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Overexpression of CUL4B is a novel predictor of prognosis for endometrial adenocarcinoma

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Summary

Purpose: It is reported that Cullin 4B (CUL4B) plays a crucial role in many physiological process. This study for the first time investigated the role of CUL4B in endometrioid adenocarcinoma (EAC). *Materials and Methods:* CUL4B mRNA and protein expression in EAC tissues and normal endometrial (NE) tissues were examined by real-time PCR and immunohistochemistry. The authors also explored the correlation between CUL4B protein expression and clinicopathological characteristics. Moreover, univariate and multivariate Cox regression analyses and Kaplan–Meier survival analyses were performed to investigate the association between TRIM62 expression and EAC patients' prognosis. *Results:* CUL4B was markedly overexpressed in EAC at both mRNA and protein levels. Immunohistochemistry assays showed that high CUL4B expression was significantly correlated with FIGO stage ($p = 0.008$) and histological grade ($p = 0.002$). Univariate and multivariate analyses revealed that CUL4B was an independent poor prognostic factor for overall and disease-free survival of EAC patients ($p < 0.05$). Furthermore, survival analyses displayed that patients with high CUL4B expression had poor prognosis. *Conclusion:* The present results demonstrated that elevated CUL4B was associated with poor outcomes of patients with EAC. CUL4B may serve as a novel prognostic marker and therapeutic target for EAC.

Key words: CUL4B; Endometrial carcinoma; Prognosis.

Introduction

Endometrial carcinoma (EC) is one of the most common malignant tumors in the female reproductive system, and ranks fourth in the female malignant tumors which is behind breast, lung, and colorectal cancers [1, 2], with 49,000 new cases reported each year in the United States. In developed countries the incidence of EC takes the first place in gynecological tumors, and it is estimated that the incidence of EC in Europe and North America is ten times higher than that in developing countries [3]. The incidence of EC in developing countries is lower than the developed countries, but the mortality rate is higher than that of developed countries [4-6]. In recent years,due to the changes in lifestyle and environmental factors, the incidence of endometrial cancer throughout the world has a rising trend, and is diagnosed at an increasingly young age [7, 8].

Endometriod adenocarcinoma (EAC) is the main pathological type of EC, accounting for more than 90%. Despite the rapid advancement of surgical and chemotherapy technologies, there are still about 20% of patients that relapse after treatment, with 80% of the five-year survival rate [9]. Although a growing number of abnormal expressed genes contributing to different prognoses in EAC have been reported,the specific etiology and mechanism remains unclear. It is considered urgent to identify novel molecular markers for early prognosis and better prediction about severity of EAC as potential targets.

The ubiquitin-proteasome system was first detected by Ciechanover *et al.*, Hershko *et al.*, and Ciehanover *et al.* [10-12], involving multiple physiological processes including growth, transcription, signal transduction, cell cycle, and DNA repair through selective degradation of target proteins [13]. It contains ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin-protein ligase(E3), and proteasome [14]. Cullin protein constitutes the skeleton structure of the ubiquitin-protein ligases complexes E3 (Cullin-RING ubiquitin ligases, CRLs) which are the key enzymes for selecting degradation of target proteins in ubiquitin-proteasome system [15, 16].

The cullin gene family is evolutionarily conserved. There are seven cullins in mammals (CUL1 to CUL3, CUL4a, CUL4b, CUL5, and CUL7). The Cullin family proteins are involved in a diverse array of functions, including cell-cycle control, DNA replication, and development. Cullin4B (CUL4B) protein belongs to the cullin family. Compared with research on other members, the study of CUL4B is very limited. In recent studies, CUL4B is recognized as a highly expressed gene in several kinds of carcinoma tissues, and functions as oncogene inhibiting the expression of tumor suppressor genes, promoting cell proliferation and migration, and finally causing the occurrence of tumor [17, 18]. So it shows that abnormal expression of CUL4B is

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closely related to the occurrence and development of tumor. So far, there is no related research about CUL4B expression and its clinical significance in EAC.

In this study, the expression level of CUL4B was detected at both mRNA and protein levels in normal endometrium (NE) and EAC tissues. Correlation analysis between CUL4B expression and clinicopathological characteristics was carried out to understand the potential role of CUL4B in EAC. In addition, Cox regression analyses and Kaplan–Meier survival analyses may provide evidence to support the use of CUL4B as a potential prognostic biomarker for EAC.

