5% CO₂, those left in the upper chamber were removed with a cotton swab. Then, cells invading across the membrane were counted under a light microscope.

Luciferase Reporter Assays

BGC-823 cells were seeded into a 24-well plate and cotransfected with miR-425-5p or control and 3'UTR-luciferase plasmids. The cells were lysed at 48 h post-transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Haiding, Beijing, China), and Renilla-luciferase was used for normalization.

Statistical Analysis

Experiments were performed in triplicate, and the results were investigated with the SPSS 22 software (IBM, New York, NY, USA). Differences between groups were compared

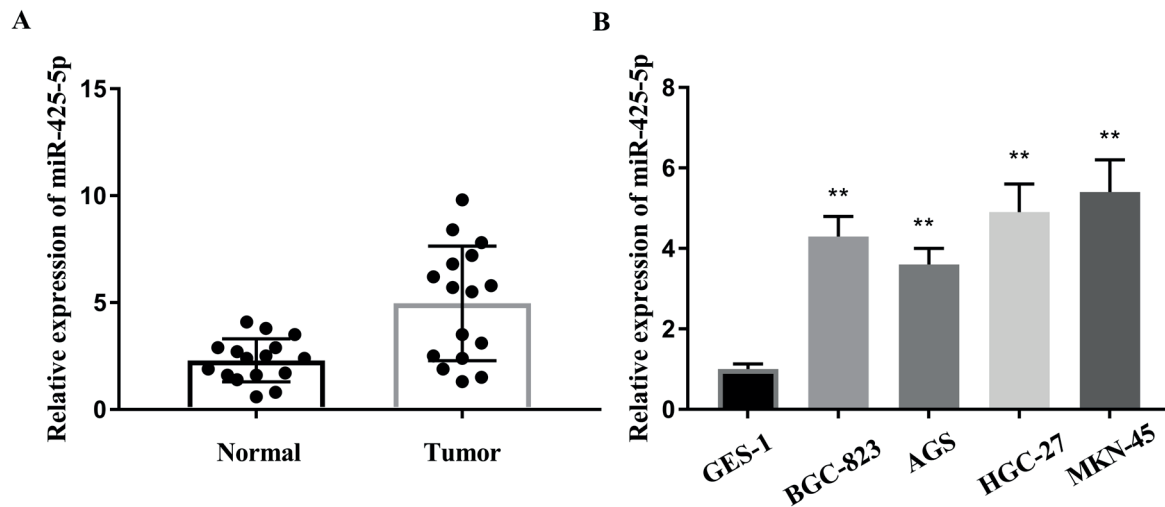


Figure 1. miR-425-5p expression is increased in GC tissues and cell lines. (A) Relative expression of miR-425-5p determined by quantitative PCR in the indicated cell lines. (B) Relative expression of miR-425-5p in a cohort of 15 human GC tissues and adjacent normal tissues. The endogenous U6 RNA was used as the internal control. ** $p < 0.01$ versus normal.

with standard deviation followed by independent-samples t -test. p -values < 0.05 were defined as significant.

Results

miR-425-5p was up-regulated in GC tissues and cell lines

Expression levels of miR-425-5p in GC samples and cell lines were analyzed by RT-PCR. As shown in Figure 1A, miR-425-5p was highly expressed in GC tissue than in normal samples. Figure 1B also showed that miR-425-5p levels were significantly increased in GC tissue compared with normal gastric cell lines.

Overexpression of miR-425-5p promoted BGC-823 cells invasion and migration

To reveal the biological role of miR-425-5p in migration and invasion, miR-425-5p mimic or miR-NC was transfected into BGC-823 cells. The qRT-PCR analysis confirmed that transfection with the miR-425-5p mimic resulted in significant overexpression of miR-425-5p (Figure 2A). Then, the effects of miR-425-5p on the invasiveness and migration of GC cells were examined by Transwell and scratch-wound assays, respectively. As shown in Figure 2B, the movement ability of BGC-823 cells was improved by miR-425-5p. Then, It was found that miR-425-5p over-expression

clearly promoted BGC-823 cells migration and invasion, compared with that observed in control cells (Figure 2C).

