European Review for Medical and Pharmacological Sciences 2019; 23: 8861-8869

MiR-181d inhibits cell proliferation and metastasis through PI3K/AKT pathway in gastric cancer

K. JIANG, L.-F. XIE, T.-Z. XIAO, M.-Y. QIU, W.-L. WANG

General Surgery, Wuxi No. 4 People's Hospital, Affiliated Hospital of Jiangnan University, Wuxi, China

Kai Jiang and *Lifei Xie* share first authorship

Abstract. – OBJECTIVE: Gastric cancer is the second highest mortality tumor and the fourth most common cancer worldwide that has high aggressiveness. MicroRNA-181d (miR-181d) has been established to be a tumor suppressor, by suppressing cell proliferation, cell cycle, and promoting apoptosis in several cancers. The purpose of this study is to explore the great roles of miR-181d in gastric cancer.

PATIENTS AND METHODS: The Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) and Western blot were applied to calculate the mRNA and protein levels of miR-181d and genes. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell assays were utilized to measure the proliferative and invasive abilities. The Kaplan-Meier method was conducted to calculate the overall survival of gastric cancer patients.

RESULTS: MiR-181d was detected to be downregulated in gastric cancer tissues and cell lines compared to the peritumoral normal tissues and normal cell line. Downregulation of miR-181d predicted poor prognosis of gastric cancer patients. Cylindromatosis gene (CYLD) was overexpressed in gastric cancer tissues, which was confirmed to be a target gene of miR-181d in gastric cancer cell line HGC-27. Moreover, miR-181d inhibited the proliferation through CYLD/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway and inhibited the invasion-mediated epithelial-mesenchymal transition (EMT) in HGC-27 cells. In addition, overexpression of miR-181d suppressed tumor growth and xenograft tumorigenesis of HGC-27 cells *in vivo*.

CONCLUSIONS: MiR-181d functioned as a tumor suppressor by inhibiting the proliferation *via* PI3K/AKT pathway *in vitro* and *in vivo* and inhibiting invasion-mediated epithelial-mesenchymal transition (EMT) by targeting CYLD in gastric cancer. The newly identified miR-181d/CYLD axis provides novel insight into the pathogenesis of gastric cancer.

Key Words:
MiR-181d, Proliferation, Invasion, EMT.

Introduction

Gastric cancer (GC), one of the gastrointestinal tract malignancies, is the second highest mortality tumor worldwide that has high aggressiveness[1,2]. The key preference for gastric cancer was a surgical operation, although the treatment modalities have improved, the 5-year overall survival rate was still poor due to cancer proliferation and invasion[3]. Therefore, it is urgent to detect novel biomarkers for the early diagnosis and treatment of gastric cancer.

MicroRNAs (miRNAs), 18-25 nucleotides non-coding RNAs in length, could down-regulate gene expression through binding to the 3′-untranslated region (3′-UTR) of target mRNA at post-transcriptional level[4]. MiRNAs have been reported to play great roles in the proliferation and metastasis of tumors and are associated with the development and progression of tumor[5]. In gastric cancer, several miRNAs were played vital roles that including miR-647, miR-29c, miR-34a, and miR-181d[6-9]. MiR-181d has been reported to act as a tumor suppressor in glioma by suppressing cell proliferation, cell cycle, and promoting cell apoptosis[10]. Similarly, the ectopic expression of miR-181d inhibited cell growth and metastasis in colorectal cancer[11]. However, little previous studies illuminated the functions of miR-181d in gastric cancer, thus, the experiments were performed to explore the vital roles of miR-181d in gastric cancer.

Cylindromatosis gene (CYLD), functions as a deubiquitinating enzyme and has been reported to inhibit cell proliferation by cleaving the ubiquitin chains from target proteins of cancer[12]. CYLD catalytic activity loss could improve the carcinogenic potency through increased cell proliferation and migration[13]. The mutation of CYLD was associated with the progression and

Corresponding Author: Kai Jiang, MD; e-mail: drraphaeljk@126.com

development of basal cell salivary neoplasms[14]. In cervical cancer, CYLD has been reported to inhibit cell proliferation, migration, and invasion, which mediated by miR-501[15]. In our study, miR-181d was discovered to inhibit cell proliferation and invasion by binding to the 3'-UTR of CYLD mRNA in gastric cancer. MiR-181d suppressed invasion-mediated epithelial-mesenchymal transition (EMT) and proliferation through CYLD/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway.

