

Cullin7 promotes epithelial-mesenchymal transition of esophageal carcinoma via the ERK-SNAI2 signaling pathway

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Abstract. Cullin7 (CUL7) is a member of Cullin protein family and exhibits a tumor-promoting role in several types of tumors, including breast, liver and ovarian. However, its roles in esophageal carcinoma (EC) have not yet been reported. In the present study, CUL7 expression in EC tissue was revealed to be significantly higher compared with nontumoral tissues, as detected by immunohistochemistry (IHC; $P=0.000$). χ^2 analysis confirmed that CUL7 expression was positively associated with invasion depth ($P=0.000$), lymph node involvement ($P=0.033$) and advanced clinical stage ($P=0.000$). Survival analysis demonstrated that CUL7 was positively associated with poor overall survival ($P=0.001$) and poor disease-free survival ($P=0.0019$). An association of CUL7 with endothelial-mesenchymal transition (EMT) was examined, and IHC results indicated that high CUL7 expression was associated with increased zinc finger protein SNAI2 (SNAI2) expression ($P=0.000$) and decreased E-cadherin ($P=0.000$). Western blot analysis demonstrated that short hairpin RNA silencing CUL7 in EC1 cells increased epithelial (E)-cadherin protein expression level, and decreased expression of Vimentin and SNAI2; cell migration was also reduced. Western blot analysis demonstrated that over expression of CUL7 in EC9706 cells increased Vimentin and SNAI2 protein expression, but decreased E-cadherin expression, and the number of migratory cells. Investigation into the potential molecular mechanisms demonstrated that over expressing CUL7 in EC9706 cells stimulated the phosphorylation of ERK. Inhibiting ERK through treatment with U0126 significantly abrogated CUL7-induced alterations in Vimentin, SNAI2 and E-cadherin expression levels. Results from the present study

demonstrated that CUL7 expression was associated with EC progression and poor prognosis. CUL7 may promote EMT via the ERK-SNAI2 pathway in EC. These data may improve our understanding of the role of CUL7 in tumors and provide supporting evidence for the development of novel therapeutic targets for EC.

Introduction

Esophageal carcinoma (EC) is a common malignant tumor worldwide, demonstrating the eighth highest incidence rate and representing the sixth most common cause of cancer associated mortality (1). The number of EC cases diagnosed annually in China account for almost half of the worldwide total (2). Considering that early symptoms in EC are frequently latent, the majority of patients are diagnosed at the advanced stage and prognosis is usually poor, owing to invasion and metastasis (3-5). Therefore, it is urgent to identify novel and effective molecular targets for the diagnosis and treatment of EC for improved patient outcomes.

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells lose their polarized organization, which is accompanied by reduced epithelial (E)-cadherin expression, and increased cellular mobility, which is accompanied by increased Vimentin expression (6,7). It has been verified that, via the EMT program, tumor cells may obtain stronger metastasis and increased drug-resistance and stemness, which may lead to tumor progression and poor prognosis (8,9). Therefore, an effective treatment that targets EMT may inhibit tumor progression.

Cullin7 (CUL7) is a member of the Cullin protein family; it is a molecular scaffold that organizes an E3 ubiquitin ligase comprising F-box protein Fbw8, S-phase kinase-associated protein 1 and ring-box 1 finger protein, and regulates cell biology functions via protein ubiquitination (10,11). A number of roles for CUL7 have been reported in numerous types of tumors. For example, in 2007, Kim *et al* (12) reported that CUL7 may promote human neuroblastoma cell proliferation by inhibiting p53-dependent or p53-independent apoptosis. In 2015, Men *et al* (13) reported high expression levels of CUL7 in lung cancer and confirmed the proliferation-inducing roles; however, the role of CUL7 in EC has not yet been reported.

The present study demonstrated for the first time, to the best of our knowledge, that CUL7 is expressed in elevated levels

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in EC tissues, as detected by immunohistochemistry (IHC), and a close association between this increased expression and invasion depth, lymph node involvement and advanced clinical stage were identified. The EMT-promoting roles of CUL7 were also investigated, and the extracellular signal-regulated kinase (ERK)-zinc finger protein SNAI2 (SNAI2) pathway was reported to be involved in this process. In addition, the present study revealed that CUL7 was positively associated with poor overall survival (OS) and disease-free survival (DFS) of patients with EC.