Knockdown of miR-425-5p suppressed BGC-823 cells invasion and migration

Moreover, we transfected BGC-823 cells with a miR-425-5p inhibitor to downexpress this miRNA, then analyzed cell migration and invasion. The qRT-PCR analysis confirmed that transfection with the miR-425-5p inhibitor resulted in significant down-regulation of miR-425-5p (Figure 3A). Moreover, the results showed that the miR-425-5p inhibitor significantly suppressed the migration and invasion abilities of BGC-823 cells (Figure 3B-3C).

miR-425-5p regulated CYLD expression by targeting 3'-UTR of CYLD mRNA

To explore the molecular mechanism of CYLD regulation, three bioinformatic algorithms (TargetScan, PicTar, and miRanda) were applied to identify potential miRNAs which may involve in the regulation of CYLD. The data suggested a putative binding site for miR-425-5p in the 3'-UTR of CYLD mRNA (Figure 4A). Thus, reporter vectors, containing luciferase complementary DNA followed by the CYLD 3'-UTR, were constructed (Figure 4A). The relative luciferase activity of the reporter containing the wild-type 3' UTR of CYLD was significantly suppressed when 425-5p was

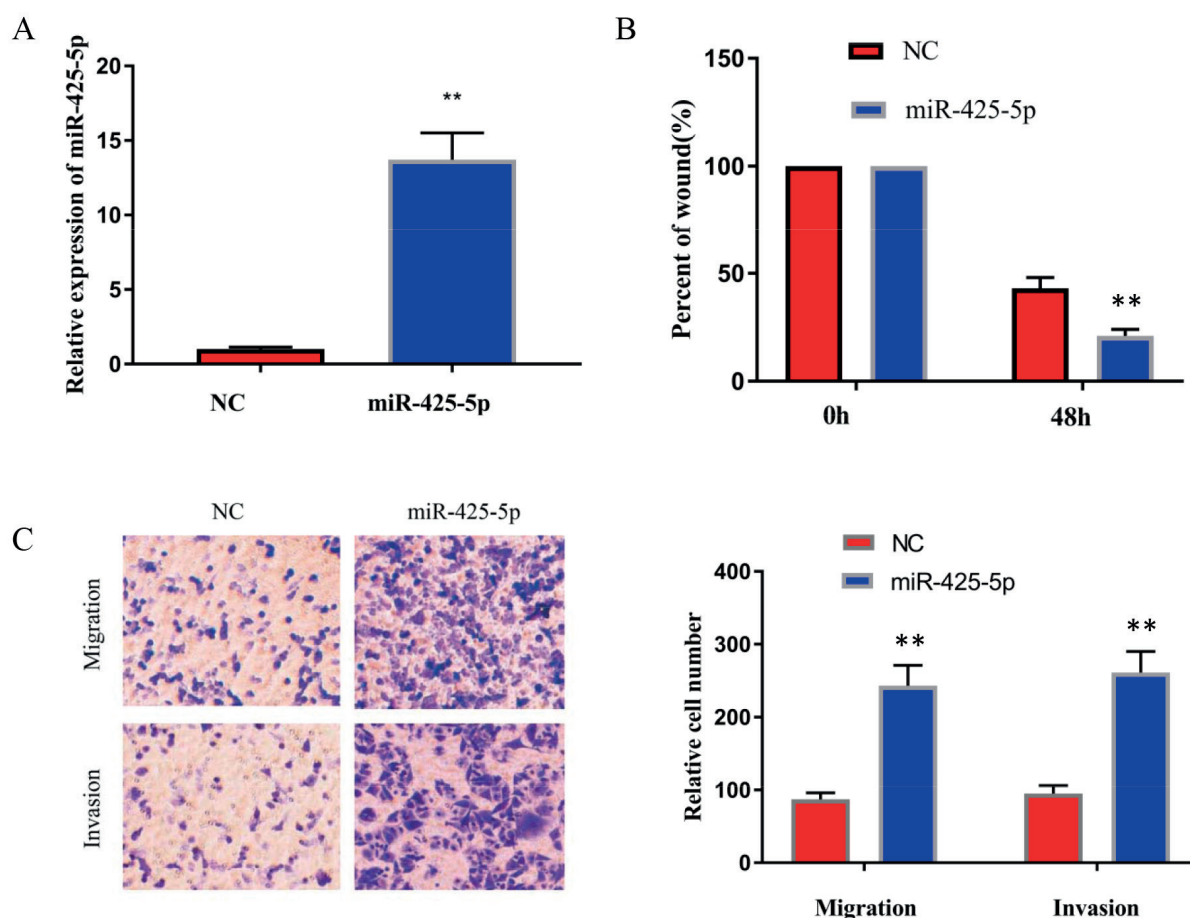


Figure 2. Up-regulation of miR-425-5p promoted migration and invasion *in vitro*. (A) Successful overexpression of miR-425-5p was confirmed by qRT-PCR. (B) The migration capacities were determined by wound scratch after being transfected with miR-425-5p or miR-NC. (C) Transwell migration and Matrigel invasion assays on BGC-823 cell transfected with miR-425-5p mimics or negative control. ** $p < 0.01$.

