JBUON 2020; 25(3): 1636-1642
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Research on mechanism of miR-130a in regulating autophagy of bladder cancer cells through CYLD

Haitao Dai[1,2], Changmao Liu[1], Yuanhua Liu[1], Zhong Zhang[1], Changwei Peng[1], Zhongyu Wang[1], Jiang Zheng[1], Chenglong Li[2], Weimin Yu[2], Fan Cheng[2]

[1]Department of Urology, the first People's Hospital of Jingzhou (The First Affiliated Hospital of Yangtze University), Jingzhou, Hubei 434000, P.R. China. [2]Department of Urology, Renmin Hospital of Wuhan University, Hubei General Hospital, Wuhan, Hubei 430060, P.R. China.

Summary

Purpose: *The study aimed to explore the regulatory mechanism of micro ribonucleic acid (miR)-130a in the autophagy of bladder cancer cells through cylindromatosis (CYLD).*

Methods: *Human bladder cancer T24 cell line was used as the objects of the study. After miR-130a was knocked down using small-interfering RNA (siRNA) in T24 cell line, the changes in expressions of miR-130a and CYLD in each group were detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The cell proliferation in each group was detected using cell counting kit-8 (CCK8) assay and flow cytometry. The changes in mRNA and protein levels of microtubule-associated protein 1 light chain 3 (LC3) and Beclin1 were determined using qRT-PCR and Western blotting. The autolysosomes were detected through acridine orange (AO)/ethidium staining bromide (ER) staining. Moreover, CYLD was knocked down using siRNA, and then the changes in mRNA expressions of miR-130a, LC3 and Beclin1 in each group were detected through qRT-PCR.*

Results: *After interference in miR-130a with siRNA, miR-130a-siRNA group had a significantly lower mRNA expression of miR-130a compared with NC-siRNA group and a significantly higher mRNA expression of CYLD ($p<0.05$), obviously inhibited cell proliferation ($p<0.05$), and decreased significantly mRNA and protein expressions of LC3 showing Beclin1 ($p<0.05$), and an evidently smaller number of autolysosomes. After knockdown of CYLD using siRNA, the mRNA expression of miR-130a had no significant changes ($p>0.05$), while the mRNA expressions of LC3 and Beclin1 declined significantly in CYLD-siRNA group compared with those in NC-siRNA group ($p<0.05$).*

Conclusion: *MiR-130 can promote the autophagy of bladder cancer cells through regulating CYLD, thus facilitating the proliferation of tumor cells.*

Key words: *miR-130a, CYLD, bladder cancer cells, autophagy*

Introduction

Bladder cancer is a malignant tumor of the urinary system, and its morbidity and mortality rates rank first among urinary system tumors in China [1]. About 1/4 of the early cases mortality rates ranks first among urinary system tumors in China [1]. About 1/4 of bladder cancer are muscle-invasive, while the remaining 3/4 of the cases are non-muscle-invasive bladder cancer, which will mostly develop into recurrent or muscle-invasive bladder cancer with the progression of disease [2]. Currently, the key to the treatment of bladder cancer is early surgery combined with postoperative intravesical chemotherapy, and this treatment strategy can reduce the recurrence rate and increase the survival to some extent, but it is still of great significance to explore more effective treatment drugs or means.

There are increasingly more studies that micro ribonucleic acids (miRs) are involved in the devel-

Corresponding author: Fan Cheng, MD. Department of Urology, Renmin Hospital of Wuhan University, Hubei General Hospital, No.238, Jiefang Rd, No. 99, Zhangzhidong Rd, Wuchang District, Wuhan, Hubei 430060, P.R. China.
E-mail: jiha77@126.com
Received: 09/12/2019; Accepted: 05/01/2020

