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ORIGINAL ARTICLE

CUL4B/KLF11 axis promotes malignant ovarian cancer progression

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Summary

Purpose: *This study aimed to explore the role and the molecular mechanism of CUL4B in the occurrence and development of ovarian cancer (OC).*

Methods: *The expressions of CUL4B in 20 pairs of OC tissue specimens and cell lines was detected by quantitative real-time polymerase chain reaction (qRT-PCR). Subsequently, the CUL4B knockdown and overexpression models were constructed in OC cell lines SKOV3 and A2780 using lentivirus, respectively. Transwell and cell scratch assays were used to analyze the effect of CUL4B on OC cell function. Furthermore, luciferase reporter gene experiment and rescue experiments were performed to explore the potential mechanism of CUL4B and downstream gene KLF11.*

Results: *The expressions of CUL4B in OC tissues was markedly higher than that of adjacent ones. Transwell and cell scratch experiments suggested that CUL4B overexpression markedly promoted the invasive and metastasis ability of OC cells. Bioinformatics and luciferase reporter gene experiments suggested that CUL4B could directly target KLF11. Rescue experiments indicated that KLF11 knockdown can reverse the inhibiton of CUL4B silencing on OC cell migration and wound healing ability.*

Conclusions: *CUL4B expression is markedly elevated in OC tissues and cell lines. CUL4B may negatively regulate the expression of KLF11, thereby enhancing the proliferative and metastatic ability of OC. In summary, CUL4B plays an important part in the progression of OC, and might serve as a new potential target and prognostic indicator for ovarian cancer treatment.*

Key words: *CUL4B, KLF11, ovarian cancer, malignant progression*

Introduction

Ovarian cancer (OC) is the gynecologic malignant tumor with the highest mortality rate. In the United States, there are over 22,000 new cases of OC annually, and more than 14,000 patients die of OC each year [1-3]. In China, there are about 52,000 new OC patients every year, and about 23,000 patients die every year [4,5]. Since the ovary is located deep in the pelvis and lacks specific clinical symptoms, only 15% of patients can be diagnosed in stage I, so more than 70% of patients are already in advanced stage (stage III or IV) when diagnosed, and the 5-year relative survival rate is only 29% [6,7]. Although these patients with advanced OC have undergone active tumor resection surgery as well as platinum-based chemotherapy, still, the 5-year survival rate is less than 25% [8]. Of all the OC, 85-90% are epithelial ovarian cancers (EOCs) and its etiology is unknown [9]. At present, EOCs are considered to have genetic and histopathologic heterogeneity in space and time [9,10]. With the deepening of ovarian tumor research, the development and application of new biomolecular technology has provided unprecedented research conditions for the discovery of the full picture of EOCs [10,11]. Molecular diagnosis and treatment can specifically target the mutations in tumor cells.

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By evaluating gene mutations of EOC subtypes, the efficacy of molecular targeted therapy can be predicted [12,13].

CUL4B belongs to the Cullin family. It is an important framework protein of ubiquitin E3 ligases (CRLs). CRLs are the largest class of ubiquitin ligases at present, which can degrade proteins in the body through the ubiquitin-proteasome pathway and are involved in many cell activities such as cell signal transduction, cycle regulation and DNA repair [14,15]. At present, the function of CUL4B is increasingly valued in tumors, and researchers believe it will be a novel carcinogen [16,17]. Previous evidence showed that CUL4B was highly expressed in cholangiocarcinoma, gastric cancer and prostate cancer, and can promote tumor invasion [17-19]. However, the study of CUL4B in tumors has just started, and it has not been reported whether CUL4B could play a certain role in OC. Therefore, we analyzed the expression of CUL4B in OC and its relationship with clinicopathologic characteristics and prognosis using clinical tissue specimens and cell models. Besides, we conducted preliminary search on the function and potential molecular mechanism of CUL4B in OC using bioinformatics data. We hoped to provide new ideas for stratification of OC, screening of patients with poor prognosis and treatment of aggressive tumors.

