CUL4A promotes proliferation and inhibits apoptosis of colon cancer cells *via* regulating Hippo pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of cullin 4A (CUL4A) on the proliferation and apoptosis of colon cancer (CC) cells, and to elucidate its regulatory relationship with the Hippo pathway.

PATIENTS AND METHODS: Paired CC tissues and adjacent normal tissues were obtained from patients. CC cells were isolated and cultured *in vitro*. CUL4A was interfered by small interfering ribonucleic acid (siRNA) (siR-CUL4A group) or overexpressed by overexpression vector (CUL4A-Vector group), with negative control (NC)-CUL4A or CUL4A –NC as the control group. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression level of CUL4A in CC tissues and cells. The proliferative ability of cells was detected by cell counting kit-8 (CCK-8) assay. Flow cytometry was applied to measure the apoptosis of cells in each group. Western blotting (WB) was conducted to determine the protein expression of CUL4A. In addition, the proliferative ability was examined *in vivo* through subcutaneous injection of cells into nude mice.

RESULTS: QRT-PCR showed that CUL4A was highly expressed in 66.67% of CC samples ($p<0.01$). *In vivo* and in vitro proliferative ability was significantly reduced in siR-CUL4A group ($p<0.01$), whereas the apoptosis rate was promoted ($p<0.01$). However, *in vivo* and *in vitro* proliferative ability increased significantly in CUL4A-Vector group ($p<0.01$), while the apoptosis rate was reduced ($p<0.01$). The protein expressions of MST1, LATS1 and p-YAP were significantly up-regulated in siR-CUL4A group ($p<0.01$), while they were remarkably down-regulated in CUL4A-Vector group ($p<0.05$, $p<0.01$).

CONCLUSIONS: CUL4A is highly expressed in CC and promotes the proliferation and inhibits the apoptosis of CC cells by regulating the Hippo pathway.

Key Words:
Colon cancer (CC), CUL4A, Hippo pathway, Proliferation, Apoptosis.

Introduction

Colon cancer (CC) is the third most common malignant tumor worldwide, which is also the fourth leading cause of cancer-related death[1]. There are more than 135,000 new CC cases each year[2]. Currently, therapeutic methods for CC have been developed rapidly, such as surgery and adjuvant chemotherapy. However, the 5-year survival rate of patients is still far from satisfactory[2-4].

With the ability to recruit substrate adaptors, cullin 4A (CUL4A) protein can be assembled into different E3 ubiquitin ligase complexes to mediate the turnover of key regulatory proteins[5]. The subfamily of CUL4 contains CUL4A and CUL4B, both of which share 83% sequence identity and functional redundancy[6]. CUL4A plays an important role in such physiological processes as cell survival, development, growth and cycle during chaperone-mediated ubiquitylation[7,8]. Meanwhile, it can interact with related genes during carcinogenesis. CUL4A has been confirmed overexpressed in various malignancies, including hepatocellular carcinoma[9], primary malignant pleural mesothelioma[10], primary human breast cancer[11], prostate cancer[12] and epithelial ovarian tumor[13]. All these findings suggest that CUL4A may function as an oncogene. However, the exact role of CUL4A in CC has not been fully elucidated.

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In this study, therefore, we investigated the role of CUL4A in regulating the proliferation and apoptosis of CC cells. All our results might help to better understand the pathogenic mechanism and potential therapeutic targets of CUL4A in CC.

Patients and Methods

Main Materials

CC samples (Yantaishan Hospital), SYBR Green real-time polymerase chain reaction (RT-PCR) Master Mix Kit (TaKaRa, Tokyo, Japan), Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F12), Opti-Minimal Essential Medium (MEM), fetal bovine serum (FBS), 0.25% trypsin + 0.02% ethylene diamine tetraacetic acid (EDTA) and phosphate-buffered saline (PBS) (Gibco, Rockville, MD, USA), Lipofectamine 2000 and TRIzol (Invitrogen, Carlsbad, CA, USA), HiPerFect Transfection Reagent (QIAGEN, Hilden, Germany), antibodies against mammalian Sterile 20-like kinase 1 (MST1), large tumor suppressor 1 (LATS1), Yes-associated protein (YAP), phosphorylated YAP (p-YAP) and β-Actin (Abcam, Cambridge, MA, USA), and 0.22 μm pinhole filter (Millipore, Billerica, MA, USA).