opment, progression, infiltration, distant metastasis and drug resistance of many different tumors, and it is even reported that miRs can be used as important markers for early diagnosis and prognosis evaluation [3,4]. There are similar reports that miR-130a has pathological expression in a variety of tumors, and it may become an important parameter for diagnosis and efficacy [5,6]. The expression and role of miR-130a varies among different organs. For example, compared with para-carcinoma tissues, miR-130a is lowly expressed in liver cancer and prostate cancer but highly expressed in gastric cancer and ovarian cancer [7]. In addition, the low expression of miR-130a is an independent risk factor for poor prognosis of patients with liver cancer and lung cancer, while its high expression is often an independent risk factor for poor prognosis of patients with gastric cancer and ovarian cancer [8]. MiR-130a has a dual character in different tumor tissues, and acts either as an oncogene or as tumor suppressor gene, and its mechanism is related to intrinsic factors of tumors, including the types of gene mutation and histological type, also indicating a diverse and unclear regulatory mechanism [9]. The end product of cylindromatosis (CYLD), as a tumor suppressor gene, is de-ubiquitinase that can regulate some important signaling pathways through de-ubiquitination of some upstream regulatory factors [10]. In addition, CYLD is also involved in maintaining the normal functions of tissues and organs and inhibiting cell cancerization, and cancerization of head and facial skin will occur in the case of interference or knockout of CYLD [11]. However, the regulatory mechanism on CYLD remains broadly unclear.

In the present study, the human bladder cancer T24 cell line was used as the main object of the study, the expression of miR-130a in T24 cell line was detected, and the related mechanism of miR-130a in regulating the autophagy of bladder cancer cells through CYLD was studied, so as to explore whether miR-130a has research prospect and important significance in the clinical diagnosis and treatment of bladder cancer.

Methods

Reagents and instruments

The following reagents and instruments were used in this study:

Cell counting kit-8 (CCK8) and DAPT inhibitor (MSU, Sigma, MO, USA), RNA extraction kit (Invitrogen Life Technologies, Carlsbad, CA, USA), quantitative reverse transcription-polymerase chain reaction (qRT-PCR) kit and acridine orange (AO)/ethidium bromide (EB) staining kit (Jiancheng, Nanjing, China), primer synthesis kits (Hanbio, Shanghai, China), Ki67, bicinchonimic acid (BCA) protein quantification kit, RIPA cell lysis buffer (Hanbio, Shanghai, China), and Beclin1, microtubule-associated protein 1 light chain 3 (LC3) and GAPDH (Cell Signaling Technology Inc., Shanghai, China).

Cell culture and transfection

Human bladder cancer T24 cell line was cultured in an incubator with 5% CO_2 at 37°C. With about 80% confluence, the cells were digested with trypsin and prepared into single cell suspension, followed by inoculation and culture in culture plates for subsequent experiments. With 60-70% of cell fusion, they were transfected with miR-130a-siRNA and NC-siRNA or CYLD-siRNA and NC-siRNA, respectively, followed by culture in the incubator for 48h.

Determination of cell proliferative activity via CCK8 assay

At 48 h after cell transfection, the cell proliferative activity was detected via CCK8 assay. The cells were inoculated into 96-well plates at a concentration of 1×10^5mL (100 μL/well) and cultured in the incubator with 5% CO_2 at 37°C for 48 h, with three replicates in each group. Then, DMEM medium was replaced, and 10 μL of CCK8 solution was added into each well. After 30 min, the optical density (OD) value of each well was assessed at 562 nm using a microplate reader.

Determination of proliferative activity of T24 cells via flow cytometry

When 60-70% of T24 cells were fused in the 6-well plates, they were transfected with miR-130a-siRNA and NC-siRNA or CYLD-siRNA and NC-siRNA, respectively. After 48 h, the cells were treated with trypsin, collected, treated with 1% Triton and labeled with Ki67, followed

Table 1. RT-PCR primer sequences

Gene	*Primer*	*Primer sequence*
MiR-130a	Forward primer	5'-CTGGAAAATTTCTGGGCCAA-3'
	Reverse primer	5'-CCAGACTGTCCTCATTCAGAAAAA-3'
Beclin1	Forward primer	5'-ACGCACGACGTCTTCCAGTA-3'
	Reverse primer	5'-CCACCTGGTTCAACTCACTCC-3'
LC3	Forward primer	5'-GCATACATTCGAAAGACC-3'
	Reverse primer	5'-CTCAGTTATCTTTTCAG-3'
U6	Forward primer	5'-GACCTCTATGCCAACACAGT-3'
	Reverse primer	5'-AGTACTTGCGCTCAGGAGGA-3'

by detection of cell proliferation via flow cytometry.