Patients and Methods

Clinical Specimens

Fifty pairs of gastric cancer tissues and the peritumoral normal tissues were obtained from Wuxi No.4 People's Hospital which undergoing surgery from June 2014 to June 2017. The tissue samples were immediately snap-frozen in liquid nitrogen and then stored at –80°C after surgery. The written informed consents were obtained from all the mentioned patients and the Ethics Committees of Wuxi No. 4 People's Hospital approved this work.

Cell Culture

The normal gastric mucosal epithelial cell line GES-1 and two human gastric cancer cell lines HGC-27, and MGC-803 were all purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). All the cells cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin sodium, and 100 μg/mL streptomycin sulfate (Sigma-Aldrich, Mo, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Transfection

The specific the miR-181d mimic or the miR-181d inhibitor and negative control plasmids were designed and synthesized from GenePharma (Shanghai, China). HGC-27 cells utilized to perform the transfection, which were seeded in a 6-well plate. In accordance with the manufacturer's instructions, the transfections were carried out utilized Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA, USA), which were diluted by Opti-MEM/Reduced serum medium (Thermo Fisher Scientific, Waltham, MA, USA). For the stable transfection, the cells were selected by Geneticin (G418; Thermo Scientific, Waltham, MA, USA), while the cells with transient transfection were harvest after transfected 48 h.

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) Assay

For the miRNAs, total miRNAs were extracted utilized a miRNeasy Mini Kit (Qiagen, Hilden, Germany) from gastric cancer tissues or cell lines. According to the manufacturer's instructions, the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was conducted to synthesize the first complementary deoxyribose nucleic acid (cDNA) chain; followed a miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) was employed to perform the qPCR. The relative levels of miRNA were derived using the $2^{-\Delta\Delta Ct}$ method with U6 small nuclear RNA as normalization.

For the mRNAs, the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract total RNAs. The Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany) was utilized to synthesize the first cDNA chain from total RNAs. The QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) was conducted to perform the RT-qPCR in a Quantitect SYBR green PCR system (Qiagen, Hilden, Germany). The $2^{-\Delta\Delta Ct}$ method was used for the mRNA quantification, which normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used for RT-qPCR analysis were listed as followed: miR-181d: 5'-CCGCTCGAGAACTTGCCAAGGGTTTGGGGGAACA-3' and 5'-CCGGAATTCATGTTCATCTACCAGTTTGCCCACT-3'; U6: 5'-CTCGCTTCGGCAGCACA-3' and 5'-GCGAGCACAGAATTAATACGAC-3'; CYLD 5'-ACGCCACAATCTTCATCACACT-3' and 5'-AGGTCGTGGTCAAGGTTTCACT-3'; GAPDH 5'-GCAAGTTCAACGGCACAG-3' and 5'-ACGCCAGTAGACTCCACGAC-3'.

Western Blot Analysis

Total proteins were lysed by radioimmunoprecipitation assay (RIPA) Lysis Buffer (Sigma, Aldrich, St. Louis, MO, USA) that contains 10% phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied to separate the proteins, and then the blots were electro-transferred to polyvinylidene difluoride (PVDF) membranes

(Millipore, Billerica, MA, USA). After being blocked by 5% fat-free milk at room temperature for 1 h, the membranes were incubated by primary antibodies. The primary antibodies were against CYLD (1:1000; Abcam, Cambridge, CA, USA), E-cadherin (1:1000; Abcam, Cambridge, CA, USA), N-cadherin (1:1000; Abcam, Cambridge, CA, USA), Vimentin (1:1000; Abcam, Cambridge, CA, USA), p-PI3K (1:1000, Cell Signaling, San Jose, CA, USA), PI3K (1:1000, Cell Signaling, San Jose, CA, USA), p-AKT (1:1000, Cell Signaling, San José, CA, USA), AKT (1:1000, Cell Signaling, San José, CA, USA). Subsequently, the blots were incubated by secondary anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Cell Signaling, San José, CA, USA). The protein signals were captured using enhanced chemiluminescence detection Kit (ECL, Pharmacia Biotech, Arlington, MA, USA).