Materials and Methods

A total of 35 EAC fresh cancer tissues were collected from patients who underwent surgical resection in the First Affiliated Hospital of Sun Yat-sen University from January 2011 to December 2011, and all the patients had not accepted chemotherapy, radiotherapy, and hormone therapy before the operation. Another 25 fresh NE tissues were obtained as control group from patients who underwent hysterectomy because of uterine fibroids during the same period. All specimens were obtained immediately after surgical resection, frozen in liquid nitrogen, and kept at -80°C until RNA extraction for quantitative real-time PCR. A total of 113 EAC and 30 NE paraffin-embedded sections between December 2006 and December 2008 were obtained for immunohistochemical research. All the tissues were confirmed by two independent pathologists. All recruited patients provided written informed consent before operation, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University.

PCR was performed according to the following procedures [19]: Total RNA of frozen tissues were isolated with TRIzol-A+ agent and treated with DNase I to remove DNA contamination. First-strand cDNA was synthesized from one microgram of total RNA. Reverse transcription was performed following the manufacturer's instructions. The cDNA templates were subjected to PCR amplification. Q-PCR was performed with a sequence detection system and SYBR green I master mix kit. GAPDH was used as an internal control. The primers for qPCR were as follows: CUL4B5'-TGGAAGTTCATTTACCACCAGAGATG-3'(forward)and5'-TTCTGCTTTTAACACACAGTGTCCTA-3'(reverse)[17]; GAPDH5'-ACTTCAACAGCGACACCCACTC-3'(forward) and5'-TACCAGGAAATGAGCTTGACAAAG-3'(reverse). The action conditions for CUL4B were as follows: pre-denaturation at 95° for five minutes; 40 cycles of denaturation at 95° for 30 seconds, annealing at 60° for 30 seconds, and extension at 72° for 30 seconds. Each reaction was repeated three times. The relative genomic expression was calculated by 2-ΔΔCt value [ΔCt = Ct (CUL4B) - Ct (GAPDH)] [20].

Briefly, 4-μm thick histological sections were cut from paraffin-embedded tissues and fixed on the slides. The next step is antigen retrieval which was performed in a citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by a ten-minute treatment with 3% H_2O_2 solution, after which the slides were incubated overnight at 4°C with the primary antibody dilution: anti-galectin-1. The slides were subsequently incubated with a detection kit. Antigen-antibody reactions were visualized with DAB. Counterstaining was performed using haematoxylin. Absence of the primary antibody was used as a negative control. All slides were reviewed by two pathologists, who were blinded to the clinical outcome. The results were evaluated according to an aggregate score of intensity of staining multiplied by the score of the area of staining. The intensity of staining was graded on the following scale: 0, no staining, 1+, mild staining, 2+, moderate staining, and 3+, intense staining. The area of staining was evaluated as follows: 0, no staining of cells in any microscopic fields; 1+, <10% of tissue stained positive; 2+, between 10% and 50% stained positive; 3+, between 51% and 80% stained positive; 4+,

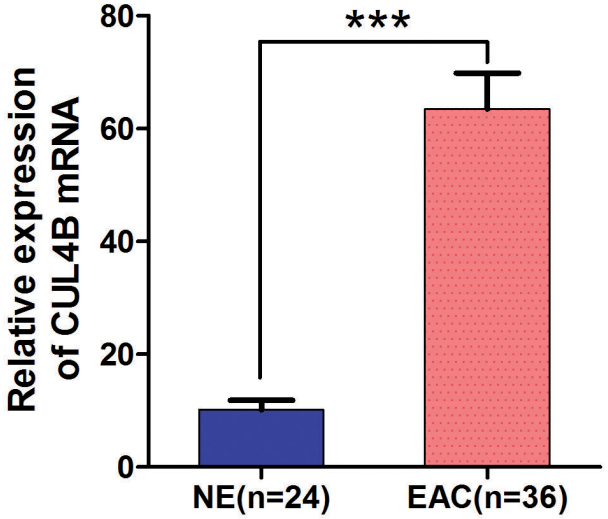

Figure 1. — The expression level of CUL4B mRNA in EAC and NE tissues. The expression level of CUL4B mRNA in EAC is significantly higher than that of NE ($p < 0.001$).

Table 1. — *The expression of CUL4B protein in two groups*

Group	CUL4B				*p*
	-	+	total	Positive rate	
NE	20	5	25	16.67%	***< 0.001***
EAC	45	68	113	60.18%	

NE: normal endometrium; EAC: endometrial adenocarcinoma.

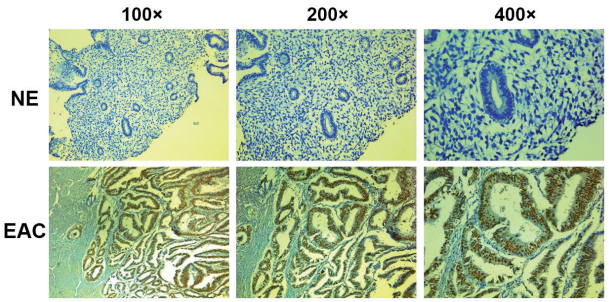

Figure 2. — The expression of CUL4B protein in EAC and NE tissues. The expression level of CUL4B protein in EAC was significantly higher compared with NE ($p < 0.001$).