Materials and methods

IHC. The present retrospective study was approved by the review board and Ethics Committee of Yidu Central Hospital of Weifang (Weifang, China); specimens were collected from 130 patients (53 males and 77 females; 40-73 years old) with primary EC who had surgical removal between January 2009 and December 2011 at Yidu Central Hospital of Weifang. Patients did not receive chemotherapy, radiotherapy or immunomodulatory therapy prior to surgery. Nontumoral tissues were used as a negative control in the present study and were obtained from outpatients who underwent gastroscopy detection.

Paraffin-embedded tissue samples were obtained from the Yidu Central Hospital of Weifang and then sectioned (4 μm), placed on slides, deparaffinized in xylene and rehydrated in a graded ethanol series. Slides were boiled in 10 mmol/l citrate buffer (pH 6.0) for 3 min at 100°C for antigen retrieval. The sections were subsequently immersed in 3% H₂O₂ for 10 min at room temperature to block endogenous peroxidase activity and incubated in goat serum blocking solution (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) at room temperature for 15 min to block non-specific antigens. Sections were incubated at 4°C overnight with primary antibodies against CUL7 (cat. no. ab115304; 1:1,000; Abcam, Cambridge, USA), E-cadherin (cat. no. ab40772; 1:1,000; Abcam) and SNAI2 (cat. no. ab85936; 1:500; Abcam), and subsequently washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse immunoglobulin G (IgG) polyclonal antibody (cat. no. KIT-7710; Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) at room temperature for 30 min. The slides were stained with 3,3'-diaminobenzidine for 5-10 sec at room temperature and counterstained with hematoxylin for 20 sec at room temperature. The evaluation scoring process was performed by two independent pathologists who were blinded to the clinical information of patients. The percentage of positive-stained cells of the total number of cells was recorded at $\times 400$ magnification in at least 5 random fields using a BX53 microscope (Olympus Corporation, Tokyo, Japan). A score representing the fraction of positive-staining tumor cells was as follows: 0, $\leq 25\%$; 1, 26-50%; 2, 51-75%; and 3, $> 75\%$. The intensity score represented the average staining intensity of positive tumor cells (0, negative; 1, weak; 2, moderate; and 3, strong). The expression levels of CUL7 were calculated using the product of the proportion score and the intensity score, and a score < 4 was considered low expression, whereas a score ≥ 4 was considered as high expression.

Cell lines and culture. The EC cell lines EC1 and EC9706 were purchased from Guangzhou Jisai Biotech Co., Ltd.

(Guangzhou, China) and cultured in RPMI-1640 Medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific Inc.).

Reverse transcription-semi-quantitative polymerase chain reaction (RT-sq PCR). Total RNA was extracted from cells ($\sim 2 \times 10^5$ /ml) using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized using a Prime Script RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocols. The primers were as follows: CUL7 forward, 5'-CCATCTCAGAGTCCCAACAC-3' and reverse, 5'-TTCAGCACCACG GCATAGFF-3'; GAPDH forward, 5'-AGAAGGCTGGGGCTC ATTTG-3' and reverse, 5'-AGGGGCCATCCACAGTCTTC-3'. The reaction mixture [5 μl mix including DNA polymerase (cat. no. D7228; Beyotime Institute of Biotechnology, Haimen, China), 1.4 μl cDNA, 1.6 μl forward primer, 1.6 μl reverse primer and 5.4 μl double distilled H₂O] was amplified at 94°C for 5 min, followed by 30 cycles at 94°C for 30 sec, 51°C for 30 sec, 72°C for 30 sec and finally 72°C for 5 min. PCR products were electrophoretically separated using 1.0% agarose gels and visualized using ethidium bromide. The results were analyzed using Lab Work software (version 4.0; UVP, Inc., Upland, CA, USA). GAPDH was used as an internal control.