Immunohistochemistry (IHC)

The expression of CYLD was evaluated by IHC utilized the Elivition™ Detection Kit (Glostrup, Denmark). In brief, the xylene and graded alcohols were utilized to deparaffinize and rehydrate the pancreas sections (4 μm). The citrate buffer (pH = 6.0) was utilized to antigen repair the sections for 25 min in a microwave oven, followed the endogenous peroxidase was blocked by the 3% H_2O_2 for 20 min. After blocked the nonspecific binding by goat serum, the sections were incubated with the primary anti-CYLD antibody at 37°C for 45 min. Once washed with phosphate-buffered saline (PBS), the sections were incubated with HRP-polymer secondary antibody successively. Subsequently, diaminobenzidine (DAB) and hematoxylin were employed to stain and re-dye the slides.

Cell Counting Kit-8 (CCK-8) Assay

The cell proliferation ability was measured by CCK-8 assay (Dojindo, Kumamoto, Japan). Briefly, HGC-27 cells transfected with special vectors were seeded in 96-well plates and cultured for 24 h, 48 h, 72 h, and 96 h at 37°C with 5% CO_2, followed 10 μL CCK-8 solution was added into each well and incubated all the cells for 2 h. Finally, the microplate reader was utilized to measure the absorbance at 450 nm.

Transwell Assay

To perform the cell invasive ability, the transwell insert (8 μm membrane; Corning, Corning, NY, USA) covered with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were placed in 24-well plate and that formed up and low two chambers. The HGC-27 cells were suspended by RPMI-1640 medium and 200 μL cell suspensions were added in the upper chamber. Meanwhile, 500 μL medium was filled into the lower chamber containing 15% FBS, which acted as inducer. After the cells were incubated at 37°C for 24 h, the non-invasive cells still on the upper surface were removed with a cotton swab. For the invasive cells, we fixed and then stained by 4% paraformaldehyde and 10% crystal violet respectively. And followed the cells of five fields that randomly selected were counted under a microscope (Olympus, Tokyo, Japan).

MiRNA Targets Prediction and Dual-Luciferase Reporter Assay

TargetScan (http://www.targetscan.org) was conducted to predict the target genes of miR-181d and CYLD was predicted to be a potential one. To validate whether miR-181d binding to CYLD in gastric cancer cell lines, the binding sequences were mutated from GAAUGUA to CUUACA. Subsequently, the wild type and the mutant 3'-UTR of CYLD were inserted into the dual luciferase reporter vectors, which were designated as WT or MUT. For the luciferase assay, pursuant to the command of the manufacturer Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) was employed to co-transfected miR-181d mimic and the WT or MUT plasmid into HGC-27 cells. Then, the luciferase activity was measured by the dual luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

All statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 16.0 software (IBM, Armonk, NY, USA) and the data were presented as mean ± SD (standard deviation). The differences between two or more groups were compared using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference). The association between the expression of miR-181d and the overall survival for gastric cancer patients were assessed by the Kaplan-Meier curve and log-rank test. $p<0.05$ was considered to be statistically significant.

Results

Downregulation of MiR-181d Predicted Poor Prognosis of Gastric Cancer

The mRNA level of miR-181d was evaluated in 50 pairs of gastric cancer and the peritumoral normal tissues. As expected, the expression of miR-181d was lower in gastric cancer tissues than that of corresponding peritumoral normal tissues ($p<0.05$) (Figure 1A). What's more, downregulation of miR-181d predicted poor 5-year survival of gastric cancer patients ($p<0.05$) (Figure 1B).

MiR-181d Inhibited the Proliferation and Invasion in HGC-27 Cells

The expressions of miR-181d were calculated in two gastric cancer cell lines (HGC-27 and MGC-803) and a normal epithelial cell line GES-1. Same with the tissues, the expression of miR-181d was higher in GES-1 cells than HGC-27 ($p<0.05$) and MGC-803 ($p<0.05$) cells (Figure 2A). To explore the function of miR-181d, the miR-181d mimic and the miR-181d inhibitor were conducted to up- ($p<0.01$) or down-regulated ($p<0.05$) miR-181d in HGC-27 cells, which were measured by RT-qPCR (Figure 2B).

MTT assay illuminated that the proliferation was suppressed by the miR-181d mimic ($p<0.05$), while promoted by the miR-181d inhibitor ($p<0.05$) in HGC-27 cells (Figure 2C). In addition, transwell assay revealed that the invasive ability was inhibited by the miR-181d mimic ($p<0.05$) whereas promoted by the miR-181d inhibitor in HGC-27 cells ($p<0.05$) (Figure 2D). All the results revealed miR-181d inhibited the abilities of proliferation and invasion in gastric cancer cell line HGC-27.

