



Dysregulation of Krüppel-like factor 12 in the development of endometrial cancer

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HIGHLIGHTS

- KLF12 is correlated with EC (Endometrial cancer) patients' prognosis.
- KLF12 is overexpressed in EC.
- Overexpression of KLF12 resulted in increased cell proliferation, decreased cell apoptosis and enhanced cell migration.
- KLF12 activates AKT signaling in EC.

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ABSTRACT

Objective. Endometrial cancer (EC) remains a malignancy with poor survival outcome. To investigate the role of Krüppel-like factor 12 (KLF12), a transcription factor, in the progression of human EC.

Methods. Immunohistochemistry, real time-PCR and western blot analysis of KLF12 expression in EC patients' tissues. Bioinformatics analysis revealed the clinical importance of KLF12 expression and survival ratio. Overexpression of KLF12 was generated using the ViraPower Adenoviral Expression System in EC cell lines. Cell viability assay, cell apoptosis assay and cell migration assay were used to determine cell proliferation, cell apoptosis and cell migration, respectively. Western blot analysis was carried out to determine the protein levels in cell lines and animal tissues.

Results. The expression of KLF12 was observed to be much higher in human EC tissues compared with normal endometrium. Moreover, KLF12 expression was correlated positively with disease recurrence and was also associated with decreased survival probability. The overexpression of KLF12 in EC cell lines resulted in increased cell proliferation, decreased cell apoptosis and enhanced cell migration. Furthermore, overexpression of KLF12 also increased tumor size in vivo. Moreover, up-regulation of KLF12 dramatically increased the expression levels of MMP2, MMP9, pAKT S473 and CCND1. Our research reveals that overexpressed KLF12 contributes the growth of EC tumor by activating AKT signaling and increasing CCND1 expression level.

Conclusions. To our knowledge, this is the first study to explore the significance of KLF12 in the development of EC, and KLF12 is expected to provide a novel potential therapeutic target for EC treatment.

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1. Introduction

Endometrial cancer (EC) is the fourth most frequent cancer among women all over the world and most common malignant tumor of the female reproductive system in developed countries [1]. At present, the incidence of EC has been increased in developing countries, and the age of diagnosis tends to be younger. Obesity, diabetes, insulin resistance,

polycystic ovary, anovulation, and endogenous estrogen synthesis caused by tamoxifen are the major risk factors for EC [2]. In addition, various types of gene mutations and abnormal activation of related signaling pathways are closely related to the occurrence and development of EC [3].

According to estrogen receptor expression, tumor differentiation, pathological types, endometrial carcinoma can be divided into two types [4]. Type I EC is the most common, estrogen-dependent, well-differentiated adenocarcinoma, which often connects to mutations of PTEN, PIK3CA, K-RAS and WNT/β-catenin and tend to have a favorable prognosis. Type II endometrial carcinoma is estrogen-independent,

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poorly-differentiated, special pathological types including adenosquamous carcinoma, papillary serous carcinoma, clear cell carcinoma, which connect to *P53* gene mutation and chromosomal stability and often have a poor prognosis [5]. Although patients with early-stage EC who undergo staging surgery have a favorable prognosis, patients diagnosed with the advanced or recurrent disease often have a worse prognosis and limited adjuvant chemotherapy and radiotherapy. Abnormal genes associated with EC were explored in the application of molecularly targeted drugs in EC treatment. Some novel targets are expected to be discovered in the diagnosis and treatment of advanced or recurrent EC.

Krüppel-like factors (KLFs) are a family of DNA-binding transcriptional regulators involved in the regulation of cellular processes, including proliferation, apoptosis, differentiation, migration, inflammation and pluripotency. Previous researches confirmed that KLFs have important tumor suppressive and oncogenic functions [6–9]. In EC, the expression of KLF4, KLF9 is reduced relative to normal endometrium. KLF9 level is tightly regulated to maintain cellular homeostasis including proliferation, extracellular matrix formation, migration and adhesion [10,11]. KLF4 plays the tumor suppressive role in EC because down-regulation of KLF4 promotes the proliferation and Epithelial-mesenchymal transition (EMT) in EC cells [12]. Overexpression of KLF17 could activate TWIST1 to induce EMT and invasive phenotypes of EC cells [13]. KLF12 is a member of KLFs family, which regulates gene transcription and tumor cell proliferation, differentiation and apoptosis through DNA binding domain at the carboxy-terminal binding to GC/CACCC rich-sequence in gene promoter [14]. Nakamura Y et al. firstly found that KLF12 plays an important role in poorly differentiated gastric cancer progression and is a potential target of therapeutic measures [15]. Increasing evidence showed that KLF12 involved the development and progression of colorectal cancer and ovarian cancer by affecting multiple biological functions of tumor cells [15–17]. However, the function of KLF12 in EC remains unclear. Our current study tries to clarify the biological role of KLF12 in the progression of EC.

