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CUL4A promotes the invasion of cervical cancer cells by regulating NF-κB signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effects of cullin 4A (CUL4A) on promoting the proliferation and inhibiting the apoptosis of cervical cancer (CC) cells by regulating the nuclear factor-kappa B (NF-κB) signaling pathway.

PATIENTS AND METHODS: The protein expressions of CUL4A and NF-κB in 75 CC tissues were detected through immunohistochemistry. The correlation between the expressions of the two proteins in CC tissues was analyzed *via* Spearman's correlation test. Meanwhile, the prognostic significance of CUL4A expression for CC patients was analyzed by Kaplan-Meier curve. CUL4A small interfering ribonucleic acid (siRNA) was transfected into CC cells (HeLa) to downregulate the expression level of CUL4A. Subsequently, the effects of CUL4A on the proliferation and apoptosis of HeLa cells were detected by methyl thiazolyl tetrazolium (MTT) assay and flow cytometry, respectively. Finally, the effect of CUL4A on the activity of the NF-κB signaling pathway was analyzed through quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RESULTS: The protein expressions of CUL4A and NF-κB in CC tissues were significantly higher than those in normal tissues ($p<0.01$). The results of the survival curve showed that the prognosis of CC patients with highly expressed CUL4A is poor ($p<0.001$). Meanwhile, lowly expressed CUL4A protein significantly inhibited the proliferation and promoted the apoptosis of HeLa cells ($p<0.01$). QRT-PCR results indicated that the relative messenger RNA (mRNA) expression levels of downstream genes of the NF-κB signaling pathway were significantly lower in CC cells than those in the control group ($p<0.001$). In addition, CUL4A expression was positively correlated with NF-κB expression in CC ($p<0.001$).

CONCLUSIONS: CUL4A promotes the invasion of CC cells through the NF-κB signaling pathway.

Key Words:
CUL4A, Cervical cancer (CC), NF-κB signaling pathway.

Introduction

Cervical cancer (CC) is one of the most common gynecological malignancies, whose mortality is relatively high[1]. The WHO defines four major types of cervical epithelial tumors, namely, cervical squamous cell carcinoma, cervical adenocarcinoma, neuroendocrine tumor and other undifferentiated epithelial tumors. Among all types of CC, cervical squamous cell carcinoma and cervical adenocarcinoma account for 70-80% and 10-15%, respectively[2]. CC is the most popular disease among women after breast cancer. It is also a major public health problem in developing countries[3]. CC has great impacts on the female reproductive system and is easy to metastasize[4]. Multiple studies have identified that there is a definitive correlation between human papillomavirus (HPV) infection and CC. HPV infection of the cervical epithelium increases the risk of precancerous lesion, eventually leading to CC development[5]. Despite great progress in the diagnosis and treatment of CC, its recurrence rate continues to increase[6,7]. Therefore, it is important to identify the molecular mechanisms of CC progression for its clinical treatment in the near future.

Cullin 4A (CUL4A), a member of the cullin protein family, primarily regulates cellular deoxyribonucleic acid (DNA) replication, cell cycle and genomic instability. CUL4A is amplified and overexpressed in breast cancer and squamous cell carcinoma. Meanwhile, overexpression of CUL4A is associated with poor prognosis of node-negative breast cancer[8,9]. Therefore, it is speculated that CUL4A may be involved in the progression of malignant tumors as an oncogene. However, the biological functions and regulatory mechanisms of CUL4A in CC have not been fully elucidated.

The members of the nuclear factor-kappa B (NF-κB) family act as transcription factors and

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play an important role in resisting inflammation and repressing tumor development[10,11]. It is reported that NF-κB protein and other proteins associated with the NF-κB signaling pathway are involved in the regulation of cell proliferation, apoptosis, and angiogenesis[12]. Due to its physiological importance, NF-κB protein has been identified as a key integrator in immunity, inflammation and tumorigenesis. The cellular level of NF-κB has been observed significantly elevated in many human tumors. Song et al[13] have demonstrated that the expression level of NF-κB in CC tissues is remarkably higher than para-cancerous tissues. Meanwhile, the positive expression of NF-κB is associated with poor prognosis of CC patients. Furthermore, various studies[14-16] have verified that NF-κB signaling plays a crucial role in the development and progression of HPV positive CC. Ubiquitination modification serves as a key player in the activation of NF-κB signaling. CUL4A is a core component of the multifunctional E3 ubiquitin ligase complex. Therefore, the CUL4A/NF-κB signaling may participate in the development of CC. The aim of this study was to investigate the effects of CUL4A on the proliferation and apoptosis of CC cells by regulating the NF-κB signaling pathway.