Detection of autolysosomes through acridine orange staining

When 60-70% of T24 cells were fused in the 6-well plates, they were transfected with miR-130a-siRNA and NC-siRNA or CYLD-siRNA and NC-siRNA, respectively. After 48 h, the cells were stained with AO/EB working solution (1 g/mL) in the dark for 20 min, and photographed under a fluorescence microscope. The cytoplasm showed bright green fluorescence, while the acidic autolysosomes showed bright red dotted fluorescence.

Detection of expressions of related RNA via qRT-PCR

After treatment, the cells in each well of the 6-well plates were added with 500 μL of TRIzol. After standing for 5 min, chloroform was added and the mixture was shaken violently for 15 s and standed still for 15 min. Then the water-phase layer was taken into another new batch of EP tubes, added with isopropanol and turned upside-down several times, followed by centrifugation. The white precipitate at the bottom of the tube was RNA. After RNA was treated with 75% ethanol and dissolved in water, its concentration and purity were assessed. The qualified RNA (A_{260}/A_{280} =1.8-2.0) was reversely transcribed into cDNA, and the mRNA expression level was detected via qRT-PCR. The primer sequences are shown in Table 1. The reaction conditions were as follows: 94°C for 5 min, 94°C for 30 s, 57°C for 30 s and 72°C for 30 s for a total of 40 cycles, and 72°C for 5 min. The data were processed using Microsoft Excel, and the relative expression level of the target gene was calculated using $2^{-\Delta\Delta Ct}$ method according to the following formula, with GAPDH as a control gene:

$$\Delta Ct_{\text{target gene}} = Ct_{\text{target gene}} - Ct_{\text{control gene}},$$

$$\Delta\Delta Ct = \Delta Ct_{\text{target gene}} - \Delta Ct_{\text{standard value}}.$$

Western blotting

After treatment, the cells in each well of the 6-well plates were added with 100 μL of RIPA lysis buffer for lysis on ice for 20 min and collected, followed by centrifugation at 12000 rpm and 4°C for 10 min. Then, the supernatant was collected into another new batch of EP tubes. The concentration of the protein extracted was quantitatively measured using the BCA kit. Twenty μg of proteins were taken and subjected to western blotting. After electrophoretic separation, the proteins were electrically transferred onto a PVDF membrane in electrophoresis buffer, sealed with 5% skim milk at room temperature for 2 h, washed on a shaker and incubated in the incubator containing the primary antibody (1:1000) at 4°C overnight. After the membrane was fully washed with tris-buffered saline (TBS), the proteins were incubated again with the secondary antibody (1:5000) at room temperature for 1 h, followed by image development with DAB in the dark. The images were scanned and recorded using a gel imager (Bio-Rad Laboratories, California, USA). Finally, the gray scale was analyzed and compared with GAPDH as an internal reference.

Statistics

The data were expressed as mean±standard deviation and processed using SPSS 17.0 software (International Business Machines Corporation, USA). One-way analysis of variance (ANOVA) was used for the statistical analysis of data. $P<0.05$ suggested that the difference was statistically significant.

Results

Effect of siRNA interference on miR-130a on CYLD

After interference in miR-130a, compared with those in NC-siRNA group, the mRNA level of miR-130a was significantly decreased ($p<0.01$), while that of CYLD was significantly increased ($p<0.01$) (Figure 1), suggesting that miR-130a may have a negative regulatory effect on the CYLD expression.

Effect of interference in miR-130a with siRNA on cell proliferative activity

The results of CCK8 assay showed that compared with that in NC-siRNA group, the cell proliferative activity declined significantly after interference in miR-130a ($p<0.01$) (Figure 2), indicating that miR-130a can obviously affect the proliferative activity of T24 cells.