Experimental Methods

Acquisition of human CC samples and isolation and culture of cells

This investigation was approved by the Ethics Committee of Yantaishan Hospital. Signed written informed consents were obtained from all participants before the study. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). Human CC tissues were obtained from patients who received surgery in our hospital. All patients were definitely diagnosed with CC. Obtained CC tissues were stored in sterile tissue preservation solution and sent to the laboratory in time for subsequent experiments. Meanwhile, a part of para-cancerous tissues was isolated and preserved in liquid nitrogen for use.

In terms of the isolation and culture of CC cells *in vitro*, CC tissues and normal para-cancerous tissues were first washed with PBS. Subsequently, the tissues were cut into tissue blocks (1 mm^3) and digested with 0.25% trypsin + 0.02% EDTA. After that, the cell suspension was collected and filtered using a cell sieve. Next, the digestion was terminated by DMEM/F12 containing 10% FBS. Cell pellets were harvested by centrifugation, and the cells were re-suspended in DMEM/F12 containing 10% FBS. Then the cells were seeded into a 100 mm culture dish at a density of 1×10^5 cells/mL and cultured in an incubator with 5% CO_2 at 37°C. On the next day, the liquid was replaced to remove non-adherent cells. The cells were cultured again by replacing the liquid every other day. When cell confluence reached about 90%, they were washed with PBS and digested into single cells using a proper amount of 0.25% trypsin + 0.02% EDTA at 37°C. Cell culture was finally terminated by DMEM/F12 + 10% FBS, and the cells were sub-cultured at 1:6.

Interference and Overexpression of CUL4A Gene

Well grown CC cells were first seeded into a 24-well plate at a density of 2×10^4 cells/mL. When cell fusion reached 90%, 5 μL of small interfering ribonucleic acid (siR)-CUL4A mother solution (20 μM) or negative control (NC)-CUL4A stock solution was added into 83 μL of serum-free Opti-MEM. After that, the mixture was mixed with 12 μL of HiPerFect transfection reagent. The transfection complex was added into the 6-well plate in drops after 10 min of incubation at room temperature. Subsequently, they were gentle mixed and cultured in an incubator with 5% CO_2 at 37°C. 48 h later, the expression level of CUL4A was determined. Similarly, 50 μL of Opti-MEM supplemented with 0.8 μg of overexpression vector (CUL4A-Vector) or CUL4A-NC plasmid was mixed with 2 μL of Lipofectamine 2000. After incubation at room temperature for 20 min, the mixture was added into cells for culture. 6 h later, the liquid was replaced with complete DMEM/F12 containing 10% FBS. The cells were cultured for another 24 h for subsequent experiments.

Extraction of Total RNA and Quantitative RT-PCR (qRT-PCR)

Tissue samples and cells were first mixed with 1 mL of TRIzol reagent. After let stand for 5 min, 200 μL of chloroform was added, followed by shaking and centrifugation at 4°C and 12,000 rpm for 10 min. Next, the supernatant was taken and added with an equal volume of isopropyl alcohol. After incubation at room temperature for 10 min, the mixture was centrifuged at 12,000 rpm and 4°C for 15 min. Then, the precipitate was washed twice with freshly prepared 75%

Table I. Primer sequences of CUL4A and GAPDH.