Patients and Methods

Experimental Materials

This study was approved by the Ethics Committee of The First People's Hospital of Wenling. Signed written informed consents were obtained from all participants before the study. A total of 75 pairs of CC tissues and corresponding para-cancerous tissues were obtained from patients who received operation in our hospital from February 2016 to March 2018. The enhanced chemiluminescence (ECL) solution was purchased from Pierce Biotechnology (Rockford, IL, USA). CUL4A small interfering ribonucleic acid (siRNA) and negative control (NC) were provided by Shanghai GenePharma (Shanghai, China). The fetal bovine serum (FBS) was purchased from Gibco (Gaithersburg, MD, USA), and the complementary deoxyribose nucleic acid (cDNA) synthesis kit was offered by TransGen Biotechnology (Beijing, China).

Experimental Groups

75 pairs of CC tissues and corresponding non-tumor tissues were divided into two groups, including: CC group and Normal group. HeLa cells were exogenously transfected with CUL4A siRNA (CUL4A siRNA group) and NC (NC group), respectively.

Immunohistochemistry (IHC)

Paraffin-embedded CC tissue sections were deparaffinized and dehydrated in xylene and alcohol, respectively. Hydrogen peroxide was then added to block the peroxidase. Subsequently, tissue sections were boiled in citrate buffer for antigen retrieval and covered with diluent of primary antibody for incubation in a wet box. The next day, the wet box was taken out and placed for 20 min. After washing away the primary antibody, the tissues were incubated with secondary antibody droplets for 30 min. The color was developed using diaminobenzidine (DAB; Solarbio, Beijing, China), and the tissues were finally counterstained, dehydrated, and cleared.

Cell Transfection

HeLa cells grown to 30-40% of confluence were transfected with CUL4A siRNA and nonsense siRNA (NC). Transfection efficiency was verified by means of Western blotting and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

QRT-PCR

The whole extraction process of total RNA in cells was performed on ice in order to prevent degradation. Specifically, total RNA in cells was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was then reversely synthesized into cDNA. Next, 2 μL of cDNA was added into a total of 20 μL of reaction mixture and analyzed using a Bio-Rad CFX96™ qPCR system (Hercules, CA, USA). β-actin was used as an internal reference. Primer sequences used in this study were shown as follows: CUL4A-F: GGAAAGCACAGTGGTCGAA, CUL4A-R: GGGACACCTGGAATTCCTTC. Interleukin-8 (IL-8)-F: GGTGCAGTTTTGCCAAGGAG, IL-8-R: TTCCTTGGGGTCCAGACAGA. Matrix metalloproteinase-2 (MMP-2)-F: CAAGGAGAGCTGCAACCTGT, MMP-2-R: TCTGGGGCAGTCCAAAGAAC. MMP-9-F: GTACTCGACCTGTACCAGCG, MMP-9-R: AGAAGCCCCACTTCTTGTCG. Vascular endothelial growth factor (VEGF)-F: CTGGAGCGTGTACGTTGGT, VEGF-R: TTTAACTCAAGCTGCCTCGC. NF-κB-F: 5'-AGTTGAGGGGACTTTCCCAGGC-3', NF-κB-R: 5'-GCCTGGGAAAGTCCCCT-

CAACT-3'. B-actin-F: CCTAGGCACCAGGTGTGAT, β-actin-R: TTGGTGACAATGCCGTGTTC.

Detection of Cell Proliferation Via Methyl Thiazolyl Tetrazolium (MTT) Assay

Transfected cells were first seeded into 96-well plates. MTT diluent (Sigma-Aldrich, St. Louis, MO, USA) was added into each well, followed by 2 h of incubation in the dark. Absorbance at the wavelength of 490 nm was measured by a micro-plate reader after 0, 24, 48, 72 and 96 h of time points, respectively. Cell proliferation rate was finally calculated.

Western Blotting

Total proteins in CC tissues and transfected cells were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Next, target proteins were separated by gel under electric current and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After sealing, the membranes were incubated with primary antibodies overnight. On the next day, the membranes were incubated with secondary antibodies. Immuno-reactive bands were finally exposed using the ECL solution.