co-transfected (Figure 4B). However, the luciferase activity of the mutant reporter didn't show this effect (Figure 4C). To further confirm that CYLD is a direct target of miR-425-5p in GC, we transfected miR-425-5p mimics into A549 cells and found that miR-425-5p mimics significantly reduced CYLD protein levels in GC cells (Figure 5A). On the contrast, we transfected miR-425-5p inhibitor into BGC-823 cells and found that miR-425-5p significantly increased CYLD protein levels in GC cells (Figure 5B). Taken together, these data indicated that CYLD was the direct target of miR-425-5p at least in GC.

Discussion

More and more evidence has shown that a number of miRNAs are involved in tumorigene-

sis and serve either as oncogenes or tumor suppressors during tumor progression^{13,14}. Previously, He et al¹⁵ showed that miR-425 expression was up-regulated in hepatocellular carcinomas cells, and over-expression of miR-425 promoted hepatocellular carcinomas cell proliferation, migration, and invasion by inhibiting CTNNA3. Liu et al¹⁶ also found that miR-425 promoted esophageal squamous cell carcinoma tumorigenesis by Targeting SMAD2. However, finding by Liu et al¹⁷ indicated that miR-425 expression was lower in human melanoma cells compared with normal cells. They also found that the miR-425 inhibits melanoma metastasis through repression of PI3K-Akt pathway by targeting IGF-1. Those results showed that miR-425 play a different role in tumors depending on the types of tumors. Recently, Zhang et al¹⁸ revealed that miR-425-5p expression was significantly upregulated in all the GC cell lines, and downregulation of