Name	Sequence
CUL4A F	5'-GGCTCCAAGAAGCTGGTCAT-3'
CUL4A R	5'-GCTCCTCGAGGTTGTACCTG-3'
GAPDH F	5'-TCAGACACCATGGGGAAGGT-3'
GAPDH R	5'-TCCCGTTCTCAGCCATGTAG-3'

ethanol and dissolved in an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water. The concentration of extracted RNA was measured using a NanoDrop spectrophotometer. Subsequently, extracted total RNA was synthesized into cDNA using the RT Master Mix kit. QRT-PCR was performed in accordance with the manufacturer's protocol of the SYBR Green RT-PCR Master Mix Kit and ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Transcription level was evaluated *via* cycle threshold (Ct) value. The expression level of gene was finally calculated by the $2^{-\Delta\Delta Ct}$ method. Primers were designed online *via* www.ncbi.nlm.nih.gov/tools/primer-blast/ and synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. Primer sequences of CUL4A and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were shown in Table I.

Cell Counting Kit-8 (CCK-8) Assay

Cells in good growth status were first inoculated into a 96-well plate at a density of 1000 cells/well. Subsequently, the cells cultured in an incubator with 5% CO_2 at 37°C for 1, 2, 3 and 4 d, respectively. 10 μL of CCK-8 detection solution was added into each well, followed by incubation at 37°C for 4 h in the dark. Optical density (OD) value at the wavelength of 450 nm was measured using a micro-plate reader. The method was repeated for 3 times, with 6 replicate wells for each treatment group. The average value was taken as the final result.

Flow Cytometry

Cells in each group were first trypsinized into single-cell suspension and washed with PBS for 3 times. After the density of cells was adjusted to 5×10^5 cells/mL, they were incubated with Annexin V and propidium iodide (PI) at 4°C in the dark for 30 min. After washing with PBS for 3 times, cell density was adjusted to 10^6 cells/mL. Cell apoptosis was detected by a flow cytometer (C6), and data analysis was achieved using CFlow Plus software.

Subcutaneous Injection of Nude Mice

CC cells transfected with siR-CUL4A or NC-CUL4A and CUL4A-Vector or CUL4A-NC were first digested into single cells by trypsin. After washing with serum-free medium twice, the cells were centrifuged. Next, the cells were re-suspended in PBS, and cell concentration was adjusted to 5×10^7 cells/mL. Then, the cells were inoculated subcutaneously into the back of nude mice (200 μL/mouse). 30 d later, all mice were sacrificed, and tumor tissues were finally collected for experiments. This invesitgation was approved by the Animal Ethics Committee of Yantaishan Hospital Animal Center.

Western Blotting (WB)

Tumor tissues or cells were lysed with cell lysis buffer at 4°C overnight, followed by centrifuged at 13.000 rpm to extract total protein. The concentration of extracted protein was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Next, the proteins were separated *via* 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. After sealing with 5% skimmed milk powder and 0.1% Tris-buffered saline-Tween 20 (TBST), the membranes were incubated with primary antibodies against MST1, LATS1, p-YAP, YAP and β-Actin by gently shaking at 4°C overnight. On the next day, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies at room temperature for 2 h. Immunoreactive bands were finally exposed using enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Experimental data were presented as mean ± standard deviation ($\bar{\chi}\pm s$). Independent-samples *t*-test was adopted for comparison between groups. $p<0.05$ was considered statistically significant.

Results

Expression Level of CUL4A in CC Tissues

A total of 15 pairs of CC tissues and normal para-cancerous tissues were obtained from patients definitely diagnosed with CC. The messenger RNA (mRNA) expression level of CUL4A was

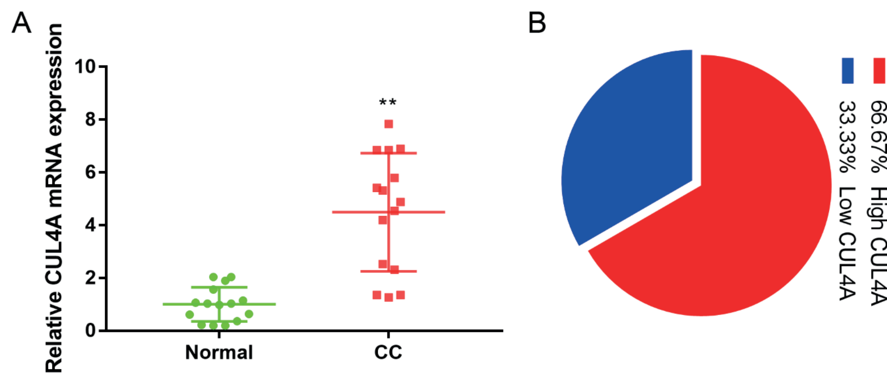

Figure 1. Expression level of CUL4A in CC tissues. **A,** Expression level of CUL4A in CC tissues and para-cancerous tissues detected via qRT-PCR. **B,** Proportions of highly and lowly expressed CUL4A in CC tissues. ** $p<0.01$: a significant difference between groups.