Table 2. — *Correlations between CUL4B protein expression and clinicopathologic characteristics in EAC.*

Characteristics	CUL4B protein		*p*
	-	+(%)	
Age (years)			0.299
> 50	23	28 (54.90%)	
< 50	22	40 (64.52%)	
Histological grade			***0.002***
G1-G2	43	49 (52.26%)	
G3	2	19 (90.48%)	
FIGO Stage			***0.008***
IA-IB	42	50 (54.35%)	
II-IV	3	18 (85.71%)	
Muscular infiltration depth			0.519
< 1/2	33	46 (58.23%)	
> 1/2	12	22 (64.71%)	
Lymph node metastasis			0.449
Negative	27	21 (43.74%)	
Positive	6	2 (25.00%)	

EAC: endometrial adenocarcinoma; FIGO: the International Federation of Gynecology and Obstetrics.

>81% stained positive. An aggregate staining score of ≤4 was considered to be a negative staining. A score between 5 and 8 was considered to be a positive staining, whereas a score between 9 and 12 was considered to be a strong staining [21].

Statistical analyses were performed by SPSS software (version 16.0), with all data expressed as means ± standard deviation (SD). The Student's *t*-test was used for evaluating the statistical significance of difference in the means between groups. The relationship between CUL4B expression and clinicopathological features was assessed using Pearson's χ^2 test, Student's *t*- test, and Fisher's exact test. The Cox proportional hazards regression model was performed for multivariate survival analysis. Survival curves were obtained with the Kaplan–Meier method, and the differences in survival of cancer patients were determined by log-rank analysis. Differences between two variables were considered to be significant when the *p* value was <0.05.

Results

The authors utilized RT-PCR assays to detect the expression level of CUL4B mRNA in 36 EAC and 24 NE fresh tissues. The result showed that the expression level of CUL4B mRNA in EAC was significantly higher, compared with NE tissues ($p < 0.001$) (Figure 1).

The products of CUL4B protein located in the nucleus. It was defined as a positive expression when the yellow particles appearing in endometrial cell nucleus. In the 30 samples with normal endometrium tissues, five were positive. with a positive expression rate of 16.67%, whereas 68 of 113 samples with EAC tissues tested positive, with a positive expression rate of 60.18%. These findings revealed that the expression of CUL4B protein in EAC was significantly higher than that of NE (p <0.001) (Table 1 and Figure 2)

Among 113 patients with EAC, the age ranged from 31 to 86 years, with the median age of 53 years. Histologic classification can be divided into three levels: G1, G2, and G3. Surgical pathology staging was classified by FIGO staging 2009. In the present study, 56 patients accepted pelvic lymphadenectomy, among which eight cases with lymph node metastasis. The present results showed that CUL4B protein expression significantly correlated with FIGO stage ($p = 0.008$) and histological grade ($p = 0.002$). There was no obvious relationship with age, muscular infiltration depth, and lymph node metastasis (Table 2).

Univariate Cox regression analyses showed that FIGO stage ($p < 0.001$), histological grade ($p < 0.001$), muscular infiltration depth ($p < 0.001$), lymph node metastasis ($p < 0.001$), and CUL4B expression ($p < 0.001$) were significant predictors of EAC. Multivariate Cox regression analyses indicated that FIGO stage ($p = 0.002$) and CUL4B expression ($p = 0.043$) were independent prognostic factors in patients with EAC (Table 3).

Kaplan–Meier survival analyses demonstrated that patients with low CUL4B expression had longer survival times, whereas those with high CUL4B expression had shorter survival times. The cumulative five-year survival rate was 82.22% (95% CI 80.72–83.73%) in the low CUL4B group, whereas it was only 71.50% (95% CI 66.20–76.80%) in the high CUL4B group ($p < 0.01$). The cumulative five-year survival rate was 80.80% (95% CI 77.81–83.79%) in the low CUL4B group, whereas it was only 68.33% (95% CI 69.23–78.12%) in the high CUL4B group ($p < 0.01$) (Figure 3).