MiR-181d Regulated the Expression of CYLD Through Binding to the 3'-UTR of CYLD mRNA

TargetScan predicted that CYLD was a target gene of miR-181d, and the binding site was located at 345-351 on 3'-UTR of CYLD mRNA. To validate miR-181d binding to the potential binding site of CYLD, the binding sequences were mutated from GAAUGUA to CUUACA, and followed evaluated the luciferase activity (Figure 3A). The luciferase reporter assay revealed that miR-181d mimic reduced ($p<0.05$) the luciferase activity of HGC-27 cells that transfected wild type CYLD 3'-UTR, while did not alter ($p>0.05$) the luciferase activity of cells transfected that mutant CYLD 3'-UTR (Figure 3B). Moreover, mRNA levels of CYLD were evaluated after transfected miR-181d mimic or miR-181d inhibitor in HGC-27 cells. As expected, overexpression of miR-181d inhibited the mRNA level of CYLD ($p<0.05$), while knockdown of miR-181d promot-

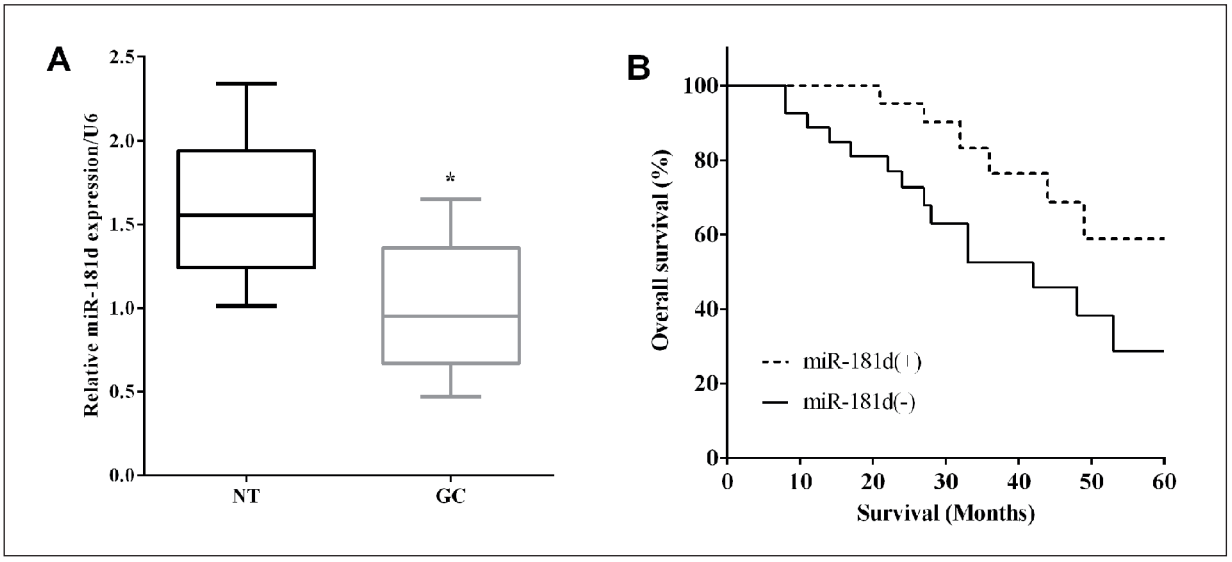

Figure 1. Downregulation of miR-181d predicted poor prognosis of gastric cancer. **A,** MiR-181d was low expressed in GC tissues compared to the corresponding peritumoral normal tissues. **B,** Overexpression of miR-181d predicted poor 5-year survival of gastric cancer patients.