measured *via* qRT-PCR. The results showed that the mRNA expression level of CUL4A in CC tissues was remarkably higher than that in normal para-cancerous tissues ($p<0.01$) (Figure 1A). Besides, 66.67% and 33.33% of CC tissues exhibited highly and lowly expressed CUL4A gene, respectively (Figure 1B).

Effect of CUL4A on Proliferation of CC Cells

CC cells were separated and cultured *in vitro*, and CUL4A was repressed by siRNA or overexpressed by overexpression vector. The results of qRT-PCR manifested that the expression level of CUL4A was significantly down-regulated in siR-CUL4A group ($p<0.01$), whereas it was notably up-regulated in CUL4A-Vector group ($p<0.01$). However, transfection of NC-CUL4A and CUL4A-NC had no significant impact on the expression level of CUL4A ($p>0.05$) (Figure 2A). Cell proliferation was determined *via* CCK-8 assay after transfection. The results indicated that the proliferative ability of CC cells was significantly weakened in siR-CUL4A group compared with control group ($p<0.01$). However, it was significantly enhanced at 3 d in CUL4A-Vector group ($p<0.01$) (Figure 2B).

Effect of CUL4A on Apoptosis of CC Cells

Flow cytometry results demonstrated that the apoptosis rate of CC cells in NC-CUL4A group (3.3±0.5)% was significantly lower than

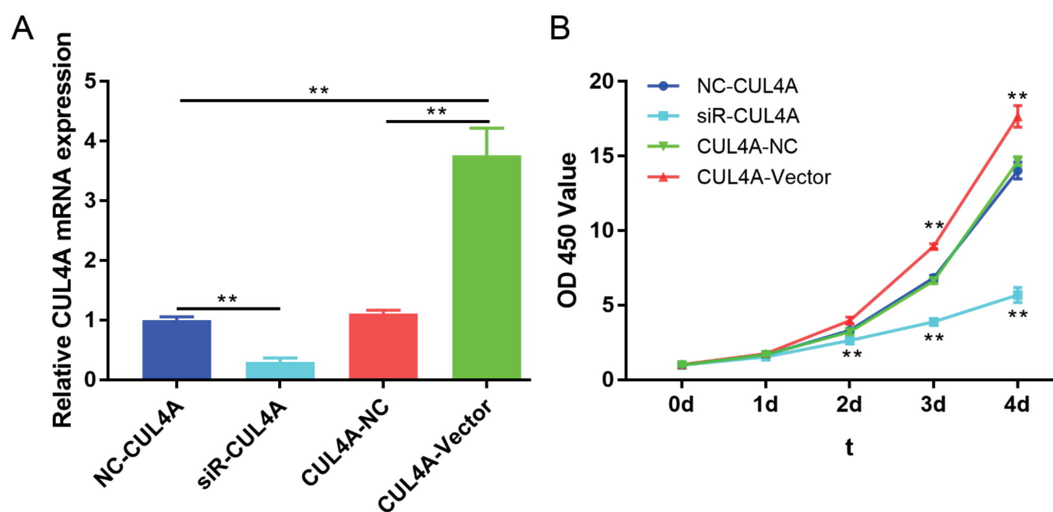

Figure 2. Effect of CUL4A on proliferation of CC cells. **A,** Expression level of CUL4A in CC cells transfected with NC-CUL4A, siR-CUL4A, CUL4A-NC and CUL4A-Vector determined via qRT-PCR. **B,** Proliferation level of cells in each group cultured *in vitro* for 1, 2, 3 and 4 d examined by CCK-8 assay. ** $p<0.01$: a significant difference between groups.