Western blot analysis. Protein was extracted from 4×10^5 /ml cells using radio immune precipitation assay buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) containing 1% protease inhibitor. Protein concentration was determined using a Bicinchoninic Acid kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 300 μg protein was loaded per well separated by 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane. Following blocking in TBS with 0.05% Tween-20 containing 5% non-fat dried milk for 1 h at room temperature, the membrane was incubated with primary antibodies at 4°C overnight and subsequently with secondary HRP-conjugated goat anti-rabbit IgG antibodies (cat. no. SA00001-2; 1:5,000; Protein Tech Group, Inc., Chicago, IL, USA) at room temperature for 1 h. Signals were detected using Enhanced Chemiluminescence Reagents (Pierce; Thermo Fisher Scientific, Inc.) and densitometric analysis was performed using Image-Pro software (version 5.1; Media Cybernetics, Inc., Rockville, MA, USA). The primary antibodies used were as follows: CUL7 (cat. no. ab115304; 1:1,000; Abcam, Cambridge, MA, USA), E-cadherin (cat. no. ab40772; 1:1,000; Abcam), Vimentin (cat. no. ab45939; 1:1,000; Abcam) SNAI2 (cat. no. ab85936; 1:1,000; Abcam), phosphorylated (p)-ERK (cat. no. 4370; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), ERK (cat. no. 9102; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (cat. no. 10494-1-AP; 1:5,000; Protein Tech Group, Inc.).

Transfection. Two different cell lines exhibiting varying expression levels of CUL7 were chosen for investigation in the present study. In the cell line exhibiting high expression of CUL7 (EC1), CUL7 expression was suppressed; in the cell line exhibiting low CUL7 expression (EC9706), CUL7 expression was over expressed. Thus, greater changes of

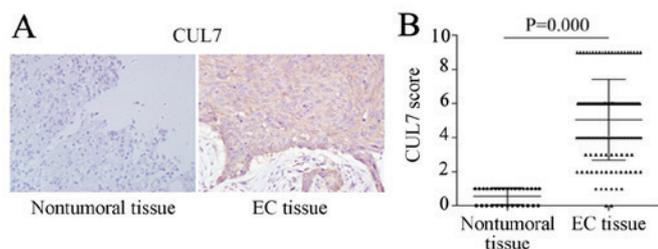


Figure 1. IHC staining of CUL7. (A) IHC staining of CUL7 protein expression in EC tissue and nontumoral tissue. Magnification, x400. (B) Difference of CUL7 expression score between two groups from (A) was analyzed using the Student's two-tailed t-test. CUL7, Cullin7; IHC, immunohistochemistry.

CUL7 expression and its effect on EC cells could be observed. A total of 1 μ g pcDNA3.1/CUL7-vector plasmid (Shanghai Gene Pharma Co., Ltd., Shanghai, China) was transfected into EC9706 cells (10^5 /ml) to upregulate CUL7 expression, and 1 μ g p-GPU6/CUL7-short hairpin (sh)RNA (Shanghai Gene Pharma Co., Ltd.) was transfected into EC1 cells to silence CUL7 expression. Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection process and 1 μ g empty negative control (nc) plasmids (Shanghai Gene Pharma Co., Ltd.) were used as controls. All procedures were performed according to the manufacturer's protocol. Following transfection, cells were incubated at 37°C for 48 h and then collected for further research. EC1 cells transfected with CUL7-shRNA and nc-shRNA cells were termed EC1-CUL7-sh and EC1-nc-sh cells, respectively; EC9706 cells transfected with CUL7-overexpression vector or nc-vector were designated EC9706-CUL7-vector and EC9706-nc-vector cells, respectively. The ERK inhibitor U0126 (cat. no. 9903; Cell Signaling Technology, Inc.) was used to investigate the molecular mechanism underlying the effect of CUL7 at 37°C for 24 h.

Migration assay. Migration assays were performed using Transwell chambers (EMD Millipore, Billerica, MA, USA). Cells (5×10^4) were seeded into the inserts of Transwell chambers in serum-free RPMI-1640 medium in the upper chamber, and RPMI-1640 medium supplemented with 20% FBS was added to the lower chamber. The cells were incubated for 48 h at 37°C, and the cells in the upper chamber were removed using a cotton swab; cells that migrated to the lower surface of the filter were fixed with 4% formaldehyde at room temperature for 15 min and stained with 0.1% crystal violet at room temperature for 20 min. Images were captured and cells were counted in 5 random fields using an XDS-200 microscope to obtain an average number of migrating cells (magnification, x200; Olympus Corporation, Tokyo, Japan).

Statistical analysis. Data are expressed as the mean \pm standard deviation, and SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. The difference between two groups was analyzed using a Student's two-tailed t-test. The association of CUL7 with clinical parameters was analyzed using the χ^2 test. Survival curves were produced using the Kaplan-Meier method and compared by means of the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Association between CUL7 and clinical parameters of patients with esophageal carcinoma.