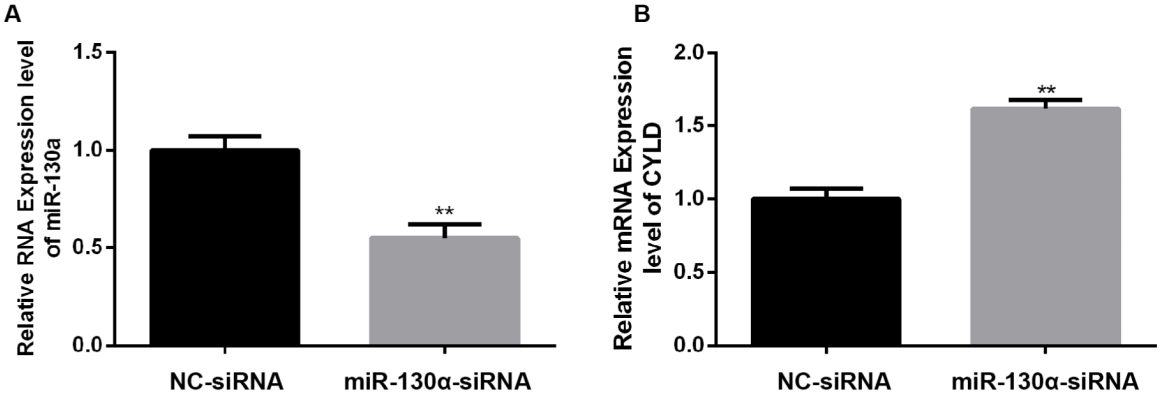

Figure 1. Effect of interference in miR-130a **(A)** and CYLD **(B)** in T24 cells detected via qRT-PCR. **p<0.01 vs. NC-siRNA group.

Effect of interference in miR-130a with siRNA on cell proliferative activity detected using Ki67

The number of T24 cells in the proliferative phase was evidently decreased after interference in miR-130a compared with that in NC-siRNA group (p<0.01) (Figure 3).

Effect of interference in miR-130a with siRNA on autophagy-related genes detected using qRT-PCR

The mRNA expression levels of Beclin1 and LC3 in T24 cells declined remarkably after interference in miR-130a compared with those in NC-siRNA group (p<0.01) (Figure 4).

Effect of interference in miR-130a with siRNA on autophagy-related proteins detected using Western blotting

Western blotting showed that the protein expression levels of Beclin1 and LC3 in T24 cells declined remarkably after interference in miR-130a compared with those in NC-siRNA group (p<0.01) (Figure 5).

Effect of interference in miR-130a with siRNA on autolysosomes

After AO/EB staining (1 g/mL) for 20 min in the dark, the cells were observed and photographed under fluorescence microscope. The cytoplasm showed bright green fluorescence, while the acidic autolysosomes showed bright red dotted fluorescence. Compared with NC-siRNA group, miR-130a-siRNA group had an obviously smaller number of acidic autolysosomes (Figure 6).

Effect of interference in CYLD with siRNA on autophagy-related genes detected through qRT-PCR

The results of qRT-PCR revealed that in T24 cells, the miR-130a mRNA expression had no sig-

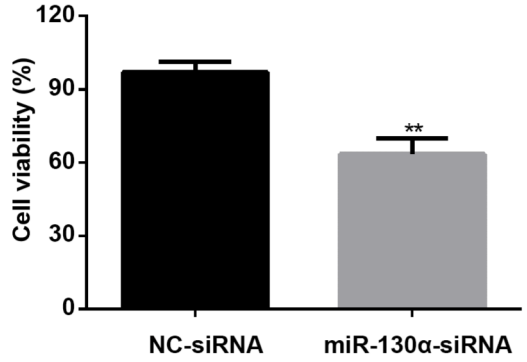

Figure 2. Cell proliferative activity after interference in miR-130a detected via CCK8 assay. **p<0.01 vs. NC-siRNA group.