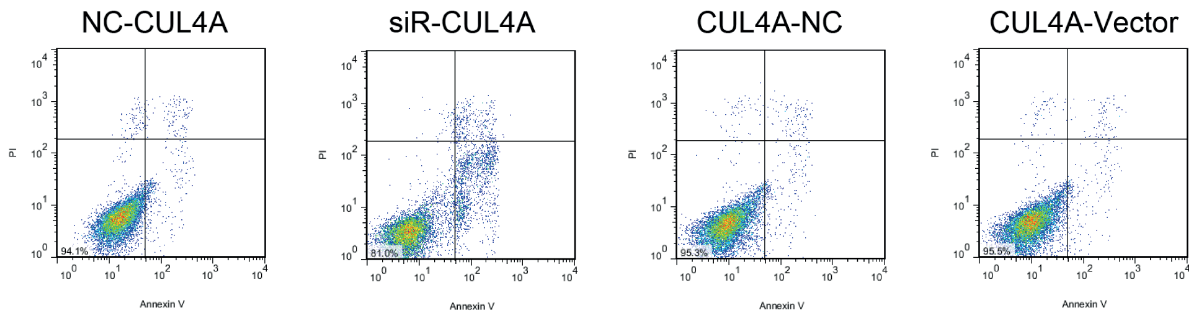

Figure 3. Apoptosis rate of CC cells analyzed *via* flow cytometry.

that in siR-CUL4A group (16.4±1.1)% (p<0.01). Besides, the apoptosis rate was (4.3±0.6)% and (1.7±0.3)% in CUL4A-NC group and CUL4A-Vector group, respectively, showing statistically significant differences (p<0.01, Figure 3).

Effect of CUL4A on Transplantation of CC Cells In Vivo

CC cells in siR-CUL4A group and CUL4A-Vector group were subcutaneously injected into nude mice separately, with CC cells as the control group. 30 d later, the tumor diameter in siR-CUL4A group was smaller but was larger in CUL4A-Vector group than that in control group (Figure 4A). WB results revealed that the protein expression of CUL4A declined prominently in siR-CUL4A group but rose remarkably in CUL4A-Vector group compared with control group (p<0.01, Figure 4B).

Regulatory Relationship Between CUL4A and Hippo Pathway

WB was performed to determine the expression levels of the Hippo pathway-related proteins MST1, LATS1, YAP and p-YAP in tumor cells at 30 d after transplantation of CC cells *in vivo* (Figure 5A). The results demonstrated that the protein expression levels of MST1, LATS1 and p-YAP were notably elevated in siR-CUL4A group (p<0.01), whereas were markedly reduced in CUL4A-Vector group (p<0.05, p<0.01). However, no significant influences of siR-CUL4A and CUL4A-Vector on the protein expression level of p-YAP were observed (p>0.05) (Figure 5B-5E)

Discussion

CC, the third most prevalent cancer in the world, shows an increasing incidence rate and younger affected population. It occurs more and