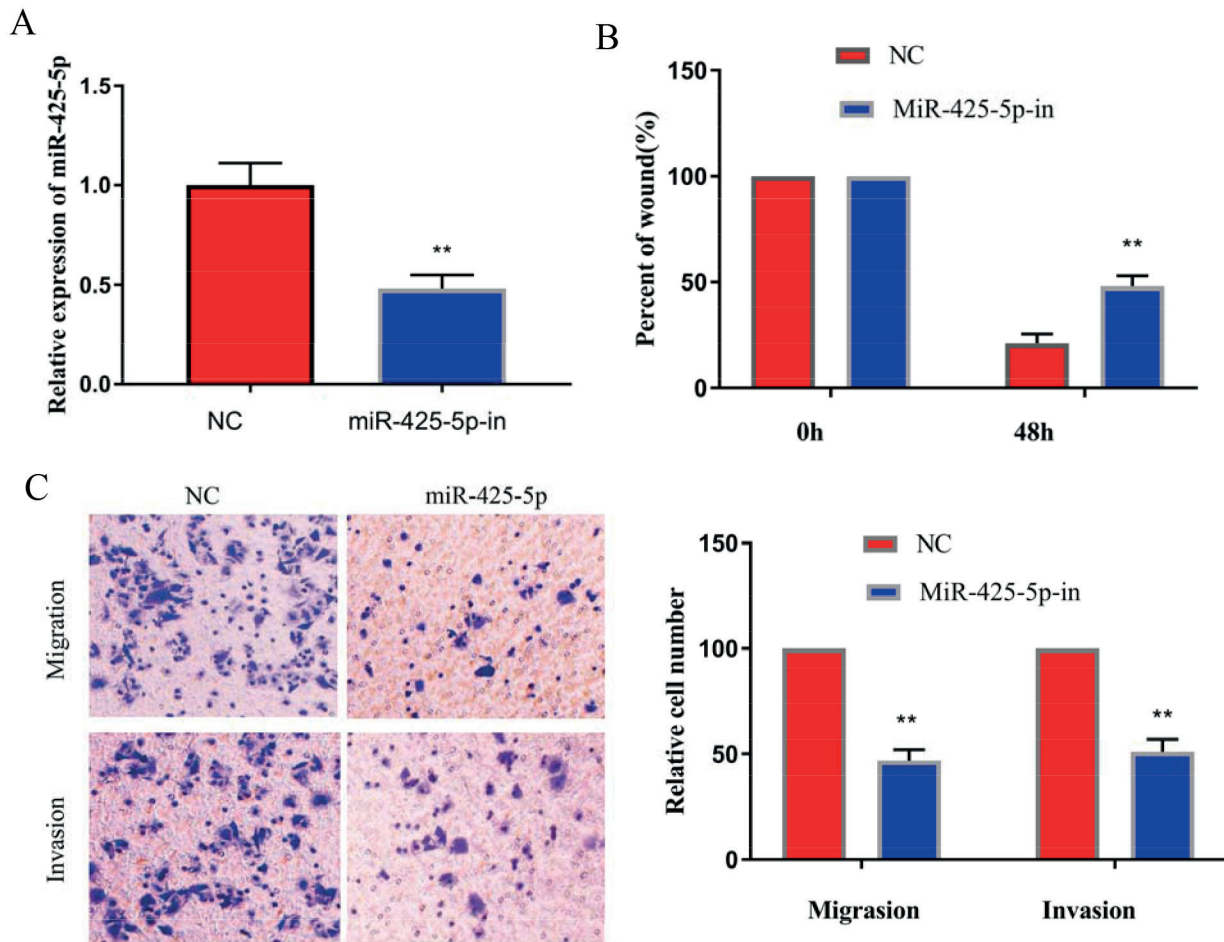


Figure 3. Down-regulation of miR-425-5p suppressed migration and invasion *in vitro*. (A) Successful knockdown of miR-425-5p was confirmed by qRT-PCR. (B) The migration capacities were determined by wound scratch after being transfected with miR-425-5p inhibitor or miR-NC. (C) Transwell migration and Matrigel invasion assays on BGC-823 cell transfected with miR-425-5p inhibitor or negative control. ** $p < 0.01$.

miR-425-5p expression inhibited GC cell proliferation, invasion, and migration, suggesting that miR-425-5p function as a tumor promoter in GC. In the present work, we further explore the underlying mechanisms of miR-425-5p-induced GC cell progression.

In the present study, we confirmed that the expression of miR-425-5p was significantly higher in GC tissues than in normal gastric tissues. Next, More importantly, miR-425-5p overexpression promoted *in vitro* cell migration and invasion. On the contrary, downregulation of miR-425-5p suppressed *in vitro* cell migration and invasion. These data confirmed that miR-425-5p exhibits tumor-promoting activity in GC.