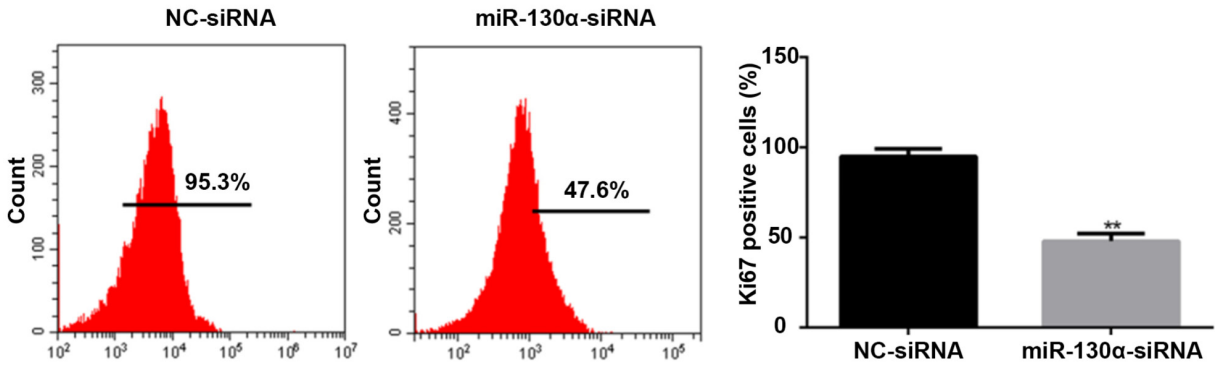

Figure 3. Cell proliferative activity after interference in miR-130a detected via flow cytometry. **p<0.01 vs. NC-siRNA group.

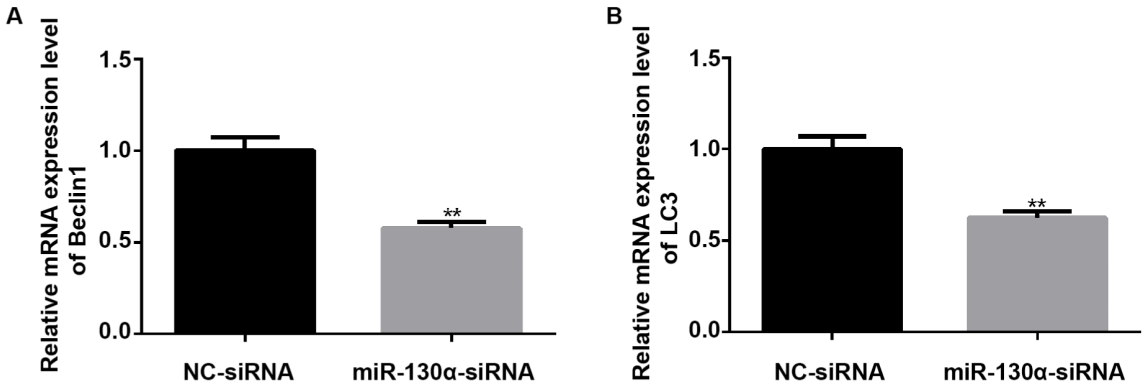

Figure 4. Effect of interference in miR-130a with siRNA on autophagy-related genes in T24 cells detected using qRT-PCR. **p<0.01 vs. NC-siRNA group.

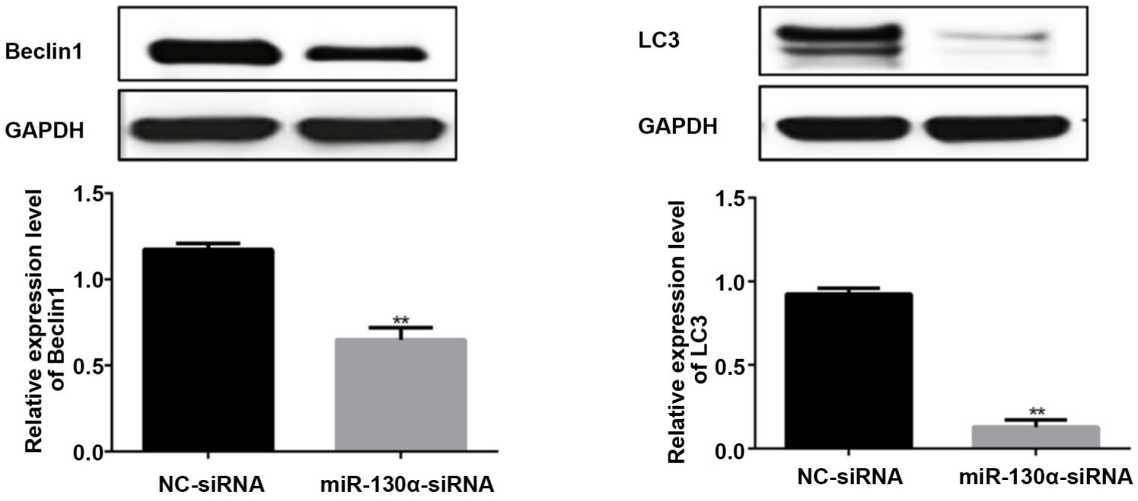

Figure 5. Effect of interference in miR-130a with siRNA on autophagy-related proteins detected using Western blotting. **p<0.01 vs. NC-siRNA group.