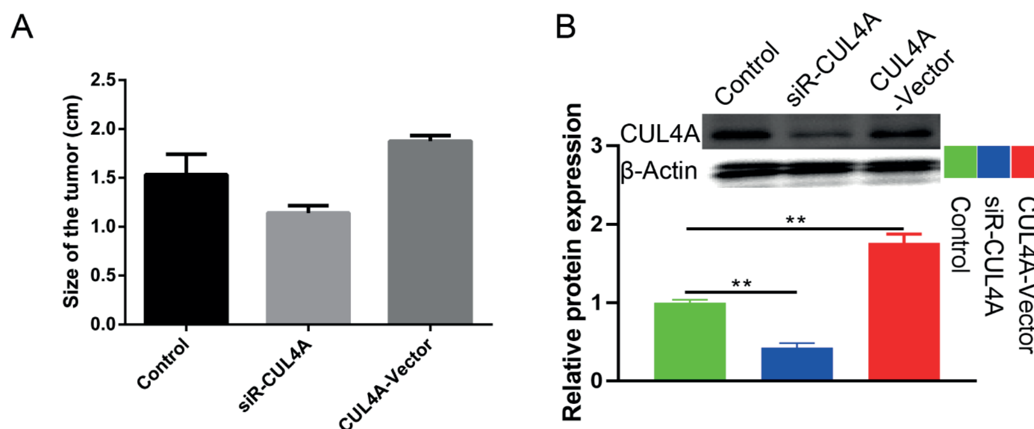

Figure 4. Effect of CUL4A on *in vivo* transplantation of CC cells. **A,** Tumor size at 30 d after *in vivo* transplantation of CC cells. **B,** Protein expression level of CUL4A in tumor cells measured by WB. **p<0.01: a significant difference between groups.

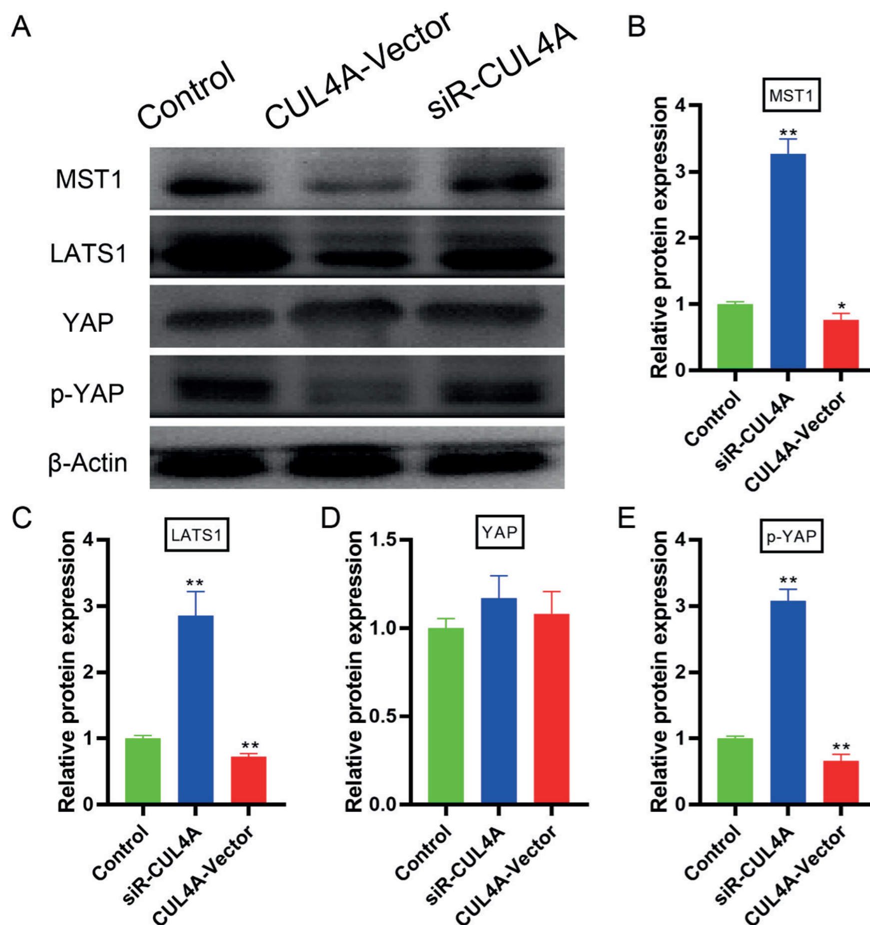

Figure 5. Regulatory relationship between CUL4A and Hippo pathway. **A,** Expressions of the Hippo pathway-related proteins MST1, LATS1, YAP and p-YAP detected through WB. **B-E,** Relative quantitative analysis of expression levels of MST1, LATS1, YAP and p-YAP. $*p<0.05$ & $**p<0.01$ *vs.* other groups, with significant differences.