Parameter	n	CUL7		χ^2	P-value
		Low	High		
Age (years)					
≤ 60	70	22	48	3.794	0.066
> 60	60	10	50		
Sex					
Male	53	16	37	1.498	0.221
Female	77	16	61		
Tumor size (cm)					
≤ 4	64	20	44	2.99	0.084
> 4	66	12	54		
Differentiation					
Well	49	14	35	0.663	0.415
Moderate/poor	81	18	63		
Invasion depth					
T1 + T2	64	28	36	24.873	0.000
T3 + T4	66	4	62		
Lymph node involvement					
N0 + N1	90	27	63	4.57	0.033
N2 + N3	40	5	35		
Clinical stage					
I-II	63	29	34	30.214	0.000
III	67	3	64		

CUL7, cullin 7.

Results

High CUL7 protein expression is positively associated with invasion depth, lymph node involvement and advanced clinical stage. IHC results revealed almost no CUL7-positive staining in nontumoral tissues, whereas strong CUL7 protein expression was observed in the cytoplasm of EC tissues (Fig. 1A). Statistical analysis demonstrated that the overall CUL7 expression score in EC tissues was significantly higher compared with nontumoral tissues (Fig. 1B). Of the 130 tissue specimens from patients with primary EC, 98 cases exhibited high expression levels of CUL7 and 32 cases exhibited low expression. Associations between CUL7 expression level and clinical parameters were also analyzed (Table I). In the low CUL7 expression group (32 cases), only 4 cases of T3+T4 were reported, which was significantly less than the 62 cases of T3 + T4 reported in the high CUL7 group (98 cases; $P = 0.000$). Furthermore, 5 cases of N2+N3 were reported in the low CUL7 expression group, which was significantly less than the 35 cases reported in the high CUL7 group ($P = 0.033$). In addition, 3 cases were reported as stage III in the low CUL7 expression group, which was significantly less than the 64 cases reported in the high CUL7 group ($P = 0.000$). However, no significant differences were identified between the CUL7 expression level and

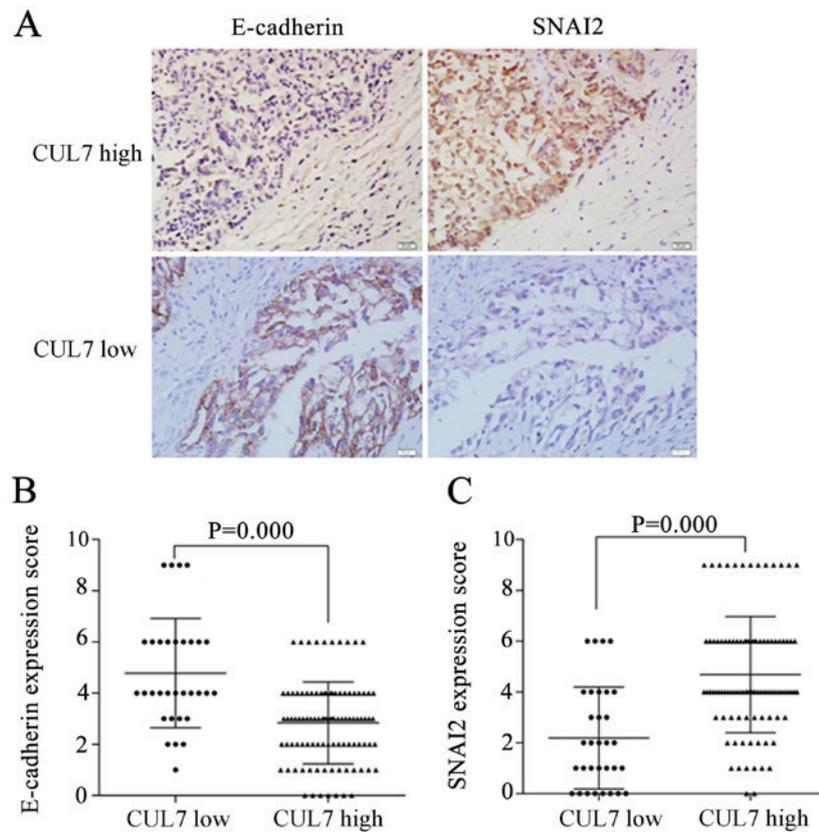


Figure 2. IHC staining of E-cadherin and SNAI2. (A) IHC staining of E-cadherin and SNAI2 in the high CUL7 expression group and in the low CUL7 expression group; magnification, x400. (B) Difference of E-cadherin expression score between the high CUL7 and the low CUL7 group was analyzed. (C) Difference of SNAI2 expression score between the high CUL7 and low CUL7 group was analyzed. CUL7, cullin7; E, epithelial; IHC, Immunohistochemistry; SNAI2, zinc finger protein SNAI2.

age, sex, tumor size or differentiation. These results indicated that CUL7 may serve a role in EC progression.