CYLD has been reported to be down-regulated in several types of cancers including colorectal cancer, breast cancer, hepatocellular carcinoma and gastric cancer, suggesting that CYLD exhibits broad tumor suppressor functions¹⁹⁻²². Using the algorithms TargetScan, and miRanda website tools, we identified CYLD as the potential target of miR-425-5p. Furthermore, we performed Luciferase reporter assays and the results showed that miR-425-5p may directly target CYLD-3'UTR with the seed sequence of 47-98 sites. The result of western blot also confirmed that over-expression of miR-425-5p could suppress the expression level of CYLD. All the above suggested that CYLD was a potential functional target of miR-425-5p.

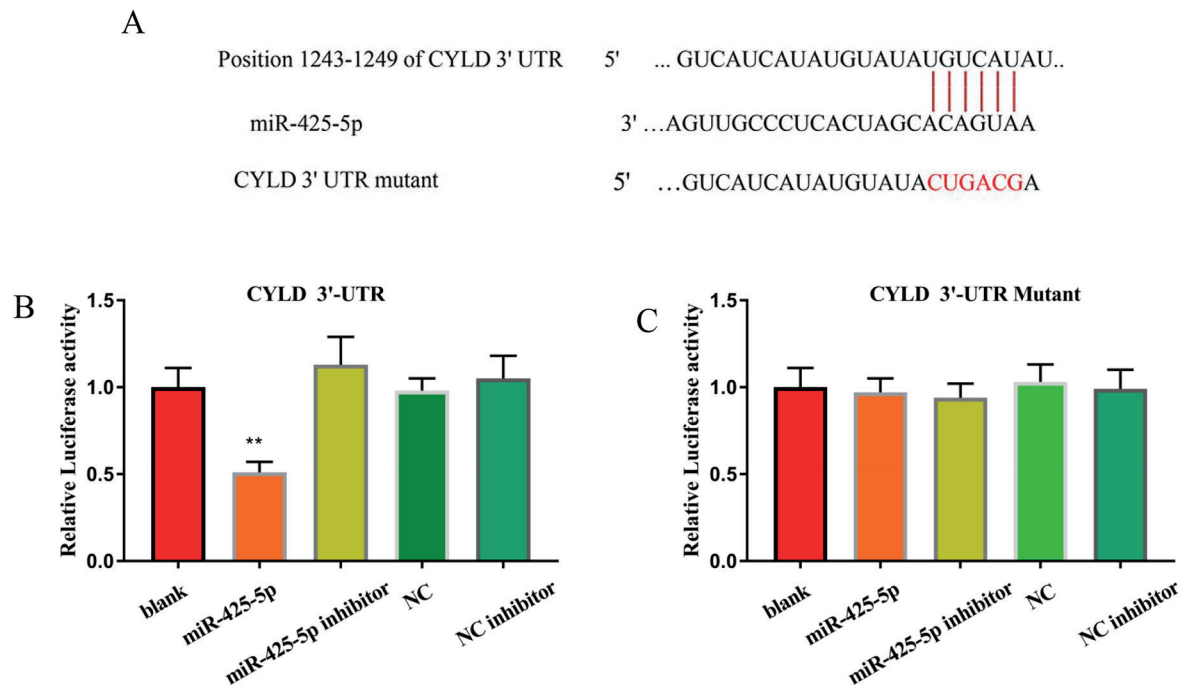


Figure 4. MiR-425-5p directly targets CYLD. (A) Predicted miR-425-5p target sequences in the 3'-UTRs of CYLD. (B,C) Relative luciferase activity was analyzed upon co-transfection with wild-type (WT) or mutant-type (mt) reporter plasmids and miR-425-5p, miR-425-5p inhibitor or miR-Ctrl in BGC-823 cells. ** $p < 0.01$.

Conclusions

Taken together, our *in vitro* experimental results demonstrated that miR-425-5p may target

CYLD to promote the invasion and metastasis of GC. miR-425-5p may serve as a novel therapeutic target for GC.