2. Materials and methods

2.1. Patient samples

Tissue samples were collected from 63 cases of endometrial carcinoma paraffin-embedded specimens, which were pathologically and clinically diagnosed as EC at the Affiliated Drum Tower Hospital of Nanjing University Medical School from 2007 to 2011. In addition, 12 cases of the freshly normal endometrium and 15 cases of EC tissues were frozen and stored in liquid nitrogen for RNA extraction. Informed consent and approval were obtained from patients for research purpose and medical ethics committee of the Affiliated Drum Tower Hospital, Nanjing University Medical School.

2.2. Immunohistochemistry (IHC) analysis

Paraffin tissue sections of 5 mm were treated with xylene and ethanol to remove the paraffin. The slides were incubated in 3% H₂O₂ at room temperature for 10 min for blocking endogenous peroxidase activity. Antigen retrieval was performed by boiling in a pressure cooker at 120 °C in 0.01 M citrate buffer solution for 10 min. Then slides were incubated with anti-KLF12 (1:150 dilution, Abcam, Cambridge, MA, USA, ab129459) at 4 °C overnight. After washing slides with phosphate-buffered saline (PBS), the slides were incubated with the HRP-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) at room temperature for 30 min. After washing with PBS, the slides were developed by DAB reagent and reaction was terminated by distilled water after stained by hematoxylin, then dehydrated in different graded ethanol and dried in room temperature. We detected the slides using a microscope and analyzed KLF12 expression. KLF12 expression was scored according to the staining intensity and quantity of tumor cells, the intensity of staining score criteria was as follow: with 0 meaning

no staining, 1 meaning weak staining, 2 meaning moderate staining, 3 meaning strong staining. The quantity of staining score criteria was as follow: 0 denoting no staining, 1 denoting < 10% of tumor cells stained, 2 denoting 10–50% of tumor cells stained, 3 denoting > 50% of tumor cells stained. The staining index score was calculated by intensity staining score multiplying quantity staining score, respectively KLF12 expression was calculated by scoring 0, 1, 2, 3, 4, 6 and 9. 63 patients' samples were tested, the median value was 4. Thus, we defined the cut-off value of IHC was 4. Scoring 4, 6 and 9 as high expression of KLF12, and Scoring 0, 1, 2 and 3 as low expression of KLF12.

2.3. Adenovirus construction and amplification

Recombinant Adenoviruses (Ad-Flag-KLF12) comprising full-length KLF12 were constructed using Ad Max system (Microbix Biosystems, Inc., Toronto, Canada) according to the manufacturer's instructions. Then recombinant viruses were amplified in HEK293A cells and titrated using Adeno-X Rapid Titer reagent (Takara, Japan).

2.4. RT-PCR (Real-time PCR) analysis

According to the manufacturer's recommendations, total RNA was extracted from freshly normal endometrium and EC tissues to compare the transcriptional expression of KLF12 using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and 1 µg of total RNA was reversely transcribed to obtain 20 µl cDNA using Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Real-time PCR was carried out using MyiQ Single Color Real-Time PCR System (Bio-Rad, Hercules, CA, USA) and primers of KLF12 and 18S rRNA. The Relative expression of KLF12 mRNA was calculated using the 2^{-ΔΔCT} method with 18S rRNA as an internal control. Reverse transcription was performed using the following primers: KLF12, 5'-CCTTCCATAGCCAGAGCAG-3' and 5'-TTGCATCCCTCAAATCACA-3'; 18S rRNA, 5'-CGGCTACCACATCCAAGGAA-3' and 5'-CTGGAATTACCGCGGCT-3'.

2.5. Cell culture and transfection

As previously reported, Ishikawa cells were kindly provided by Professor L.H. Wei (Peking University People's Hospital, China), and HEC-1B were purchased from the Shanghai Cell Collection. Both cell lines were maintained in DMEM-F12 medium (Invitrogen, Carlsbad, CA, USA) added with 10% fetal bovine serum (FBS) and 1% double antibiotics (Invitrogen, Carlsbad, CA, USA) at a 37 °C, 5% CO₂ humidified incubator. When cells intensity reached 60% ~ 70%, they were infected at various multiplicities of adenovirus expressing KLF12 or control adenovirus after starvation treatment.