Detection of Cell Apoptosis Via Flow Cytometry

After HeLa cells were transfected with CUL4A siRNA and NC, cell apoptosis was observed in strict accordance with Annexin V-FITC apoptosis kit. The differences in cell apoptosis were analyzed *via* FACSCalibur flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Statistical Analysis

GraphPad Prism 8.0 (La Jolla, CA, USA) was used for statistical analysis and plotting. Experimental data were represented as mean ± Standard Deviation (SD). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $*p<0.05$ was considered statistically significant.

Results

CUL4A Expression Was Upregulated in CC Tissues

First of all, the changes in the protein expression of CUL4A in 75 pairs of CC tissues and adjacent normal tissues were examined by IHC staining. The results showed that CUL4A expression increased significantly in 56 (75%) CC tissues when compared with adjacent normal tissues ($**p<0.01$). Meanwhile, the survival time of CC patients with highly expressed CUL4A was remakably shorter than that of patients with lowly expressed CUL4A ($**p<0.01$). These results suggest that high expression of CUL4A is related to poor prognosis of CC patients ($***p<0.001$) (Figure 1).

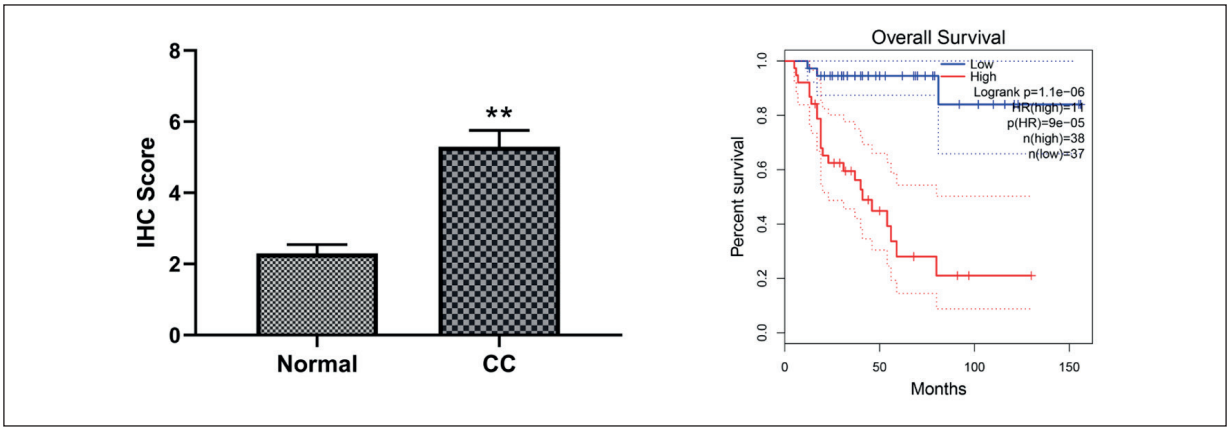

Figure 1. Expression of CUL4A in CC detected *via* IHC. **A,** Expression level of CUL4A in CC analyzed using IHC staining. The expression of CUL4A increased remarkably in CC group compared with Normal group ($**p<0.01$). **B,** Significance of CUL4A expression for prognosis of CC patients analyzed using survival curve. The overall survival rate of CC patients with highly expressed CUL4A was lowered ($***p<0.001$) ($**p<0.01$ *vs.* Normal group, $***p<0.001$ *vs.* Normal group).

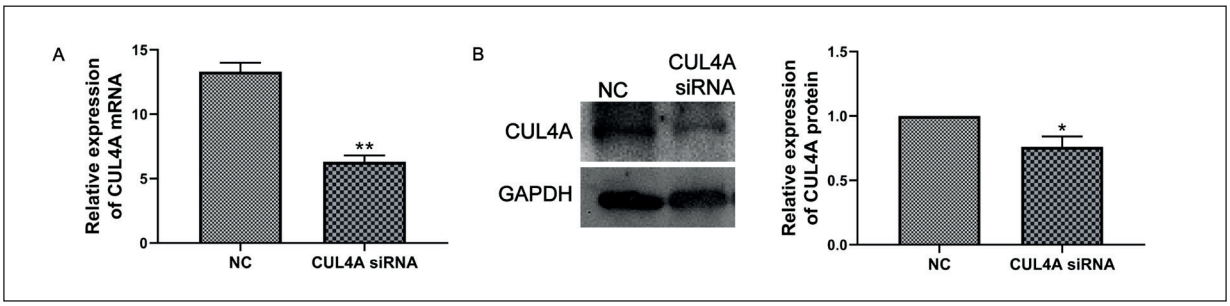

Figure 2. Verification of CUL4A expression in HeLa cells inhibited by CUL4A siRNA. **A,** Changes in mRNA expression of CUL4A in transfected cells. The mRNA expression of CUL4A in CUL4A siRNA group was lower than that in NC group (**p<0.01). **B,** Protein expression of CUL4A measured *via* Western blotting. CUL4A siRNA group exhibited a notably lower expression of CUL4A than NC group (*p<0.05) (*p<0.05 *vs.* NC group, **p<0.01 *vs.* NC group).