Methods

Patients and OC tissues

20 cases of surgically resected OC tissues and normal tissues were collected in The First Hospital of Hangzhou Fuyang. All specimens were stored at -80°C. All enrolled patients were diagnosed by postoperative pathologic analysis, and had not received any anti-tumor therapy before surgery. This study was approved by the Ethics Committee of The First Hospital of Hangzhou Fuyang. Besides, all patients had signed an informed consent form.

Cell lines and reagents

Human ovarian cancer cells 3AO, A2780, CAOV3 and SKOV3 and a normal human ovarian surface epithelial cells HOSEPiCs were purchased from American Type Culture Collection (ATCC) company (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a 37°C, 5% CO_2 incubator.

Transfection

The blank control group (sh-NC or NC) and the lentivirus (sh-CUL4B or CUL4B) containing CUL4B overexpression or knockdown sequences were purchased from Shanghai Jima (Shanghai, China). The cells were plated in 6-well plates and cultured until the cell density reached 30-50%. Transfection was performed according to the manufacturer instructions. After 48 h, the cells were collected for subsequent experiments.

Transwell cell migration assay

The serum-free medium diluted transfected cells (5×10^5) were inoculated into the upper layer of the matrix gel-containing chamber, and 700 µL of 20% FBS medium was placed as a chemical attractant in the lower compartment of the chamber. The transwell was incubated for indicated time, then the lower layer penetrating cells were collected, fixed with methanol for 15 min, and stained with 0.2% crystal violet for 20 min. After washed with phosphate buffered saline (PBS), cells were subjected to penetrating cell counting in 10 random fields.

Cell wound healing assay

A horizontal and a vertical line was drawn across on the back of the sterilized 6-well plates. A single-cell suspension of 5×10^5 cells was prepared and seeded in 6-well plates. When the cells were 90% confluent, the inner surface of cells were scratched with a 10 µL sterile

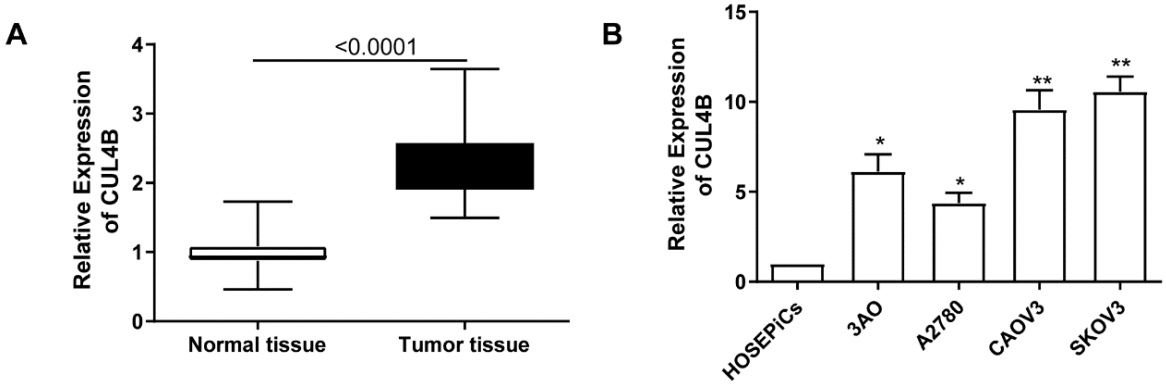

Figure 1. CUL4B is highly expressed in ovarian cancer (OC) tissues and cell lines. **A:** Relative expression of CUL4B in OC tissues compared with normal tissues. CUL4B expression was examined by qPCR and normalized to GAPDH expression. **B:** Analysis of CUL4B expression levels in OC cell lines (3AO, A2780, CAOV3 and SKOV3) compared with the normal human ovarian surface epithelial cells (HOSEPiCs) by qRT-PCR; Data are average ± SD, *p<0.05, **p<0.01.

pipette perpendicular to the horizontal line, and then washed twice with PBS to remove the scratches. 2 mL of 1% FBS medium was added per well. After taking photos, cells were then placed in the incubator for 24 h. Then, the crawling state of the cells was compared and recorded.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from OC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the RNA was reverse transcribed into complementary DNA (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Japan). QRT-PCR reaction was performed using SYBR® Premix Ex Taq ™ (TaKaRa, Otsu, Japan), and StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The following primers were used in the qRT-PCR reaction (5'-3'): CUL4B: F: GCTGGCAAAACCATTGCAGA, R: CCATGTCCCAAATGGAGGGT; KLF11: F: CCGCGCCGGTTGACAT, R: TGACCCCAGGAGCTCATACA; GAPDH: F: CCTGGCACCCAGCACAAT, R: GCTGATCCACATCTGCTGGAA.