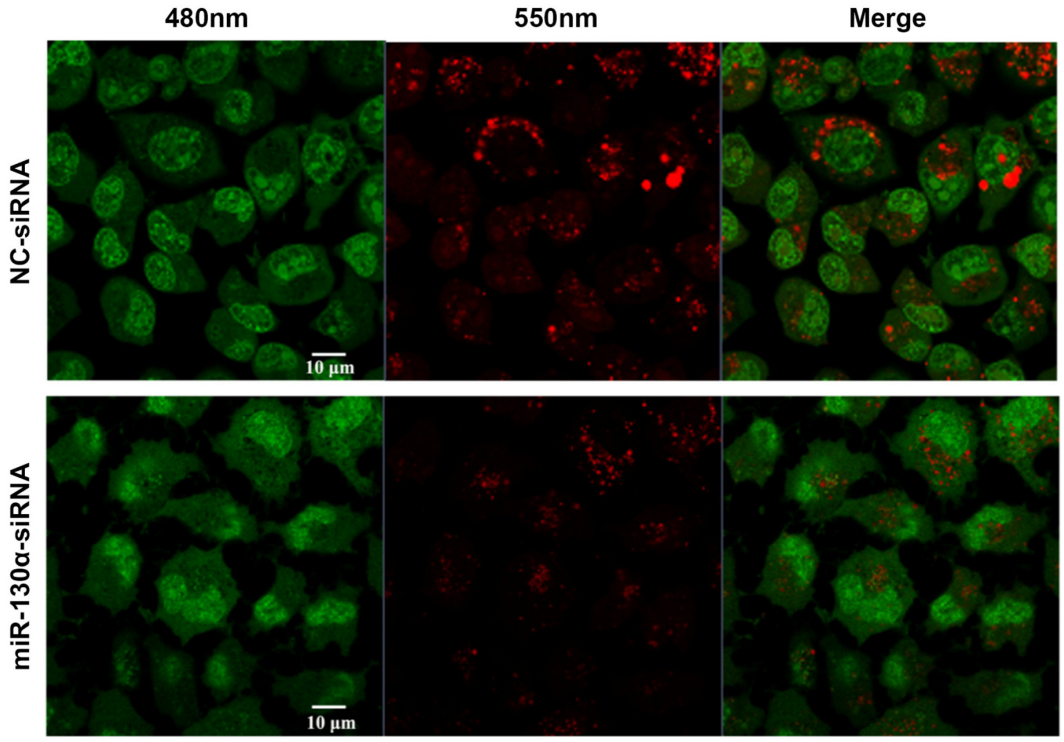

Figure 6. Effect of interference in miR-130a with siRNA on autolysosomes using acridine orange staining.

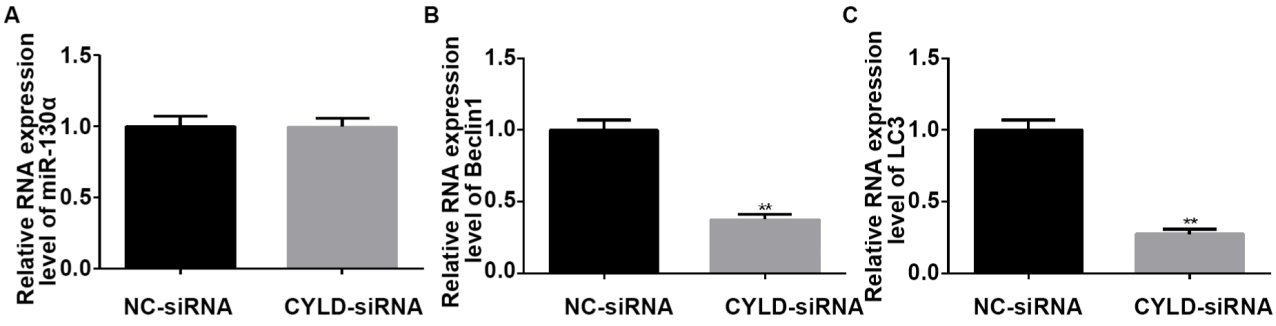

Figure 7. Effect of interference in CYLD with siRNA on autophagy-related genes detected through qRT-PCR. **p<0.01 vs. NC-siRNA group.

nificant changes, while the mRNA expression levels of Beclin1 and LC3 declined significantly after treatment with CYLD-siRNA compared with those in NC-siRNA group ($p<0.01$) (Figure 7), demonstrating that miR-130a is at the upstream of CYLD, thus regulating the changes in autophagy.