2.6. Western blotting

Protein was extracted from Ad-KLF12-transfected EC cells to perform Western blotting analysis. Cells were rinsed with PBS twice and then were lysed in lysis buffer (1% Nonidet P-40, 50 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl, 0.1% sodium dodecyl sulfate) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and protein concentration was measured as previously described [18]. Equal amounts of protein (20 µg) were electrophoresed in 10% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in PBS containing 5% non-fat milk at room temperature for 1 h and incubated with following primary antibodies at 4 °C overnight: antibody against KLF12 (1:2000 dilution, Rabbit polyclonal, Abcam, Cambridge, MA, USA129459); antibody against β-actin (1:5000 dilution, Rabbit polyclonal, Bioworld Technology, Ap0060); antibody against Flag (1:2000 dilution, Mouse monoclonal, Cell Signaling Technology, 8146 s), antibody against AKT (1:1000 dilution, Mouse monoclonal, Cell Signaling Technology, 2967 s), antibody against pAKT S473 (1:1000 dilution, Rabbit polyclonal, Santa Cruz, sc135651),

antibody against CCND1 (1:1000 dilution, Rabbit polyclonal, Abgent, F008508), The membranes were incubated with secondary horseradish peroxidase (HRP)-conjugated after rinsing three times (10 min for every time) with PBS containing 0.1% Tween. Protein bands were detected by chemiluminescence imaging system (Amersham Biosciences Corp, Piscataway, NJ, USA).

2.7. Immunostaining

For immunostaining of Ishikawa cells, the KLF12 stable expressed cells or control cells were fixed with 4% formaldehyde in PBS for 30 min at room temperature, respectively. After three quick washes with PBS, non-specific binding was blocked with culture medium (10% fetal bovine serum in PBS). Cells were then incubated with Flag antibody (Mouse monoclonal, Protein-tech, 8146 s) and GFP antibody (Rabbit Polyclonal, Cell Signaling Technology, 50,430-2-AP) in culture medium at 1:200 for 1 h, washed with culture medium for 15 min three times, and incubated with Alexa-conjugated secondary antibodies at 1:500 for 1 h, followed by three washes with culture medium. To permeabilize cells, 0.1% Triton X-100 was added to the culture medium during fixing, antibody incubation and washing. Immunostained samples were imaged with a Leica confocal microscope.

2.8. Cell viability assay

5×10^3 cells per well were seeded in ninety-six-well and five plates were arranged in parallel in every group. After incubating in serum-free medium for 12 h, cells were transfected with Ad-Flag-KLF12 or Ad-LacZ (at a multiplicity of infection (MOI) of 20) for 48 h and then detected the absorbance value at 450 nm using Cell Counting Kit-8 (CCK8, Dojindo, Kumamoto, Japan) and the absorbance value at 450 nm determined cell viability in accordance with the manufacturer's protocol.

2.9. Colony formation assay

Two EC cells (Ishikawa and HEC-1B) were transfected with Ad-Flag-KLF12 or Ad-LacZ (at a multiplicity of infection (MOI) of 20) for 24 h. Then transfected cells were plated in a six-well plate with 1×10^3 cells per well and cultured at 37 °C in 5% CO₂. 14 days later, cells were washed with PBS and stained with 0.1% crystal violet in Methanol for 20 min. The colonies were counted with an inverted phase contrast microscope.

2.10. Cell apoptosis assay

Cell apoptosis was monitored using Cell Death Detection ELISA Kit (Roche Applied Science, Germany). Two EC cells were seeded in 12-wells with 5×10^4 cells per well. After incubating in serum-free medium for 12 h, cells were transfected with Ad-Flag-KLF12 or Ad-LacZ (at a multiplicity of infection (MOI) of 20) for 48 h. According to the manufacturer's instructions, cells were lysed and centrifuged at 200 ×g for 10 min before harvesting the supernatant and the mono- and oligonucleosomes in the supernatants were quantified using antibodies against DNA and histone-biotin. The absorbance at 405 nm was measured and the percentage of cell apoptosis and necrosis were assessed from the ratio of absorbance in the Ad-Flag-KLF12 treated cells to that of the controls.

2.11. Cell migration assay

Wound-healing assay was used to evaluate cell migration ability. As previously recorded, the transfected EC cells were seeded in 6-well plates and artificial wounds were created when cells were grown to confluent. At the same time, PBS was added to clear cell debris and cells were cultured in serum-free medium for 48 h. cell migration

gaps were photographed and measured with an inverted phase contrast microscope at the 0 h and 48 h.

2.12. Subcutaneous xenograft model

Female nude mice (four-week-old) were purchased from the Laboratory Animal Center of the Drum Tower Hospital Affiliated to Nanjing University Medical School (Nanjing, China). The use of animals was approved by the Ethics Committee of Drum Tower Hospital Affiliated to Nanjing University Medical School. Ishikawa cells (1×10^7) were subcutaneously injected to the right flank of female nude mice. 9 days later, mice with tumor formation were randomly divided into two groups and Ad-LacZ (2.0×10^6 MOI), Ad-Flag-KLF12 (2.0×10^6 MOI) were intratumorally injected into two groups of nude mice ($n = 8$ mice/group) every 3 days. The tumors of nude mice were observed every 3 days and tumor volume was measured using caliper with the traditional formula: $V = 1/2 \times \text{length (mm)} \times \text{width}^2 \text{ (mm)}$. Four weeks later, the nude mice were sacrificed and tumor tissues were stored for western blotting analysis.