ABI Step One software (Applied Biosystems, Foster City, CA, USA) was used for data analysis, and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression level.

Western blot

Cells were lysed using cell lysate radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China). 5 × loading buffer was added into total protein lysate at the ratio of 1:4, then the protein was boiled for 5-10 min. 20 μg of protein were subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After incubating with blocking solution for 1 h, the membranes were added with the primary antibody (CUL4B, KLF11, GAPDH) and incubated at 4°C overnight. Then, the membranes were added with the horse radish peroxidase (HRP)-labeled secondary antibody, and the electrochemiluminescence (ECL) chemiluminescence detection kit was examined for luminescence.

Dual-luciferase reporter assay

After mutating the binding site of CUL4B and KLF11, wild-type CUL4B and mutant CUL4B were cloned into pMIR. OC cells were seeded in 24-well plates and co-transfected with pcDNA3.1-KLF11 and WT CUL4B or mutant pMIR luciferase reporter plasmids. 48 h after

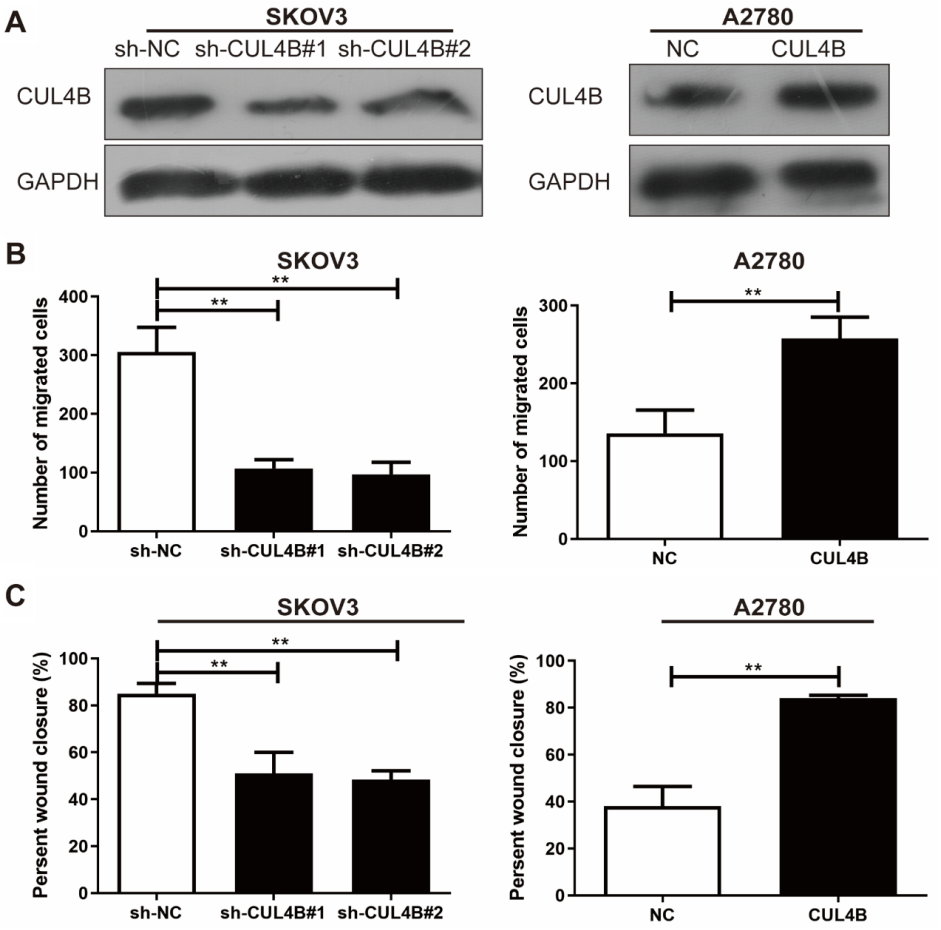

Figure 2. CUL4B can promote the metastatic ability of ovarian cancer (OC) cells. **A:** Western blot verification of CUL4B expression after transfection of CUL4B knockdown or overexpression in OC cell lines SKOV3 and A2780. **B:** Representative results of Transwell migration assays in OC cell lines SKOV3 and A2780 transfected with sh-CUL4B or CUL4B. **C:** Cell scratch test was used to detect the healing ability of cells transfected with sh-CUL4B or CUL4B. Data are average ± SD, **p<0.01.