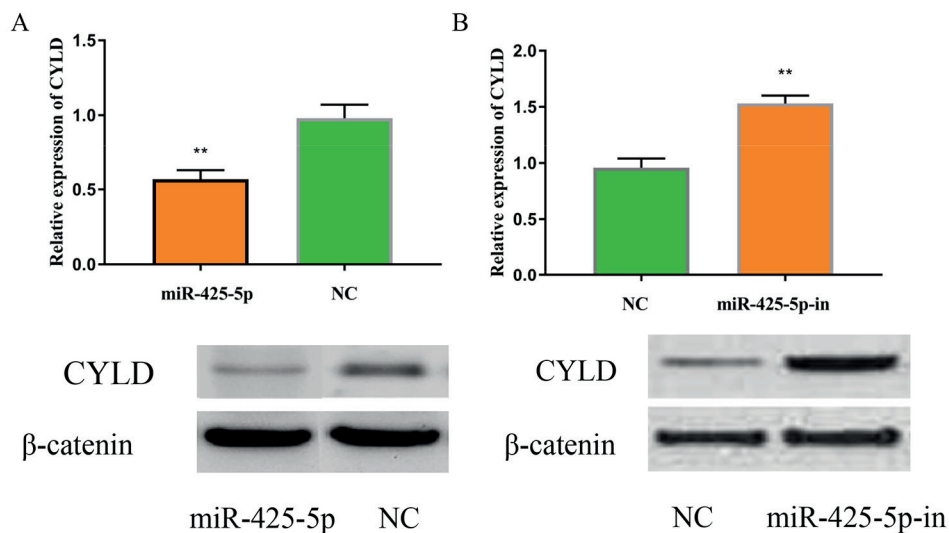


Figure 5. The effects of miR-425-5p on the expression of CYLD protein in GC cell line. (A) Levels of CYLD in BGC-823 cells. Cells were transfected with miR-425-5p mimetic or NC-miR for 24 h. Levels of CYLD were determined by Western blot. (B) Levels of CYLD in BGC-823 cells. Cells were transfected with miR-425-5p inhibitor or NC-miR for 24 h. Levels of CYLD were determined by Western blot. ** $p < 0.01$.

Conflict of interest

The authors declare no conflicts of interest.