2.13. Statistical analysis

χ^2 test was used to evaluate the associations between overexpression of KLF12 with related clinicopathological factors. The Kaplan-Meier method was used to compare the disease-free survival rate between different groups. Statistical analysis was performed using SPSS version 17.0 (SPSS, Chicago, IL, USA) and $P < 0.05$ was considered to indicate a statistically significant difference. All assays were independently performed for 3 times.

3. Results

3.1. Overexpression of KLF12 in EC and correlated with poor prognosis of EC patients

To compare the relative expression of KLF12 in EC with normal endometrium, we first detected the mRNA expression of KLF12 between 15 cases of EC tissues and 12 cases of normal endometrium by Real time-PCR analysis. The results showed that KLF12 transcriptional expression was higher in EC tissues compared with the normal endometrium ($P = 0.0041$) (Fig. 1A). As shown in Fig. 1B, KLF12 protein expression in paraffin-embedded specimens of EC tissues were also evaluated using immunohistochemistry. We further analyzed the relationship between KLF12 expression and clinicopathological factors of EC patients and found the expression level of KLF12 was significantly related to disease recurrence ($P = 0.033$, Table 1). KLF12 gene expression and patient survival data of 552 EC cases were accessed from the Human Protein Atlas (Supplementary material). Receiver operating characteristic (ROC) curve analyses, including the area under the curve (AUC) and the calculation of optimal cut-off values, were performed for KLF12 expression to determine the prognostic factors with the best predictive accuracy. The Kaplan-Meier survival analysis indicated that the survival rate was significantly higher in tissues with high KLF12 expression compared with tissues with low KLF12 expression (Fig. 1C). This finding suggested that KLF12 is associated with poor prognosis in human EC.

3.2. Generation of KLF12 expression in Ishikawa and HEC-1B cells

The endogenous KLF12 protein expression in HEC-1B cells was higher than Ishikawa cells (Fig. 2A). The Ishikawa cells and HEC-1B cells were both transfected with Ad-Flag-KLF12 or Ad-LacZ, Ad-GFP used as a control was transfected at the same time. RT-PCR and western blotting analysis were used to evaluate the efficiency of transfection and results showed that KLF12 mRNA and protein expression were highly expressed after transfection with Ad-Flag-KLF12 (Fig. 2B and C). Cell