transfection, the dual luciferase reporter assay system (Promega, Madison, WI, USA) was used to normalize the reporter luciferase activity to the control firefly luciferase activity.

Statistics

Continuous variables were analyzed using *t*-test, and categorical variables were analyzed using x^2 test or Fisher's exact probability method. Kaplan-Meier method was used to evaluate the prognostic survival time of patients, and log-rank test was used to compare the differences between different curves. SPSS 22.0 software (IBM, Armonk, NY, USA) was used to process the program, and the data were expressed as mean ± standard deviation. P<0.05 was considered statistically significant.

Results

CUL4B was highly expressed in ovarian cancer tissues and cell lines

Firstly, we detected the expression of CUL4B in 20 pairs of OC tissues and normal tissues by qRT-PCR, which suggested that CUL4B expression was up-regulated in OC tissues (Figure 1A). In addition, compared with normal stromal cells (HOSEPiCs), CUL4B was markedly overexpressed in OC cell lines (Figure 1B). Therefore, CUL4B might be used as a new biological prognosis indicator of OC.

CUL4B promoted the migration and invasion in ovarian cancer cells

To explore the functional effect of CUL4B in OC cells, the CUL4B overexpression and knockdown models were first constructed in SKOV3 and A2780, and the transfection efficiency was verified by Western Blot and q-PCR assays (Figure 2A). Subsequently, Transwell experiments showed that knockdown of CUL4B remarkably reduced the number of transmembrane OC cells while overexpression could increase the cell migration ability of OC cells (Figure 2B). The cell scratch assay also revealed that the healing ability of OC cells