References

- JEMAL A, BRAY F, CENTER MM, FERLAY J, WARD E, FORMAN D. Global cancer statistics. *CA Cancer J Clin* 2011; 61: 69-90.
- BERRETTA S, BERRETTA M, FIORICA F, DI FRANCIA R, MAGISTRI P, BERTOLA G, FISICHELLA R, CANZONIERI V, DI BENEDETTO F, TARANTINO G. Multimodal approach of advanced gastric cancer: based therapeutic algorithm. *Eur Rev Med Pharmacol Sci* 2016; 20: 4018-4031.
- FERRO A, PELETEIRO B2, MALVEZZI M3, BOSETTI C3, BERTUCCIO P3, LEVI F4, NEGRI E3, LA VECCHIA C5, LUNET N6. Worldwide trends in gastric cancer mortality (1980-2011), with predictions to 2015, and incidence by subtype. *Eur J Cancer* 2014; 50: 1330-1344.
- CHEN W, ZHENG R, ZENG H, ZHANG S, HE J. Annual report on status of cancer in China, 2011. *Chin J Cancer Res* 2015; 27: 2-12.
- LAI EC. Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat Genet* 2002; 30: 363-364.
- CALIN GA, CROCE CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; 6: 857-866.
- Meister G. miRNAs get an early start on translational silencing. *Cell* 2007; 131: 25-28.
- ZHANG Y, WEN X, HU XL, CHENG LZ, YU JY, WEI ZB. Downregulation of miR-145-5p correlates with poor prognosis in gastric cancer. *Eur Rev Med Pharmacol Sci* 2016; 20: 3026-3030.
- REN C, CHEN H, HAN C, FU D, ZHOU L, JIN G, WANG F, WANG D, CHEN Y, MA L, ZHENG X, HAN D. miR-486-5p expression pattern in esophageal squamous cell carcinoma, gastric cancer and its prognostic value. *Oncotarget* 2016; 7: 15840-15853.
- ZHAO B, DONG AS. MiR-874 inhibits cell growth and induces apoptosis by targeting STAT3 in human colorectal cancer cells. *Eur Rev Med Pharmacol Sci* 2016; 20: 269-277.
- ZHANG S, ZHAO Y, WANG L. MicroRNA-198 inhibited tumorous behaviors of human osteosarcoma through directly targeting ROCK1. *Biochem Biophys Res Commun* 2016; 472: 557-565.
- ZHAO XQ, LIANG TJ, FU JW. miR-494 inhibits invasion and proliferation of gastric cancer by targeting IGF-1R. *Eur Rev Med Pharmacol Sci* 2016; 20: 3818-3824.
- IORIO MV, FERRACIN M, LIU CG, VERONESE A, SPIZZO R, SABBIONI S, MAGRI E, PEDRIALI M, FABBRI M, CAMPIGLIO M, M  NARD S, PALAZZO JP, ROSENBERG A, MUSIANI P, VOLINIA S, NENCI I, CALIN GA, QUERZOLI P, NEGRINI M, CROCE CM. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 2005; 65: 7065-7070.
- GARZON R, CALIN GA, CROCE CM: MicroRNAs in cancer. *Annu Rev Med* 2009; 60: 167-179
- HE B, LI T, GUAN L, LIU FE, CHEN XM, ZHAO J, LIN S, LIU ZZ, ZHANG HQ. CTNNA3 is a tumor suppressor in hepatocellular carcinomas and is inhibited by miR-425. *Oncotarget* 2016; 7: 8078-8089
- LIU L, ZHAO Z, ZHOU W, FAN X, ZHAN Q, SONG Y. Enhanced Expression of miR-425 Promotes Esophageal Squamous Cell Carcinoma Tumorigenesis by Targeting SMAD2. *J Genet Genomics* 2015; 42: 601-611.
- LIU P, HU Y, MA L, DU M, XIA L, HU Z. miR-425 inhibits melanoma metastasis through repression of PI3K-Akt pathway by targeting IGF-1. *Biomed Pharmacother* 2015; 75: 51-57.
- ZHANG Z, LI Y, FAN L, ZHAO Q, TAN B, LI Z, ZANG A. miRNA-425-5p is upregulated in human gastric cancer and contributes to invasion and metastasis *in vitro* and *in vivo*. *Exp Ther Med* 2015; 9: 1617-1622.
- HELLERBRAND C, BUMES E, BATAILLE F, DIETMAIER W, MASSOUMI R, BOSSERHOFF AK. Reduced expression of CYLD in human colon and hepatocellular carcinomas. *Carcinogenesis* 2007; 28: 21-27.
- HUTTI JE, SHEN RR, ABBOTT DW, ZHOU AY, SPROTT KM, ASARA JM, HAHN WC, CANTLEY LC. Phosphorylation of the tumor suppressor CYLD by the breast cancer oncogene IKKepsilon promotes cell transformation. *Mol Cell* 2009; 34: 461-472.
- WELTE S, URBANIK T, ELSSNER C, KAUTZ N, KOEHLER BC, WALDBURGER N, BERMEJO JL, PINNA F, WEISS KH, SCHEMMER P, JAEGER D, LONGERICH T, BREUHAHN K, SCHULZE-BERGKAMEN H. Nuclear expression of the deubiquitinase CYLD is associated with improved survival in human hepatocellular carcinoma. *PLoS One* 2014; 9: e110591.
- ZHU M, ZHOU X, DU Y, HUANG Z, ZHU J, XU J, CHENG G, SHU Y, LIU P, ZHU W, WANG T. miR-20a induces cisplatin resistance of a human gastric cancer cell line via targeting CYLD. *Mol Med Rep* 2016; 14: 1742-1750.