Discussion

Endometrial carcinoma is still a world wide threat for women health even if the treatments have been greatly improved. A number of endometrial carcinoma patients unfortunately progress to relapse and develop distant metastasis after surgery. The molecular mechanism of endometrial carcinoma pathogenesis remains unknown. Ubiquitin proteasome degradation pathway is closely related to tumor development in a manner of regulating the cell cycle protein and protein kinase [22, 23]. Cullin protein acts as a scaffold protein of the E3 ligase complex which is the key enzyme of ubiquitination [15, 16]. The CUL4 subfamily is composed of two members: CUL4A and CUL4B. Various studies have shown that CUL4A played a critical role in cancer pathogenesis [24, 25]. As CUL4B shared extensive sequence homology with CUL4A, CUL4B was considered exerting similar function as CUL4A. A recent study [26] reported that the mutations of CUL4B was associated with x-linked mental retardation syndrome. In another study, CUL4B was involved in DNA damage repair process, which might lead to malignant transformation if this process had an exception [27]. Hu *et al.*'s study [17] showed that CUL4B was overexpressed in many cancer tissues, such as esophageal, lung, colorectal, pancreatic, breast, cervical, and ovarian cancers. Overexpression of CUL4B inhibited the expression of H2AK119 and H3K27, which eventually led to the promotion of cell proliferation

Table 3. — *Univariate and multivariate analyses of different prognostic factors associated with overall survival in EAC.*

Variables	Univariate analysis		Multivariate analysis	
	HR(95%CI)	*p*	HR(95%CI)	*p*
Age (years)	1.032 (0.994-1.072)	0.098	1.019 (0.976-1.064)	0.400
FIGO stage	5.683 (3.305-9.773)	***< 0.001***	3.552 (1.616-7.805)	***0.002***
Histological grade	6.152 (2.935-12.893)	***< 0.001***	1.899 (0.648-5.565)	0.189
Muscular infiltration	5.806 (2.205-15.289)	***< 0.001***	1.900 (0.730-4.951)	0.242
Lymph node status	11.086 (4.165-29.507)	***< 0.001***	1.827 (0.563-5.930)	0.316
CUL4B expression	13.683 (1.826-102.451)	***0.011***	8.724 (1.067-71.340)	***0.043***

EAC: endometrial adenocarcinoma; FIGO: the International Federation of Gynecology and Obstetrics.

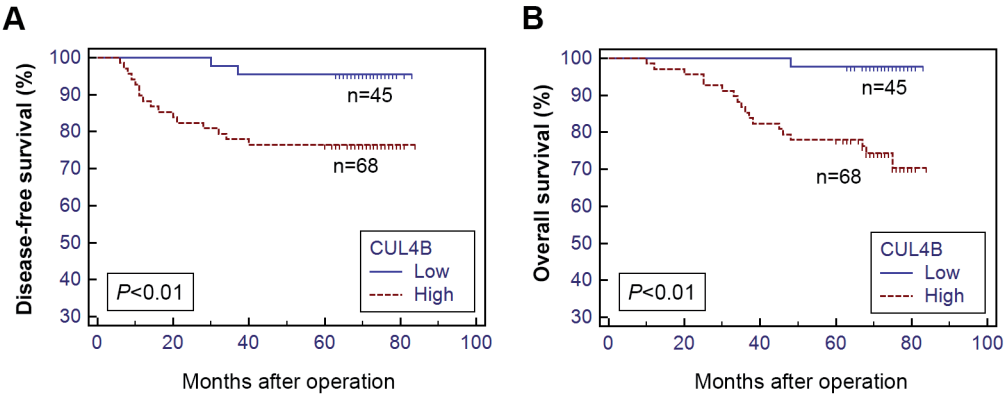

Figure 3. — Kaplan-Meier survival curves according to CUL4B protein expression in EAC patients.
(A) and (B) Kaplan–Meier survival analyses demonstrate that patients with high CUL4B expression have shorter OS ($p < 0.01$) and DFS ($p < 0.01$).

and migration [17, 18]. Additionally, CUL4B served as an oncogene via cell signaling pathways such as NF-KB pathway, epidermal growth factor pathway, and P27 pathway [28-30].

To the best of the present authors' knowledge, this study for the first to explore the relationship between CUL4B expression and EAC. The authors examined the mRNA and protein expression of CUL4B in both EAC and NE tissues. It was demonstrated that CUL4B expression in EAC was significantly upregulated, compared to NE. Correlation analyses were utilized to explore the relationship between CUL4B expression and clinicopathologic characteristics in EAC patients. The present results showed that CUL4B expression was markedly correlated with FIGO stage and histological grade. Furthermore, on the basis of the univariate and multivariate analyses, it was indicated that CUL4B was an independent prognostic factor in patients with EAC. Kaplan–Meier survival analyses demonstrated that patients with high CUL4B expression had shorter OS and DFS. However, specific molecular mechanism indicating on how CUL4B promotes the occurrence and development of EAC requires further research.

Conclusion

In conclusion, the present results revealed that CUL4B was overexpressed in EAC and may serve as a novel prognostic marker and therapeutic target for EAC.

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