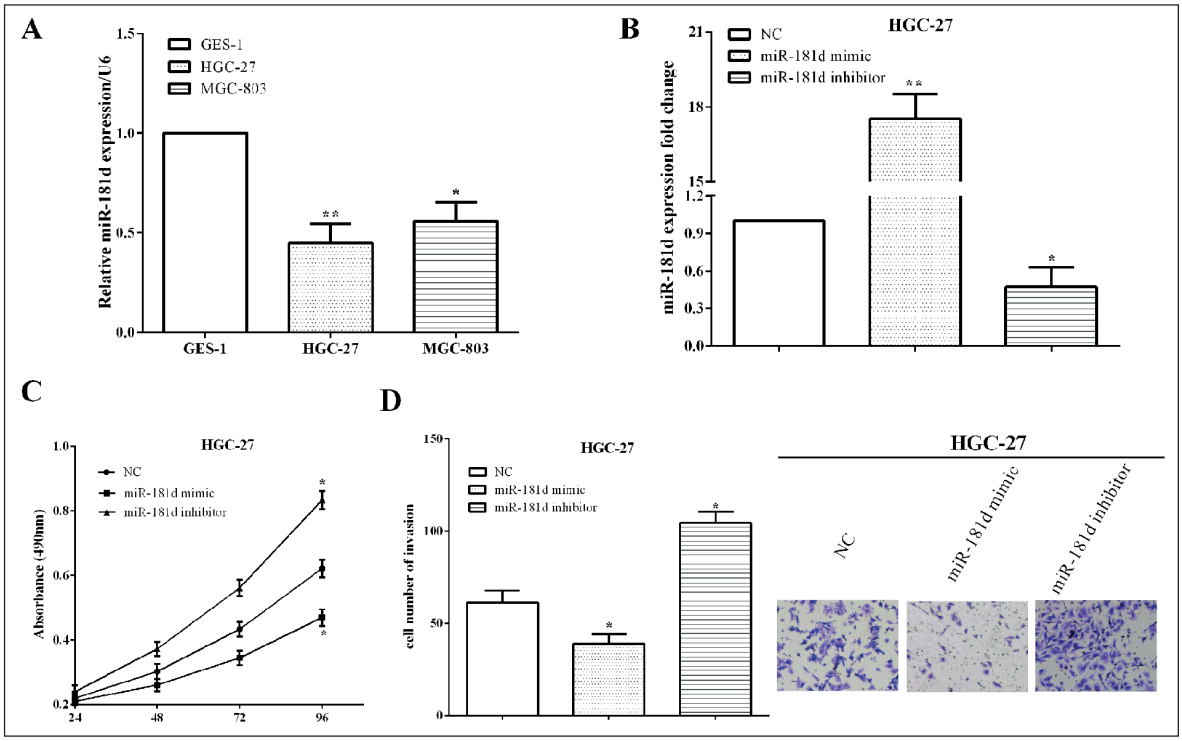

Figure 2. MiR-181d inhibited the proliferation and invasion in HGC-27 cells. **A,** Expression of miR-181d was higher in GES-1 cells than HGC-27 and MGC-803 cells. **B,** MiR-181d mimic and the miR-181d inhibitor were conducted to up- or down-regulate miR-181d in HGC-27 cells. **C,** MTT assay illuminated the proliferative ability was suppressed by the miR-181d mimic, while it was promoted by the miR-181d inhibitor in HGC-27 cells. **D,** Transwell assay revealed that miR-181d mimic inhibited the invasive ability and it was promoted by miR-181d inhibitor in HGC-27 cells (magnification: 40×).