Specific siRNA Inhibited CUL4A Expression in HeLa Cells

The effects of CUL4A on CC cell biology were then investigated. HeLa cells were transfected with siRNA to downregulate the expression of CUL4A *in vitro*. The analysis results of Western blotting and qRT-PCR manifested that CUL4A siRNA transfection could markedly inhibit the protein and messenger RNA (mRNA) expression levels of CUL4A in HeLa cells (*p<0.05) (Figure 2).

Lowly Expressed CUL4A Repressed the Proliferation and Induced the Apoptosis of HeLa Cells

MTT assay and flow cytometry were applied to determine the influences of CUL4A knockdown on the proliferation and apoptosis of CC cells. The results of MTT assay indicated that absorbance at the wavelength of 490 nm decreased remarkably in CUL4A siRNA group in comparison with NC group (*p<0.05). Flow cytometry demonstrated that the percentage of apoptotic cells was evidently raised in CUL4A siRNA group compared with NC group. The above results suggest that knocking down CUL4A can partially inhibits the proliferation and promotes the apoptosis of CC cells (**p<0.01) (Figure 3).

CUL4A Protein Regulated the Expressions of Downstream Proteins of the NF-ĐB Signaling Pathway

The function of CUL4A in the NF-κB signaling pathway was further explored. It was indicated that the protein expression of NF-κB declined distinctly in CUL4A siRNA group (*p<0.05). Additionally, the mRNA expressions of downstream genes of the NF-κB signaling pathway, including VEGF, MMP-2, MMP-9 and IL-8, in cells with CUL4A downregulation were evaluat-

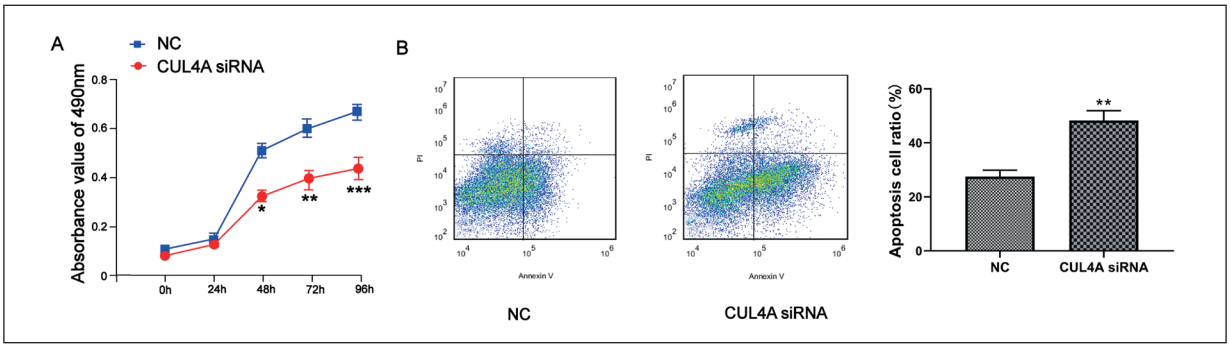

Figure 3. Impacts of CUL4A on CC cell proliferation and apoptosis. **A,** Changes in the proliferation of HeLa cells with low CUL4A expression detected by MTT assay. Absorbance at the wavelength of 490 nm decreased remarkably in CUL4A siRNA group in comparison with NC group (*p<0.05). **B,** Change in apoptosis of HeLa cells with low CUL4A expression determined *via* flow cytometry. The percentage of apoptotic cells was raised evidently in CUL4A siRNA group compared with that in NC group (**p<0.01) (*p<0.05 *vs.* NC group, **p<0.01 *vs.* NC group).