CUL7 promotes EMT of EC cells. IHC staining of serial tissue sections revealed that, in the high CUL7 expression group, E-cadherin protein expression was weak and SNAI2 expression was strong (Fig. 2A); in the low CUL7 group, E-cadherin expression was strong and SNAI2 expression was weak. Statistical analysis results indicated that high CUL7 expression was associated with low E-cadherin expression ($P=0.000$; Fig. 2B) and with high SNAI2 expression ($P=0.000$; Fig. 2C). These data suggested an association of CUL7 expression with EMT. In cell culture experiments, CUL7 mRNA and protein expression levels in EC1 cells were significantly higher compared with EC9706 cells ($P<0.05$; Fig. 3A and B). CUL7 expression was altered by transfecting EC1 cells with CUL7-targeted shRNA, which demonstrated that silencing CUL7 expression significantly inhibited the protein expression levels of SNAI2 and Vimentin, whereas E-cadherin expression was upregulated (Fig. 3C). Over expression of CUL7 in EC9706 cells significantly promoted SNAI2 and Vimentin expression, but inhibited E-cadherin expression (Fig. 3D). These results provided further evidence of EMT induction by CUL7. In addition, cell migration experiments demonstrated that, following CUL7 silencing, the number of EC1 cells migrating to the lower surface of the filter were significantly lower compared with nc-sh transfected cells (18 ± 6 vs. 35.33 ± 5.13 cells/field, respectively; $p<0.05$; Fig. 3E and F).

Following CUL7 overexpression, the number of EC9706 cells migrating to the lower surface of the filter was significantly higher compared with nc-vector transfected cells (14.67 ± 3.51 vs. 30.67 ± 7.02 , respectively; $p<0.05$; Fig. 3E and G).

ERK-SNAI2 pathway participates in CUL7-induced EMT. EMT is a complicated process and is regulated by numerous signaling pathways under various biological and pathological conditions (6,7). ERK phosphorylation in EC9706 cells was upregulated in CUL7-overexpressing cells compared with nc-vector cells (Fig. 4A). Furthermore, in EC9706 cells co-treated with the CUL7 over expression vector and the ERK inhibitor U0126 at 37°C for 48 h, Vimentin and SNAI2 protein expression levels were notably reduced, whereas E-cadherin expression was notably increased compared with CUL7-vector treated cells co-treated with dimethylsulfoxide (Fig. 4B). These results suggested that CUL7 may promote EMT via the ERK-SNAI2 signaling pathway.

CUL7 is associated with poor OS and DFS. To further investigate the roles of CUL7 in patient prognosis, survival analysis was performed using the Kaplan-Meier method. Of the 30 patients in the low CUL7 group, 12 cases succumbed to mortality, with an OS rate of 60% at 60 months (Fig. 5A). Of the 40 patients in the high CUL7 expression group, 30 cases succumbed to mortality with an OS rate of 25% at 60 months. In addition, 16 of the 30 cases in the low CUL7 group exhibited recurrence, with a DFS rate of 46.67% at 60 months (Fig. 5B); 32 of the