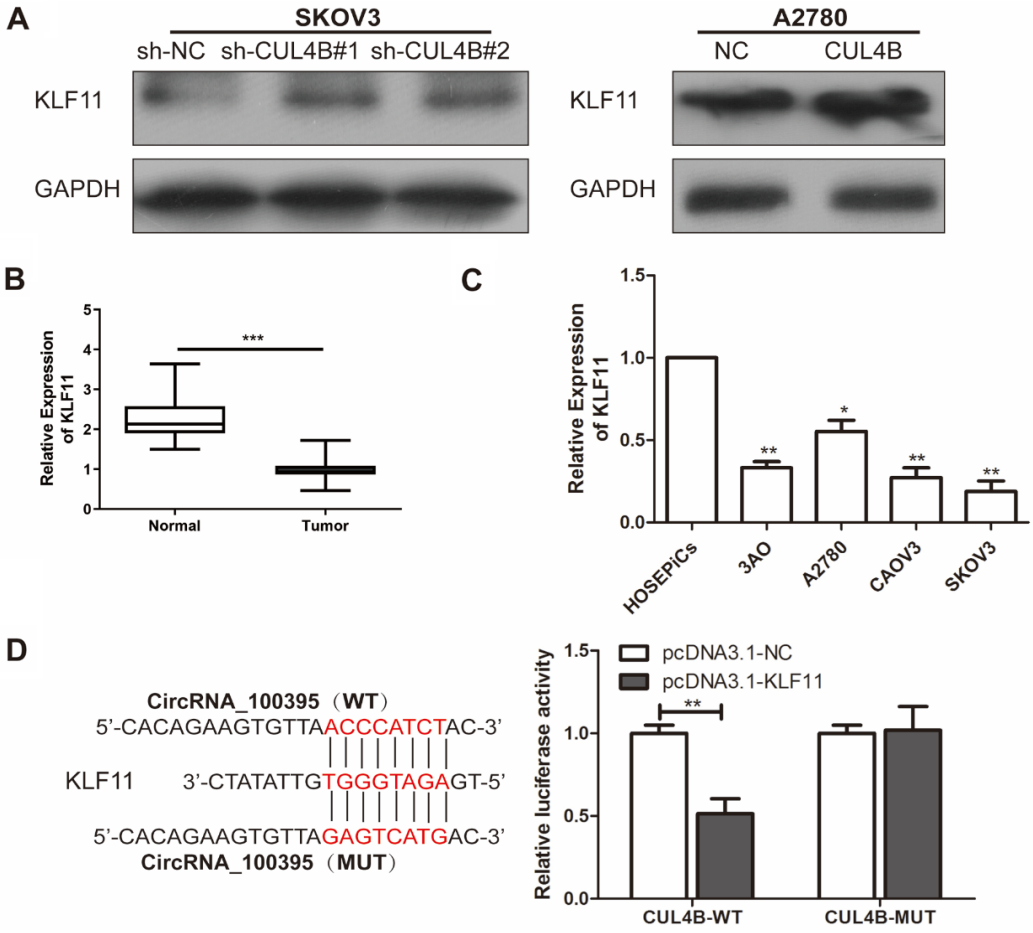

Figure 3. CUL4B can regulate KLF11. **A:** Western blot verification of the expression level of KLF11 protein after transfection of CUL4B knockdown or overexpression in ovarian cancer (OC) cell lines SKOV3 and A2780. **B:** qRT-PCR detection of KLF11 expression in tumor tissues and normal tissues in patients. **C:** The expression levels of KLF11 in OC cell lines detected by qRT-PCR. **D:** Luciferase reporter gene experiment was performed to evaluate the binding between CUL4B and KLF11. Data are average ± SD, *p<0.05, **p<0.01, ***p<0.001.

was markedly reduced after CUL4B knockdown, whereas CUL4B overexpression significantly increased the healing ability of OC cells (Figure 2C). Those above results suggested that CUL4B could promote the migration ability and invasion in OC cells.

CUL4B was bound to KLF11

The bioinformatics website suggested that CUL4B can be targeted to KLF11.Therefore, we detected the protein levels of KLF11 after knockdown and overexpression of CUL4B in OC cells. The results showed that KLF11 was remarkably increased after CUL4B knockdown, while CUL4B overexpression could markedly reduce the KLF11 level (Figure 3A). Moreover, qRT-PCR analysis of OC tissues and normal ones revealed that KLF11expression was markedly reduced (Figure 3B). In addition, KLF11 expression was also down-regulated in OC cells (Figure 3C). Further luciferase reporter gene experiment indicated that CUL4B can be targeted by KLF11 through a specific binding site (Figure 3D).

KLF11 modulated CUL4B in ovarian cancer cell lines

To further explore the regulation between CUL4B and KLF11 in OC cells, we transfected sh-KLF11 or KLF11 in CUL4B-knockdown or CUL4B-