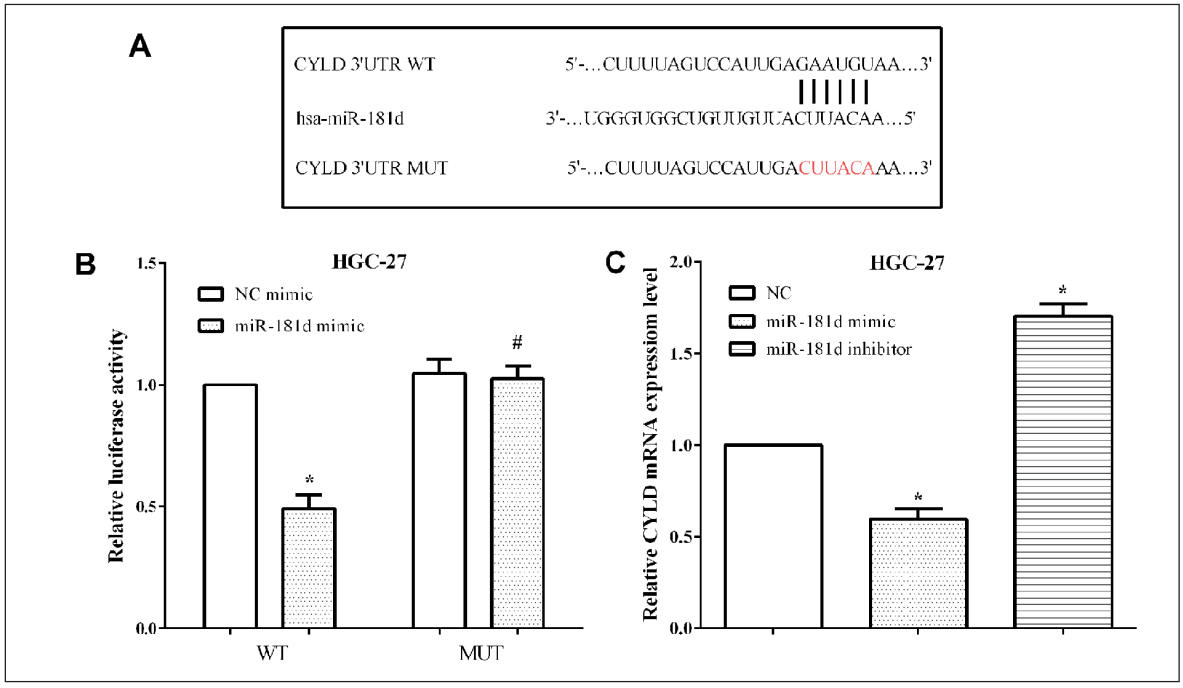

Figure 3. MiR-181d regulated the expression of CYLD by directly binding to the 3'-UTR of CYLD mRNA. **A,** TargetScan predicted CYLD was a direct target gene of miR-181d. **B,** MiR-181d mimic reduced the luciferase activity of HGC-27 cells that transfected wild type CYLD 3'-UTR. **C,** MiR-181d overexpression inhibited the mRNA level of CYLD, while knockdown of miR-181d promoted the expression of CYLD in HGC-27 cells.