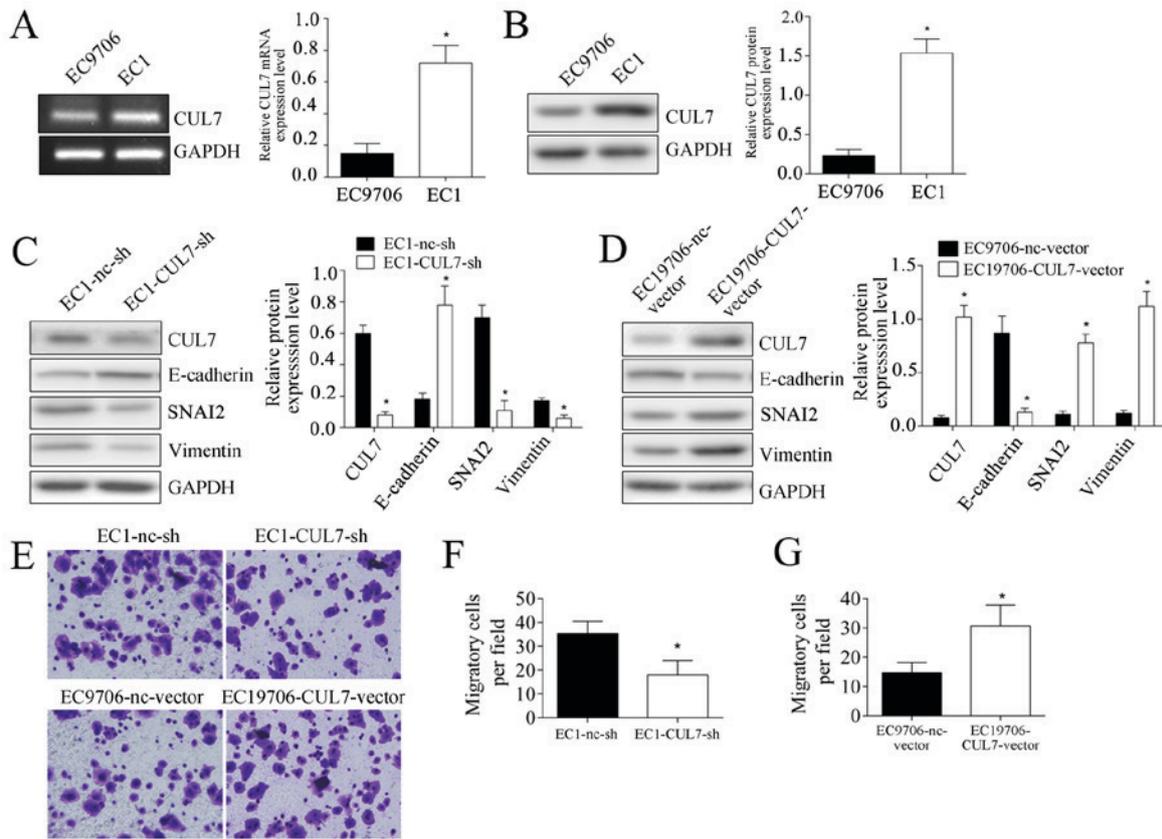


Figure 3. CUL7 promotes epithelial-mesenchymal transition of EC cells. (A and B) CUL7 mRNA and protein expression in EC1 and EC9706 cells was detected using (A) reverse transcription-polymerase chain reaction and (B) western blotting. (C and D) CUL7 expression was altered (C) in EC1 cells by CUL7-targeted shRNA transfection and (D) in EC9706 cells by CUL7-overexpression vector transfection, and the subsequent effects on protein expression levels of CUL7, E-cadherin, Vimentin and SNAI2 were detected by western blotting. (E-G) The effects on cell migratory ability were detected using a Transwell chamber in EC1 cells treated with CUL7-targeted shRNA transfection and in EC9706 cells treated with CUL7-overexpression vector. *P<0.05. CUL7, cullin7; E, epithelial; EC, epithelial carcinoma; nc, negative control; sh, short hairpin RNA; SNAI2, zinc finger protein SNAI2.

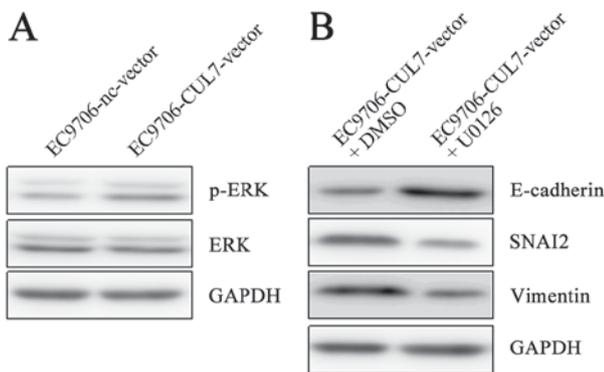


Figure 4. ERK-SNAI2 signaling participates in epithelial-mesenchymal transition induced by CUL7. (A) ERK phosphorylation levels were examined by western blotting in EC9706 cells transfected with CUL7 over expression vector. (B) In EC9706 cells co-treated with CUL7 over expression vector and with the ERK inhibitor U0126, alterations in E-cadherin, SNAI2 and Vimentin protein expression levels were analyzed. CUL7, cullin7; DMSO, dimethylsulfoxide; E, epithelial; ERK, extracellular signal-regulated kinase; nc, negative control; p, phosphorylated; SNAI2, zinc finger protein SNAI2.