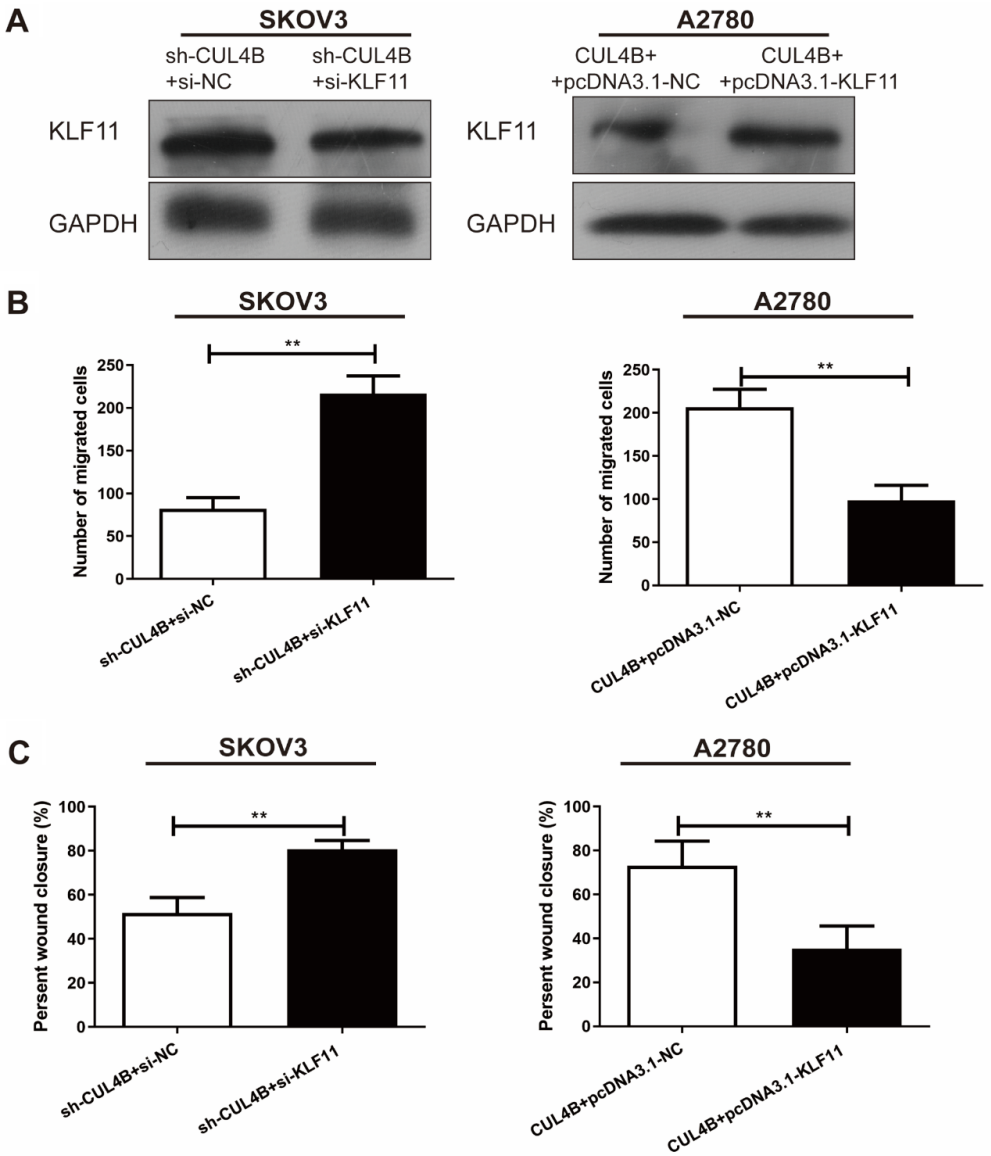

Figure 4. CUL4B can regulate the mechanism of action of KLF11 in ovarian cancer (OC) cell lines. **A:** Western blot detection of KLF11 expression levels after co-transfection of CUL4B and KLF11 knockdown or overexpression vectors in OC cell lines SKOV3 and A2780. **B:** Representative results of Transwell migration assays in in OC cell lines SKOV3 and A2780 after co-transfection of CUL4B and KLF11 knockdown or overexpression vectors. **C:** Cell scratch test was used to detect the cell healing ability after co-transfection of CUL4B and KLF11 knockout in OC cell lines SKOV3 and A2780. Data are average ± SD, **p<0.01.

overexpression cells, respectively (Figure 4A). Subsequently, Transwell assays demonstrated that knockdown of KLF11 can counteract the inhibitory effect of CUL4B knockdown on OC cell migration, while overexpression of KLF11 can reverse the promotion effect of overexpression of CUL4B on migration of OC cells (Figure 4B). Similarly, cell scratch test showed that the decreased healing ability of OC cells caused by knocking down CUL4B can be reversed by knocking down KLF11 and *vice versa* (Figure 4C). In sum, we concluded that KLF11 modulated CUL4B in OC.