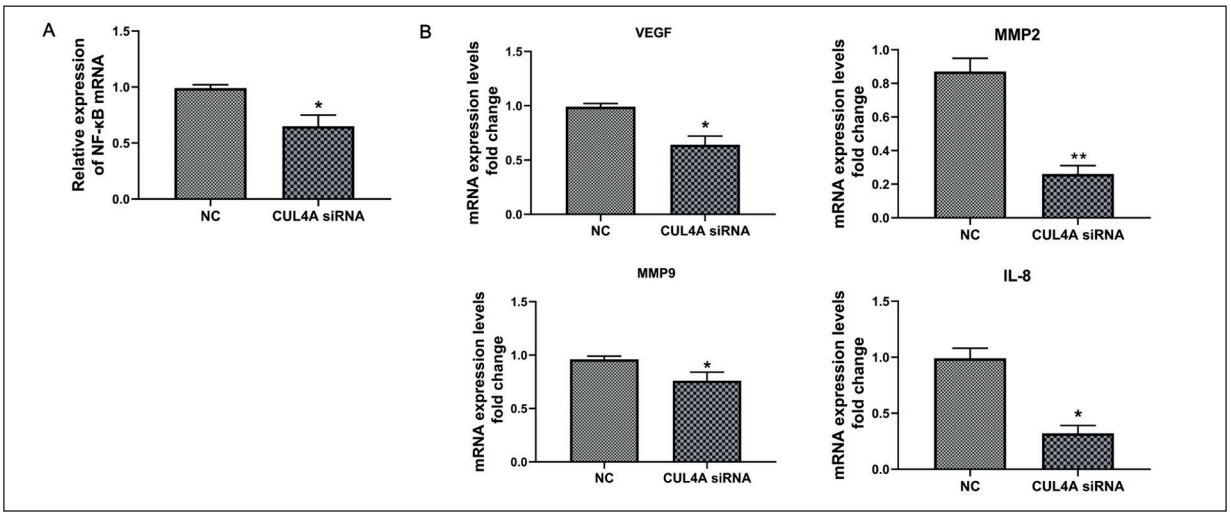

Figure 4. Effects of CUL4A on the NF-κB signaling pathway. **A,** Changes in the protein expression of NF-κB in cells transfected with CUL4A siRNA. The expression of NF-κB declined distinctly in CUL4A siRNA group in contrast with NC group (**p*<0.05). **B,** Expressions of downstream genes VEGF, MMP-2, MMP-9 and IL-8 of NF-κB analyzed using qRT-PCR. Compared with NC group, CUL4A siRNA group had prominently lowered mRNA expression levels of VEGF, MMP-2, MMP-9 and IL-8 (**p*<0.05) (**p*<0.01 *vs.* NC group).

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CUL4A Protein Regulated CC Cell Proliferation and Apoptosis Through the NF-ÐB Signaling Pathway

The expression of NF-κB in 75 CC tissues was examined by means of IHC. According to the results, there was more evident staining of NF-κB protein in CC group than that in Normal group, implying that NF-κB protein was upregulated in CC tissues (**p*<0.05). Besides, Spearman's correlation test indicated that the protein expression of CUL4A was positively correlated with that of NF-κB in CC (****p*<0.001) (Figure 5).

Discussion

In the present study, the role of ubiquitin ligase CUL4A in CC cells was investigated. The experimental results verified that CUL4A expression