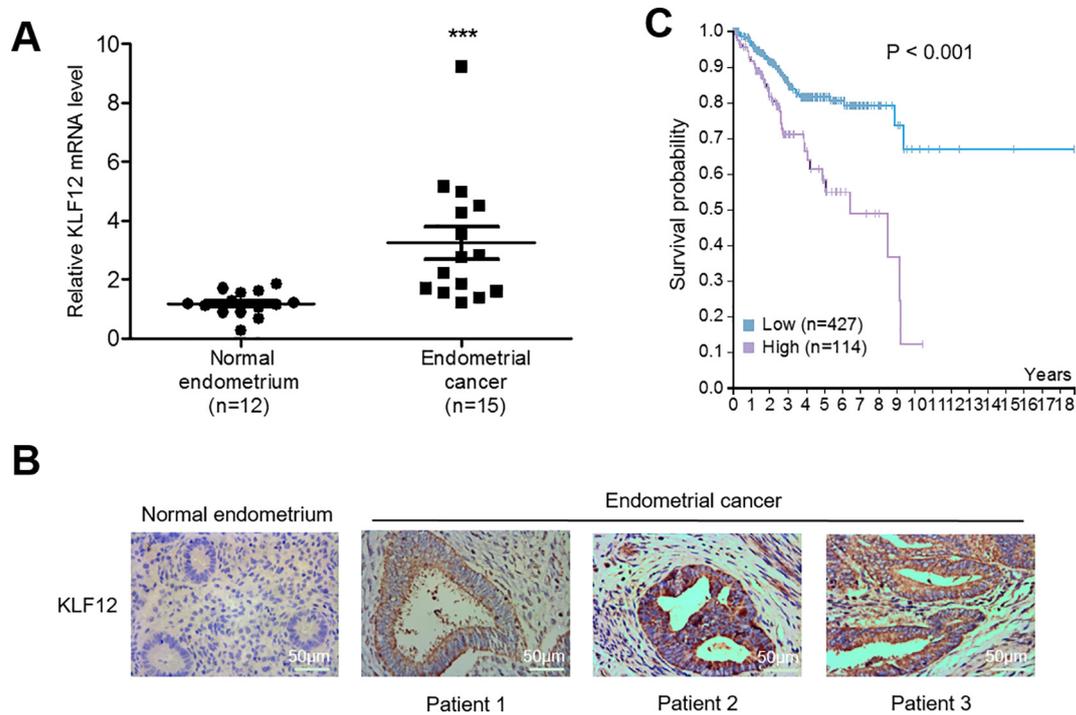


Fig. 1. Expression of KLF12 in tissues from endometrial cancer (EC) patients and the association between KLF12 expression and patients' survival rates. (A) Up-regulation of KLF12 mRNA was found in EC tissues (n = 15) compared with normal endometrium (n = 12). ***P < 0.001. Student's *t*-test. (B) Immunohistochemistry (HC) staining of KLF12 expression in EC tissues. KLF12 was positively stained in the cytoplasm and nucleus of the majority of tumor tissues. Normal endometrium and sample of patient 1 were negative for overexpression of KLF12. Samples of patient 2 and patient 3 were evaluated as overexpression of KLF12 (scale bars, 50 µm). (C) Clinical analysis and Kaplan–Meier curves were made to show the survival rate of EC patients with different KLF12 expression level. The cut-off value of KLF12 mRNA expression was 584.49.

immunofluorescence targeting KLF12 (red) in Ishikawa cell was mainly expressed in the nucleus (Fig. 2D).

3.3. Effects of KLF12 on proliferation, apoptosis and migration in EC cells

CCK-8 and colony formation assays were conducted to explore the role of KLF12 in regulating the proliferation of EC cells, Fig. 3A showed up-regulation of KLF12 promoted cell proliferation in Ishikawa cells ($P = 0.0053$) and HEC-1B cells ($P = 0.0191$). Meanwhile, colony formation capacity of Ishikawa cells and HEC-1B cells were significantly enhanced when KLF12 were upregulated ($P < 0.001$) (Fig. 3B and C). In addition, western blotting analysis in transfected cells was carried out to detect the protein levels of the related proliferation or apoptosis genes. Wound-healing assays were performed to evaluate migration capability changes and the statistical results suggested that KLF12 could enhance the migration ability of EC cells (Fig. 3D and E). Cell Death

Table 1
Association of KLF12 expression with clinicopathological parameters from EC patients.^a

KLF12 expression	Low (n = 23)	High (n = 40)	P value
FIGO stage			
I–II (n = 49)	20 (40.8%)	29 (59.2%)	0.184
III–IV (n = 14)	3 (21.4%)	11 (78.6%)	
Histological type			
EA (n = 51)	18 (22.2%)	33 (77.8%)	0.680
Non-EA (n = 12)	5 (41.6%)	7 (58.4%)	
Tumor grade			
G1/G2 (n = 49)	17 (34.6%)	32 (65.4%)	0.576
G3 (n = 14)	6 (42.8%)	8 (57.2%)	
Recurrence			
Yes (n = 15)	2 (13.3%)	13 (86.7%)	0.033
No (n = 48)	21 (43.7%)	27 (56.2%)	

EA, endometrioid adenocarcinoma; Non-EA, non-endometrioid adenocarcinoma.

^a Values are given as number (percentage), unless indicated otherwise.

Detection Elisa was performed to find whether KLF12 involves tumor cells apoptosis. As shown in Fig. 3F, the results indicated that the forced expression of KLF12 significantly decreased the proportions of apoptotic and dead cells ($P = 0.03$, $P = 0.092$).

3.4. MMP2, MMP9, pAKT S473 and CCND1 are up-regulated in the cells of over-expressing KLF12

Since the PI3K/AKT signaling pathway has been demonstrated to play a vital role in the development and progression of EC, phosphorylation of AKT was associated with tumor cell proliferation and apoptosis [19]. We detected the expression of phosphorylation of AKT and results showed that up-regulation of KLF12 evidently increased the expression levels of pAKT S473 and caused up-regulation of CCND1 protein (Fig. 4), a result consistent with the previous report that CCND1 could be regulated by PI3K/AKT [20]. Of particular note, the total AKT did not change after KLF12 expression, but the phosphorylation form increased significantly, suggesting that KLF12 does not affect AKT expression directly, but promotes AKT phosphorylation to regulate the pathway. In addition, accumulating evidence confirmed MMP-2 and MMP-9 expression levels are involved in the epithelial-mesenchymal transition (EMT) and cell migration and invasion [21]. The migration gaps were enhanced in KLF12-transfected cells, thus, we detected the expression of MMP2, MMP9, and found that KLF12 could significantly increase the expression of MMP2 and MMP9 (Fig. 4). Wound-healing assays were performed to evaluate migration capability changes and the statistical results suggested KLF12 could enhance the migration ability of EC cells.