Discussion

OC is the fourth most common cause of cancer deaths among women in developed countries [1-3]. Due to the hidden onset of OC patients and the lack of early diagnosis indicators, about 75% of patients have already experienced distant metastasis when diagnosed [3,4]. Although 75% of patients with advanced OC have undergone cytoreductive surgery and platinum-based chemotherapy and other effective treatments, and the cancer has achieved clinical remission, most patients will experience disease recurrence and eventually death. The 5-year survival rate of OC is about 30% [4-6]. The degree of peritoneal spread is related to the poor prognosis of patients with advanced OC [6-8]. The molecular mechanism by which OC cells invade and regenerate on the peritoneal surface has not been thoroughly studied [7,8]. Therefore, a better understanding of the molecular events leading to tumor invasion and metastasis is crucial for the development of new OC treatment strategies [8-13].

CUL4B belongs to the Cullin family and participates in the formation of the largest number of ubiquitin ligases in the human body, Cullin-RING ubiquitin ligase [14,15]. A number of studies in recent years have shown that CUL4B can regulate tumorigenesis and development of various tumors through epigenetic mechanisms [16-19]. This study explored the levels of CUL4B in OC tissues as well as cell lines, and also explored the effect of CUL4B on the development of OC. Firstly, CUL4B expression was verified in 20 pairs of OC tissues and normal ones, which showed that CUL4B expression was markedly up-regulated, suggesting that CUL4B may serve as an oncogene in OC. Metastasis of OC is a complex process involving multiple genes and steps. At present, the molecular mechanism of OC invasion and metastasis has not been thoroughly studied [20,21]. In order to further explore the impact of CUL4B on oc cell function, lentivirus was used to construct CUL4B overexpression or knockdown expression models. Transwell and cell scratch experiments showed that CUL4B can promote the invasion and metastasis ability of OC, but its specific molecular mechanism remained still unclear.

In order to further elucidate the underlying molecular mechanism of CUL4B functioning, we used bioinformatics software to predict the potential targets of CUL4B and discovered KLF. The KLF family regulates the function of cells by controlling the expression of genes, including cell proliferation, apoptosis, differentiation, and the generation of new organisms [22]. Several studies have shown that KLF11, a member of the KLF family, participates in and regulates cell proliferation, cell cycle, and apoptosis in various life processes [23,24]. In this experiment, the expression level of KLF11 protein was markedly increased after CUL4B knockdown and the expression level of KLF11 protein was markedly decreased after CUL4B overexpression. The luciferase reporter gene experiment results showed that CUL4B can be targeted by KLF11 through a specific binding site. Through rescue experiments, it was found that knocking down or overexpressing KLF11 can counteract the biological effect of CUL4B knocking down or overexpressing in OC cells. With the deepening of research, further understanding of the biological function of CUL4B and KLF11 genes and their role in the development of OC will be more conducive to the diagnosis, treatment and prognosis assessment of OC.

Conclusions

CUL4B is highly elevated in OC tissues as well as in cell lines. CUL4B may negatively regulate the expression of KLF11, thereby enhancing the proliferative and metastatic ability of OC. In summary, CUL4B is involved in the occurrence and development of OC, and may become a new potential target and prognostic indicator for OC treatment.

Conflict of interests

The authors declare no conflict of interests.