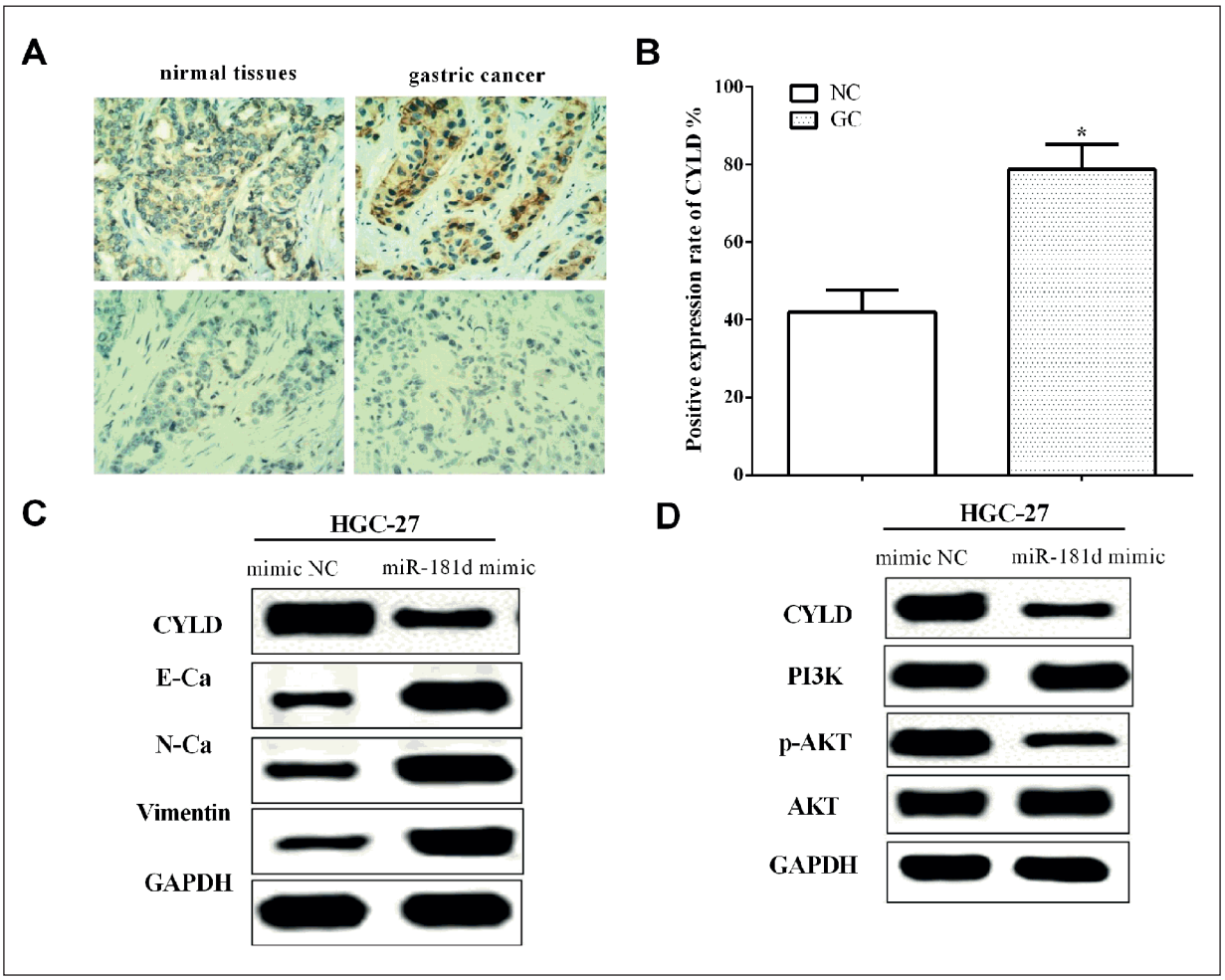

Figure 4. MiR-181d inhibited the invasion-mediated EMT and proliferation through PI3K/AKT signal pathway. **A,** Subcellular localization of CYLD was located at cytoplasm (magnification: 200×). **B,** Relative positive rate of CYLD in gastric cancer tissues and peritumoral normal tissues was calculated. **C,** Expression of CYLD was lower in HGC-27 and MGC-803 cell lines than GES-1 cell line. **D,** MiR-181d inhibited cell invasion-mediated EMT and proliferation through CYLD/PI3K/AKT signal pathway.

ed (p<0.05) that expression of CYLD in HGC-27 cells (Figure 3C). All the results indicated that miR-181d regulated the expression of CYLD in gastric cancer cell line HGC-27.

MiR-181d Inhibited the Invasion-Mediated EMT and Proliferation Through PI3K/AKT Signal Pathway

The CYLD protein expression was evaluated using IHC assay, and we discovered that CYLD was upregulated in gastric cancer tissues compared with the peritumoral normal tissues. The subcellular localization of CYLD was located at cytoplasm, and the positive and negative expression in gastric cancer tissues and the peritumoral normal tissues were shown in Figure 4A. The relative positive rate of CYLD was calculated and the positive rates in gastric cancer tissues were higher than that in peritumoral normal tissues (p<0.05) (Figure 4B). Moreover, Western blot was employed to calculate the expression of proteins that associated EMT and the pathway in HGC-27 cells. We discovered that miR-181d mimic suppressed EMT ability by downregulating the expression of CYLD and E-cadherin, but upregulating the expression of N-cadherin and Vimentin in HGC-27 cells (Figure 4C), which suggested that miR-181d inhibited the EMT through regulating the expression of CYLD. In addition, overexpression of miR-181d suppressed p-PI3K and p-AKT expression in HGC-27 cells (Figure 4D), which illuminated that miR-181d inhibited the proliferation through CYLD/PI3K/AKT pathway. All the results revealed that miR-