Discussion

Both morbidity and mortality rates of bladder cancer, one of the malignant tumors of the urinary system, are increasing year by year, and the disease is regulated by multiple genes, in which the CYLD signaling pathway has been paid extensive attention and widely studied. However, the regulatory mechanism on CYLD signaling pathway in bladder cancer is not clear.

MiRs are a kind of evolutionarily highly conserved endogenous single-stranded non-coding RNAs with about 18-25 nucleotides in length. An increasing number of studies have demonstrated that miRs are involved in the occurrence and development of many diseases, and they play important roles in the gene regulation of tumors [12]. The targeted regulation of miRs on downstream genes has become an important research hotspot, such as that in the field of cardiovascular diseases and cancer [13,14]. There are reports that miR-130b is involved in the occurrence of gastric cancer, liver cancer and colorectal cancer [15,16], and it is also found that miR-130b is highly expressed in esophageal cancer tissues, whose mechanism is that miR-130b can facilitate the proliferation and metastasis of esophageal squamous cell carcinoma cells through the targeted regulation on PTEN gene [17]. In addition, miR-130b is also highly expressed in hepatocellular carcinoma, which can promote the migration and invasion of liver cancer cells through down-regulating PPAR gene expression and inducing its epithelial-mesenchymal transition [18]. However, the biological function of miR-130a and its mechanism of action remain unclear. The results of this study also confirmed that highly-expressed miR-130a in bladder cancer can regulate the expression of CYLD.

The end product of CYLD, as a tumor suppressor gene, is a kind of de-ubiquitinase that can regulate Wnt/catenin, TGF-β, NF-κB and JNK signaling pathways through de-ubiquitination of some upstream regulatory factors [19]. In addition, CYLD plays an important role in maintaining the normal function of tissues and organs and inhibiting tumorigenesis. The down-regulation or deletion of CYLD protein expression mainly leads to cancer of head and facial skin [20], such as multiple family trichoepithelioma. Moreover, the down-regulation of CYLD expression is related to the occurrence of a variety of tumors. Studies have shown that the mRNA level of CYLD is significantly down-regulated in breast cancer tissues compared with para-carcinoma tissues, and after knockdown of CYLD using siRNA, NF-κB is over-activated, and the proliferation, migration and invasion of tumor cells are clearly enhanced [21].

In the present report, the bladder cancer T24 cell line was used as the main object of the study, and miR-130a was knocked down using siRNA in T24 cells. The results showed that compared with NC-siRNA group, miR-130a-siRNA group had a significantly lower mRNA expression of miR-130a and a significantly higher mRNA expression of CYLD, an obviously inhibited cell proliferation rate, obviously decreased mRNA and protein expressions of LC3 and Beclin1, and an evidently smaller number of autolysosomes. After knockdown of CYLD using siRNA, the mRNA expression of miR-130a had no significant changes, while the mRNA and protein expressions of LC3 and Beclin1 declined significantly in cells. Similarly, studies have shown that miR-130a is highly expressed in gastric cancer, and the higher expression corresponds to poorer prognosis [22]. The cell proliferation is remarkably inhibited after interference in miR-130a in ovarian cancer cells [23], consistent with this study. In addition, CYLD, as a tumor suppressor, is lowly expressed in various cancer tissues, such as colon and liver cancer [24], also consistent with the results in this study.

In conclusion, miR-130 can promote the autophagy of bladder cancer cells through regulating CYLD, thus facilitating the proliferation of tumor cells.

Authors' contributions

HD wrote the manuscript. HD and CL were responsible for cell culture and transfection. YL, ZZ and CP contributed to CCK8 assay and flow cytometry. ZW, JZ and CL helped with PCR and western blot. WY and FC worked on statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the ethics committee of the First People's Hospital of Jingzhou (The First Affiliated Hospital of Yangtze University).

Conflict of interests

The authors declare no conflict of interests.

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