3.5. Up-regulation of KLF12 promotes tumor growth in vivo

We injected Ad-Flag-KLF12 or Ad-LacZ into the tumor of nude mice to reveal the effect of KLF12 on EC growth in vivo, we found that tumor volume was significantly bigger in KLF12 group than control group, which suggested tumorigenesis of Ishikawa cells in vivo were

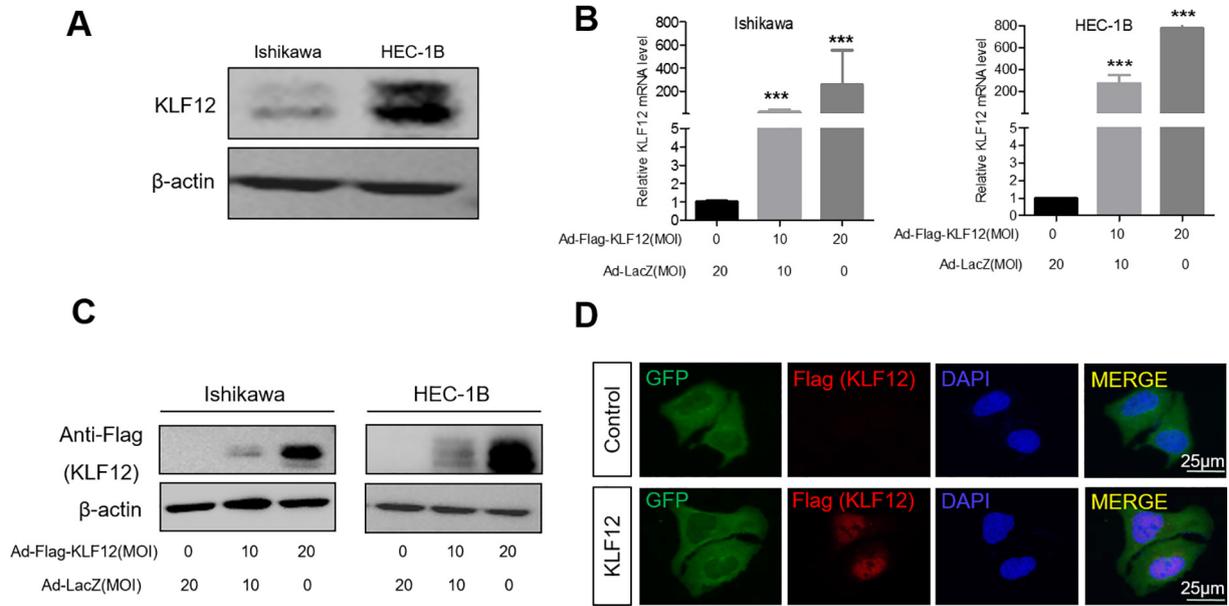


Fig. 2. Detection of KLF12 expression in Ishikawa and HEC-1B cells transfected with Ad-KLF12. (A) Endogenous KLF12 protein expression was detected in Ishikawa and HEC-1B cells using western blotting. (B–C) Relative mRNA and protein expression of KLF12 were measured by RT-PCR and Western blotting in Ishikawa and HEC-1B cells after transfected with Ad-KLF12. Data are expressed as means ± SEM. n = 3, ***P < 0.001, one-way ANOVA. (D) The cellular location of expressed KLF12 (Flag-tagged) in Ishikawa cells was analyzed by immunofluorescence staining via confocal microscopy. GFP was used for a cytoplasmic marker, DAPI was used for nuclear staining. Scale bars = 25 μm.

significantly promoted by upregulated-KLF12 (Fig. 5A and B). To verify the regulation of KLF12 to pAKT S473 and CCND1 in vivo, we examined the related protein expression in tumor tissues of nude mice and demonstrated the up-regulation of KLF12 also increased the protein expression of pAKT S473, CCND1 in vivo (Fig. 5C).

4. Discussion

KLF12 was identified as one of the members of the Krüppel-like factors (KLFs) family, which regulate gene transcription and tumor cell proliferation, differentiation and apoptosis through DNA binding