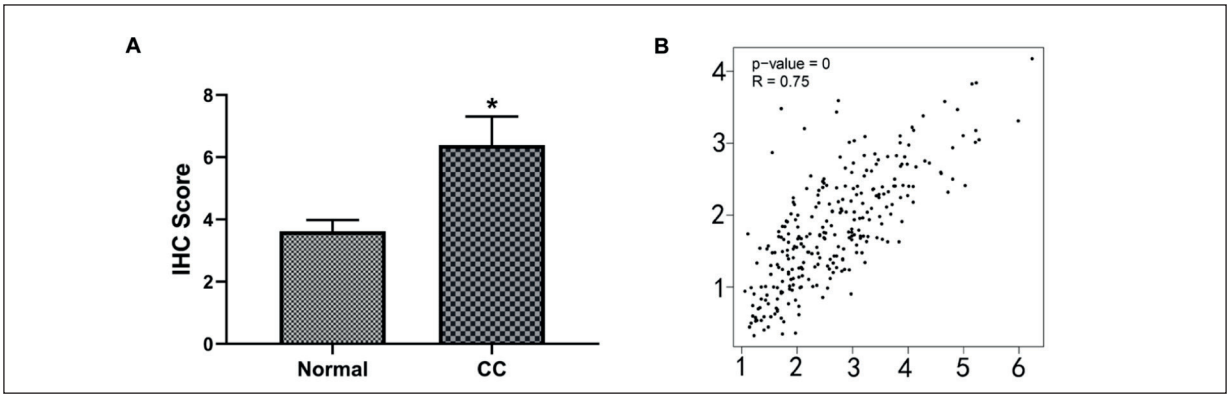

Figure 5. Protein expression of NF-κB in CC tissues. **A,** Difference in the protein expression of NF-κB in CC tissues and normal tissues detected *via* IHC. The protein expression of NF-κB in CC group was significantly higher than that in Normal group (**p*<0.05). **B,** Correlation between the expressions of CUL4A and NF-κB in CC tissues. The protein expression of CUL4A had a significantly positive correlation with that of NF-κB (****p*<0.05) (**p*<0.05 *vs.* NC group, ****p*<0.001 *vs.* NC group).

rose remarkably in CC tissues. The downregulation of CUL4A could remarkably facilitate the apoptosis and repress the proliferation of CC cells. CUL4A was able to activate the protein expression of NF-κB, moreover, the knockdown of CUL4A might weaken the expressions of NF-κB and its downstream genes (e.g., MMP-2, MMP-9 and IL-8) in CC cells. In addition, the expressions of CUL4A and NF-κB in CC tissues were measured. It was found that CC group displayed overexpressed CUL4 and NF-κB in comparison with Normal group. Based on the analysis of IHC score, the expression of CUL4A was positively correlated with NF-κB expression. All these results illustrate that the dysregulation of CUL4A participates in the proliferation and apoptosis of CC cells *via* the NF-κB signaling pathway.

Schindl et al[17] have demonstrated that the overexpression of CUL4A is associated with the overall survival and disease-free survival of breast cancer patients. Similarly, it has been discovered that CUL4A expression is significantly upregulated in gastric cancer (GC) tissues and cell lines. Meanwhile, CUL4A is correlated with poor prognosis of GC patients[18]. High-level of CUL4A expression has also been detected in other types of tumors. Therefore, it can be inferred from previous studies that CUL4A is a vital player in the proliferation and apoptosis of malignant tumors. In early studies, CUL4A is regarded as a potential oncogene based on the ability to ubiquity late and degrade several well-known tumor suppressor genes, such as p21, p27, DDB2 and p53[19,20]. For example, Li et al[21] have denoted that ubiquitin ligase CUL4A degrades p27 in a targeted manner and accelerates the proliferation of erythroid progenitor cells and proerythroblasts. In this study, it was testified that exogenous reduction of CUL4A expression significantly inhibited the proliferation but induced the apoptosis of CC cells by suppressing the expressions of NF-κB and downstream genes of the NF-κB signaling pathway. To our knowledge, it was reported for the first time in our study that CUL4A might be implicated in the proliferation and apoptosis of CC cells by regulating the NF-κB signaling pathway.

Usually, the recurrence rate is high after cancer treatment, so is that of CC. One reason for the relapse is radiation. High-level radiation activates another NF-κB signaling pathway, and NF-κB is an important transcription factor that plays a role in inhibiting inflammatory responses and malignant phenotypes of tumor cells. Moreover, NF-κB affects the physiological function of cells and participates in the regulation of apoptosis, proliferation, and angiogenesis[22]. In this study, it was found that the protein expression of NF-κB was significantly upregulated in CC tissues and was positively correlated with CUL4A expression. Besides, the expressions of NF-κB and downstream genes of the NF-κB signaling pathway, such as MMP-2, MMP-9 and IL-8, decreased and were significantly inhibited after downregulation of CUL4A. MMPs belong to the endopeptidase family, which play an important role in the degradation of extracellular matrix. Meanwhile, they are associated with the invasive and metastatic phenotypes of tumors[23]. In addition, IL-8 from monocytes, macrophages and endothelial cells is an effective pro-inflammatory chemokine that promotes the adhesion, migration, invasion and chemoresistance of CC cells[24,25]. The above experimental results suggest that the CUL4A/NF-κB signaling pathway is involved in the migration and invasion of CC cells, which requires further confirmation.

Conclusions

Shortly, it is demonstrated in the present study that CUL4A promotes the proliferation and inhibits the apoptosis of CC cells by activating the NF-κB signaling pathway. The novelty of this study was that targeting the CUL4A/NF-κB signaling pathway may be a promising treatment strategy for CC. Moreover, CUL4A is probably a biomarker and therapeutic target for predicting CC progression, but its clinical value still needs to be verified by a large number of CC samples.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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