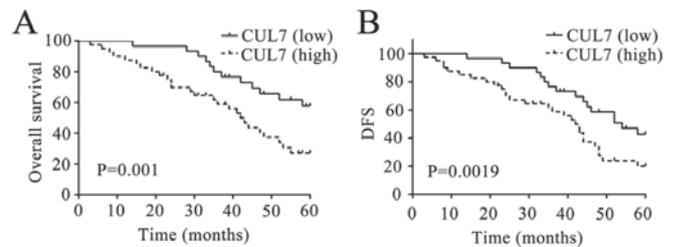


Figure 5. Survival analysis of patients with esophageal carcinoma. (A) Overall survival rates of patients in the low and high CUL7 expressing groups were analyzed. (B) DFS rates were also analyzed in patients from the two groups. CUL7, cullin7; DFS, disease-free survival.

Discussion

The CUL7 gene is located at the 6p21.1 locus (14); it is a member of Cullin protein family, and previous studies have examined CUL7 expression in tumors. For example, it has been reported that CUL7 may promote the proliferation, invasion and metastasis of liver carcinoma cells, which suggested that CUL7 is a novel gene that is associated with liver carcinogenesis and progression (15,16). Xi *et al* (17) reported on the clinical significance of CUL7 in ovarian cancer and suggested that CUL7 was positively associated with clinical staging and lymph node metastasis. However, to the best of our knowledge,

40 cases in the high CUL7 group exhibited recurrence, with a DFS rate of 20% at 60 months. The log-rank test revealed that the differences in OS and DFS rates between the two groups were significantly different, which further suggested a role for CUL7 in EC prognosis.

of the limited types of tumors that have been used in studies investigating CUL7, there have been no reports of CUL7 in EC. In the present study, high expression levels of CUL7 were detected in EC tissues and this increased level of expression was positively associated with invasion depth, lymph node involvement and advanced clinical stage, which was in line with the previously reported roles of CUL7 in liver cancer and ovarian cancer (15-17). However, a significant association between CUL7 and tumor size was not reported in the present study, which was inconsistent with the pro-proliferation roles of CUL7 reported previously (15,16). This may be explained by variations in organ origin, experimental conditions and operation in experiments.

The EMT program is an important factor in tumor progression (6,7). On the basis of the associations between CUL7 expression and invasion depth, lymph node involvement and advanced clinical stage, CUL7 may promote EMT in EC cells. SNAI2 is a transcription factor that has been reported to be upregulated in various types of tumors and is associated with decreased expression of the epithelial marker E-cadherin and increased expression of the mesenchymal marker Vimentin, and may thus serve important roles in the regulation of EMT (18). In the present study, results from IHC and cell culture experiments suggested that CUL7 may enhance the expression levels of SNAI2 and Vimentin, and inhibit E-cadherin expression, and increase cell migration. These data provided evidence for the hypothesis of the present study that CUL7 may promote EMT in EC cells. This corresponds with previous reports of the invasion- and metastasis-promoting roles of CUL7 in liver carcinoma (15,16), which further indicated a close association between CUL7 and EC progression. The ERK signaling pathway is also considered an important factor in various types of cell behaviors, including proliferation, migration and drug resistance (19,20), and results from the present study indicated that CUL7 may promote EMT via the ERK-SNAI2 signaling pathway, which may suggest novel targets for the treatment of EC.

In clinical work, clinical staging has been considered to be essential for the prediction of prognosis as well as representing a basis to decide which therapeutic option (such as surgery, chemotherapy or radiotherapy) should be performed (21,22). As a positive association between CUL7 expression and clinical stage was identified, the present study aimed to investigate the relationship between CUL7 and prognosis. The results revealed that high CUL7 expression was associated with poorer OS and DFS, which was consistent with the EMT-promoting roles of CUL7 and suggested that CUL7 may be a marker of EC progression and poor prognosis.

The present study investigated the roles of CUL7 in progression, EMT and poor prognosis of EC patients, as well as the potential molecular mechanism. These data may improve our current knowledge of the roles of CUL7 in tumors and may provide a novel target for the treatment of EC.

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Competing interests

The authors declare that they have no competing interests.

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