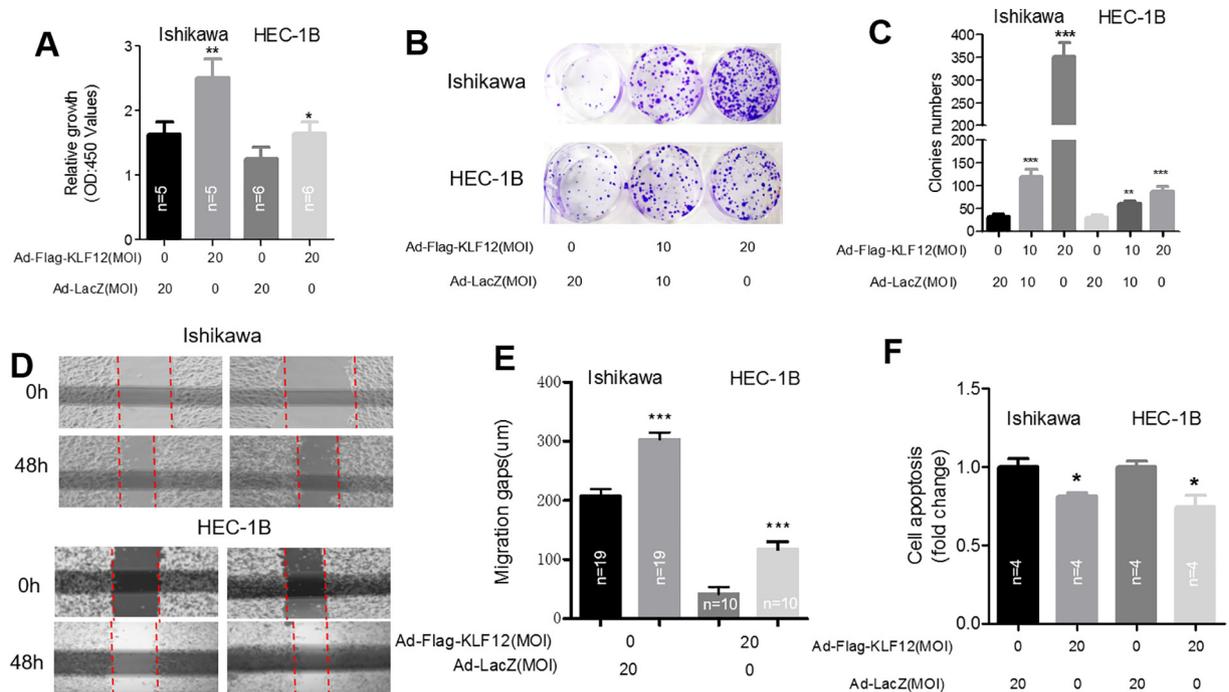


Fig. 3. KLF12 promoted cell proliferation and migration, and inhibited cell apoptosis. (A) Ishikawa and HEC-1B cells viability were assayed 48 h after transfected with Ad-KLF12 using CCK8. Data are expressed as means ± SEM. *P < 0.05, **P < 0.01, Student's t-test. (B–C) Representative micrographs and quantification colonies were determined by colony formation assay. Data are expressed as means ± SEM. n = 3, **P < 0.01, ***P < 0.001, one-way ANOVA. (D–E) Phase-contrast microscopy images and statistical results demonstrated Ishikawa and HEC-1B cells' migration gaps were increased after transfected with Ad-KLF12. Data are expressed as means ± SEM. ***P < 0.001, Student's t-test. (F) Cell migration was determined using Cell Death Detection Elisa Kit. Data are expressed as means ± SEM. ***P < 0.001, Student's t-test.

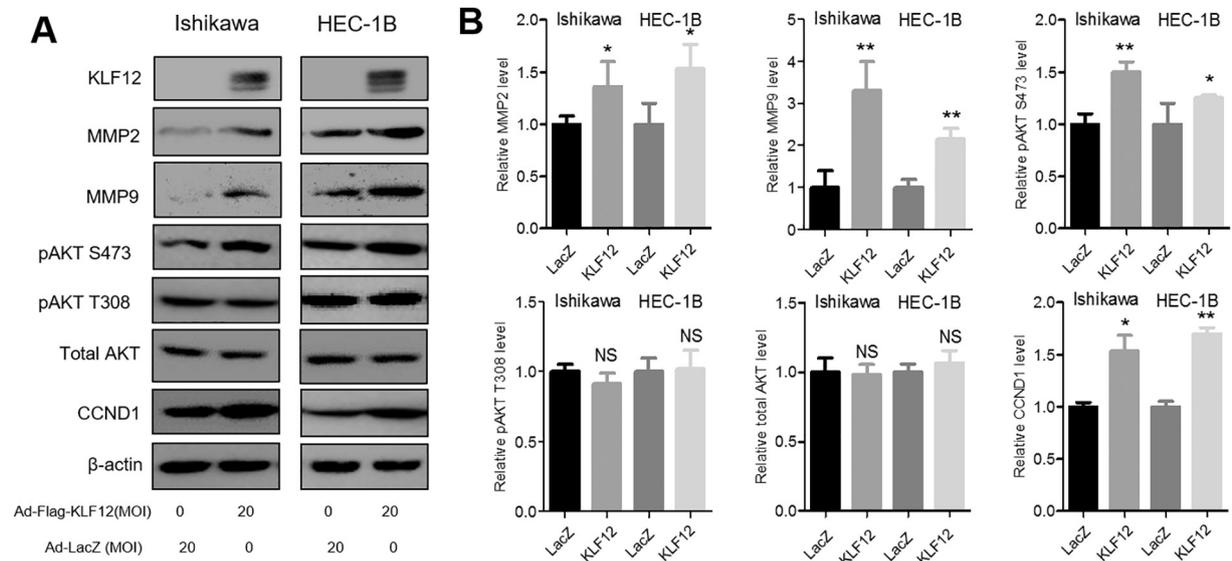


Fig. 4. MMP2, MMP9, pAKT S473 and CCND1 are up-regulated in the cells of over-expressing KLF12. (A) Representative immunoblot of multiple proteins from Ishikawa and HEC-1B cells transfected with KLF12, β -Actin was used as loading control. (B) Quantitative graphs of the proteins level. Data are expressed as means \pm SEM. $n = 4$; * $P < 0.05$, ** $P < 0.01$, Student's t -test.

domain at the carboxy-terminal binding to GC/CACCC rich-sequence in gene promoter [22]. Previous studies have shown that KLFs involved in the development and progression of endometrial carcinoma by affecting the proliferation and migration of EC cells. Recent researches suggested KLF12 could be the downstream target gene of miR-137, miR-382, miR-205 which are associated with the development of gastric cancer, osteosarcoma and breast cancer [23–25]. However, the function of KLF12 on EC remains unclear.