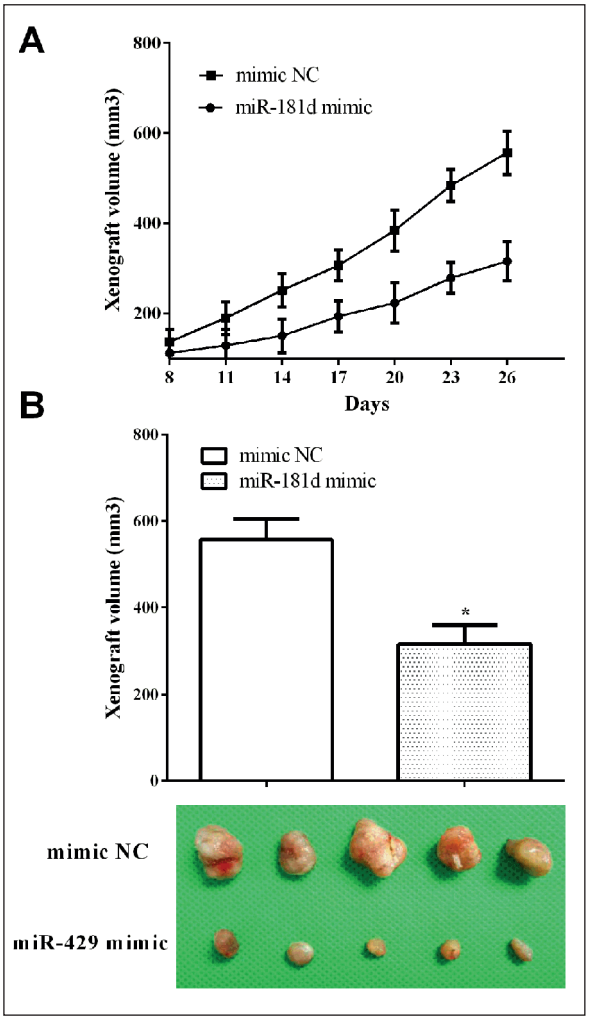

Figure 5. MiR-181d suppressed the xenograft growth *in vivo*. **A,** It had a smaller tumor volume of cells that overexpressed miR-181d than the control group. **B,** Overexpression of miR-181d inhibited the growth of gastric cancer.

181d inhibited the invasion-mediated EMT and proliferation through CYLD/PI3K/AKT signal pathway in HGC-27 cells.

MiR-181d Suppressed the Xenograft Growth In Vivo

HGC-27 cells stably transfected the miR-181d mimic or control plasmid were applied to inject into the nude mice at subcutaneous. This study was approved by the Animal Ethics Committee of Wuxi No. 4 People's Hospital Animal Center. The xenograft tumors volume was calculated every 3 days and the group of transfecting the miR-181d mimic had a slower growth rate than control group (Figure 5A). Also, it had a smaller tumor volume of cells overexpressed miR-181d then the control group, which indicated that overexpression of miR-181d inhibited xenograft growth of gastric cancer cells ($p<0.05$) (Figure 5B).

Discussion

Gastric cancer, the most frequent gastrointestinal tract malignancy, has high morbidity and mortality[1]. The 5-year overall survival rate was still poor due to cancer proliferation and invasion[3]. Therefore, it is urgent to explore the novel biomarkers for the early diagnosis and treatment of gastric cancer.

MiRNAs play great roles in the proliferation and metastasis of tumors and are associated with the development and progression of tumor[5]. MiR-181d has been reported to act as a tumor suppressor, inhibited the proliferation, migration and arrested the cell cycle in esophageal squamous cell carcinoma[10]. Similarly, knockdown of miR-181d suppressed the proliferation, migration, and invasion by impairing glycolysis in CRC cells[16]. Consistent with all above, we first proposed that miR-181d was downregulated in gastric cancer tissues compared to the peritumoral normal tissues and downregulation of miR-181d predicted poor prognosis of gastric cancer patients. MiR-181d inhibited the proliferation through CYLD/PI3K/AKT pathway and inhibited the invasion-mediated EMT in HGC-27 cells. What's more, overexpression of miR-181d suppressed the xenograft growth of HGC-27 cells. However, miR-181d promoted neurite outgrowth in spinal cord injury[17], thus, we believe that miR-181d may have tissue-specificity.

CYLD has been reported to function as a deubiquitinating enzyme, catalytic activity loss of CYLD improved the carcinogenic potency through increased cell proliferation and migration[13]. CYLD has been elucidated to be a target gene of miscellaneous miRNAs that including miR-130b, miR-19a, miR-1228 and miR-181d[18-21]. In lung adenocarcinoma, CYLD was indicated to be a target of miR-197, and inhibited the proliferation and promoted cell apoptosis[22]. Similarly, CYLD was a target of miR-767 and suppressed cell proliferation in melanoma[23]. Consistent with Iliopoulos et al[24], we first proposed that CYLD was a direct target gene of miR-181d in gastric cancer. The expression of CYLD was mediated by miR-181d through directly binding to its 3'-UTR of mRNA. What's more, overexpression

of miR-181d inhibited the EMT through directly binding to the 3'-UTR of CYLD mRNA in HGC-27 cells. MiR-181d suppressed the proliferation through CYLD/PI3K/AKT pathway in HGC-27 cells.

Conclusions

We showed that MiR-181d was downregulated in gastric cancer tissues and cell lines compared to the normal tissues and normal cell line. What's more, downregulation of miR-181d predicted poor prognosis in gastric cancer. MiR-181d directly targeted to the 3'-UTR of CYLD mRNA in gastric cancer. MiR-181d inhibited the proliferation through CYLD/PI3K/AKT pathway and inhibited the invasion-mediated EMT in HGC-27 cells. In addition, overexpression of miR-181d suppressed the growth of gastric cancer *in vivo*.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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