References

1. Li G, Zhang K, Gong F, Jin H. A study on changes and clinical significance of blood glucose, blood lipid and inflammation in patients with ovarian cancer. JBUON 2019;24:2322-6.
2. Hunn J, Rodriguez GC. Ovarian cancer: etiology, risk factors, and epidemiology. Clin Obstet Gynecol 2012;55:3-23.
3. Lukanova A, Kaaks R. Endogenous hormones and ovarian cancer: epidemiology and current hypotheses. Cancer Epidemiol Biomarkers Prev 2005;14:98-107.
4. Li L, Ji J, Wang JB, Niyazi M, Qiao YL, Boffetta P. Attributable causes of breast cancer and ovarian cancer in China: reproductive factors, oral contraceptives and hormone replacement therapy. Chin J Cancer Res 2012;24:9-17.
5. Zheng C, Yang R. RCD24, B7-H4 and PCNA expression and clinical significance in ovarian cancer. JBUON 2019;24:715-9.
6. Ozols RF. Management of advanced ovarian cancer consensus summary. Advanced Ovarian Cancer Consensus Faculty. Semin Oncol 2000;27:47-9.
7. Berek JS, Bertelsen K, du Bois A et al. Advanced epithelial ovarian cancer: 1998 consensus statements. Ann Oncol 1999;10 (Suppl 1):87-92.
8. Kochli OR. [Ovarian cancer. Screening, therapy and follow-up]. Gynakol Geburtshilfliche Rundsch 1994;34:123-9.
9. Risch HA. Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. J Natl Cancer Inst 1998;90:1774-86.
10. Bapat SA, Mali AM, Koppikar CB, Kurrey NK. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 2005;65:3025-9.
11. Provencher DM, Lounis H, Champoux L et al. Characterization of four novel epithelial ovarian cancer cell lines. In Vitro Cell Dev Biol Anim 2000;36:357-61.
12. Boljevic I, Malisic E, Milovic KM, Jovanic I, Jankovic R. Expression levels of genes involved in cell adhesion and motility correlate with poor clinicopathological features of epithelial ovarian cancer. JBUON 2020;25:1911-7.
13. Yang S, Si L, Jia Y et al. Kaempferol exerts anti-proliferative effects on human ovarian cancer cells by inducing apoptosis, G0/G1 cell cycle arrest and modulation of MEK/ERK and STAT3 pathways. JBUON 2019;24:975-81.
14. Vulto-van SA, Nakagawa T, Bahi-Buisson N et al. Variants in CUL4B are associated with cerebral malformations. Hum Mutat 2015;36:106-17.
15. Ravn K, Lindquist SG, Nielsen K, Dahm TL, Tumer Z. Deletion of CUL4B leads to concordant phenotype in a monozygotic twin pair. Clin Genet 2012;82:292-4.
16. He YM, Xiao YS, Wei L, Zhang JQ, Peng CH. CUL4B promotes metastasis and proliferation in pancreatic cancer cells by inducing epithelial-mesenchymal transition via the Wnt/beta-catenin signaling pathway. J Cell Biochem 2018;119:5308-23.
17. Qi M, Jiao M, Li X et al. CUL4B promotes gastric cancer invasion and metastasis-involvement of upregulation of HER2. Oncogene 2018;37:1075-85.
18. Zhao M, Qi M, Li X et al. CUL4B/miR-33b/C-MYC axis promotes prostate cancer progression. Prostate 2019;79:480-8.
19. Li P, Zhang L, Yang M, Qi M, Jin X, Han B. Cul4B is a novel prognostic marker in cholangiocarcinoma. Oncol Lett 2017;14:1265-74.
20. Lengyel E. Ovarian cancer development and metastasis. Am J Pathol 2010;177:1053-64.
21. Naora H, Montell DJ. Ovarian cancer metastasis: integrating insights from disparate model organisms. Nat Rev Cancer 2005;5:355-66.
22. Zhang D, Zhang XL, Michel FJ, Blum JL, Simmen FA, Simmen RC. Direct interaction of the Kruppel-like family (KLF) member, BTEB1, and PR mediates progesterone-responsive gene expression in endometrial epithelial cells. Endocrinology 2002;143:62-73.
23. Ellenrieder V, Buck A, Harth A et al. KLF11 mediates a critical mechanism in TGF-beta signaling that is inactivated by Erk-MAPK in pancreatic cancer cells. Gastroenterology 2004;127:607-20.
24. Buck A, Buchholz M, Wagner M, Adler G, Gress T, Ellenrieder V. The tumor suppressor KLF11 mediates a novel mechanism in transforming growth factor beta-induced growth inhibition that is inactivated in pancreatic cancer. Mol Cancer Res 2006;4:861-72.