more frequently in people under 50 years old[14]. CUL4A is a critical player in cell cycle regulation and genomic stability, whose dysregulated copies or expression may have a profound influence on cells. Besides, highly expressed CUL4A up-regulates the expressions of genes that probably cause tumorigenesis. CUL4A has been identified as a potential candidate gene for cancer progression[5]. For example, CUL4A acts as an oncogene in the progression of IL-6-induced colorectal cancer through ZEB1[15]. The JAK-STAT3 pathway triggers DICER1 to degrade proteasomes by the ubiquitin ligase complex of CUL4A-DCAF1, thus promoting CC development[16]. Li et al[7] have indicated that the protein expression of CUL4A in CC tissues is significantly higher than that in normal tissues. Patients with overexpressed CUL4A and protein accumulation suffer from relapse and die within a short time after surgery. However, EMT progression, proliferation, migration and invasion of CC cells *in vitro* and tumor growth *in vivo* can be prominently inhibited by knocking down CUL4A *via* siRNA. Yang et al[17] have shown that the growth of tumor cells is obviously inhibited by knocking down CUL4A, CUL 4B or damage-specific DNA-binding protein 1 (DDB1) both *in vitro* and *in vivo*. The proliferation, colony formation and invasion of CC cells decrease significantly through the destruction of the interaction between CUL4A and DDB1 by compound NSC1892. In this study, the results revealed that CUL4A was highly expressed in 66.67% CC tissues. After interference of CUL4A expression, the proliferation ability of CC cells was notably down-regulated *in vitro* and *in vivo*. However, the apoptosis rate was significantly promoted *in vitro*. However, overexpressed CUL4A facilitated cell proliferation and tumor formation *in vitro* and *in vivo*.

The Hippo kinase cascade is a growth inhibition pathway, which regulates tissue growth and cell fate. It consists of genes such as MST1/2, LATS1/2, protein salvador homolog 1 (SAV1) and Mps one binder (MOB). In the case of CC, the resistance of cells to 5-fluorouracil is enhanced by the uncontrolled Hippo pathway[18]. MST1/2 activated by upstream signaling factors binds to the regulatory protein SAV1, and phosphorylates LATS1/2 and MOB, further phosphorylating YAP. After that, phosphorylated YAP is retained in the cytoplasm after binding to the protein, thereby inhibiting transcription[19]. YAP has been recognized as an oncogene located in human cancer chromosome 11q22. Multiple studies have found that it is often overexpressed in common human cancers[20]. In addition, YAP is a downstream effector of the signal cascade of Hippo signaling pathway, which promotes gene expression by enhancing the activity of transcription factors. Meanwhile, it has been identified as a key regulator of cell proliferation and apoptosis in Drosophila[21]. Zheng et al[22] have illustrated that circPPP1R12A-73aa promotes tumorigenesis and metastasis of CC by activating the Hippo-YAP signaling pathway[22]. Reciprocal activation between YAP and NF-κB has been observed in human CC cells. Moreover, the survival rate of patients with high expressions of YAP and p-p65 is significantly reduced[23]. Based on the research on gastric cancer, CUL4A controls the expression of YAP, and overexpressed CUL4A can distinctly repress the phosphorylation of YAP. Additionally, YAP enters the nuclei to accelerate the proliferation of cancer cells. The expression of p-YAP is up-regulated in cancer cells with disturbed CUL4A expression, and the proliferative ability of cells is significantly reduced[24]. In the present study, the expressions of the Hippo pathway-related proteins were analyzed. The results revealed that there were regulatory relationships between CUL4A and MST1, LATS1 and p-YAP. After interfering in CUL4A expression by siRNA, the inhibition of the Hippo pathway was alleviated, and the expressions of MST1, LATS1 and p-YAP were promoted by cascade kinases. Furthermore, the role of YAP in entering the nucleus and promoting cell proliferation was reduced.

Conclusions

Summarily. the novelty of this study was that CUL4A is highly expressed in CC, which promotes CC cell proliferation and inhibits cell apoptosis by regulating the Hippo pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

Yantai Municipal Science and Technology Project (2015YD006); Shandong Province Medical and Health Science and Technology Project (2015WS0038).

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