In our study, we aimed to investigate the KLF12 levels in EC samples, the correlation with clinicopathologic features and effects on the cellular biological function of two endometrial epithelial cancer cells (Ishikawa and HEC-1B). First of all, we tried to evaluate KLF12 expression in EC tissues and our results suggested KLF12 were overexpressed

in EC tissues compared to normal endometrium both at the mRNA and protein levels. Furthermore, combined immunohistochemistry results with clinical data analysis, we observed the KLF12 expression level was correlated with poor prognosis in EC patients which indicated that KLF12 protein expression may be an important prognostic factor for EC patients. We further confirmed increasing KLF12 promoted proliferation and migration and suppressed apoptotic and death in EC cells. Moreover, we also demonstrated that upregulated-KLF12 contributed to EC cell tumor growth in vivo. Our results are consistent with previous studies on the oncogene role of KLF12 in gastric cancer and colorectal cancer [15,16].

However, the specific regulatory mechanism of KLF12 on the biological behavior of EC is not clear. PI3K/AKT is an important regulatory axis

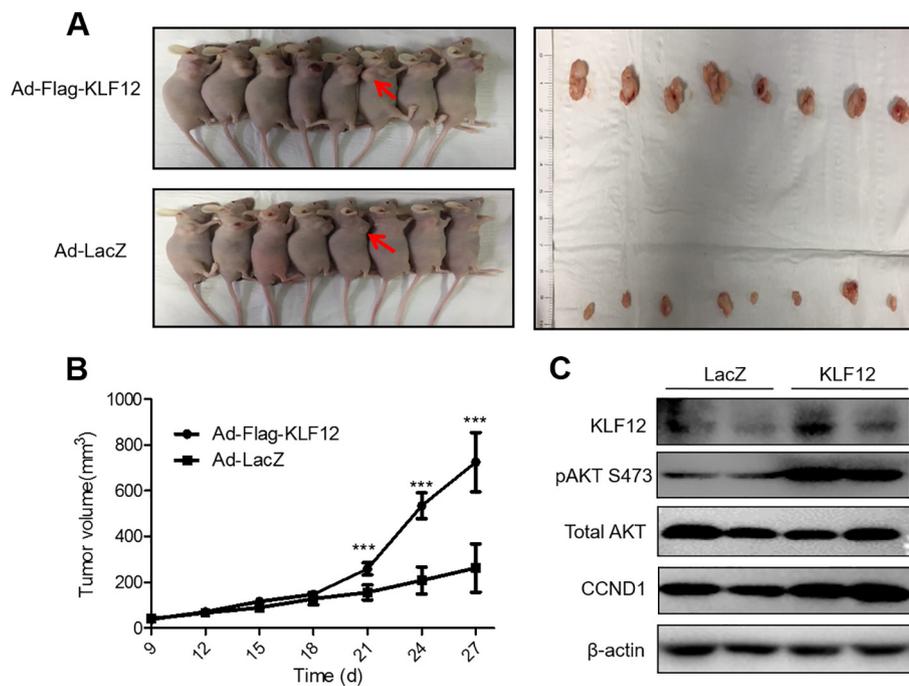


Fig. 5. KLF12 promoted tumor growth in vivo. (A) Representative photos of tumorigenesis of mice treated with Ad-KLF12 and Ad-LacZ. Tumors are marked with red arrow. (B) Tumor volumes were evaluated every 3 days, and tumors were harvested and tumor growth curves were made after 27 days. Data are expressed as means \pm SD. $n = 8$; **** $P < 0.001$, Student's t -test. (C) Representative immunoblot of the level of pAKT S473 and CCND1 in the tumors.

that regulates cell proliferation and differentiation, and its abnormal activity is closely related to the development of EC [3,26]. Based on the important regulatory role of AKT activation in cell proliferation and apoptosis, we tried to explore whether AKT was activated when KLF12 promoted the malignant proliferation of EC cells. Our study confirmed up-regulation of KLF12 enhanced the expression of phosphorylation of AKT in Ser473 both in the EC cells and tumor tissues. Previous researches have indicated the activation of PI3K/AKT signaling pathway shows the effect on promoting cell proliferation, metastasis, angiogenesis and inhibiting apoptosis by causing downstream effector molecules [27–29]. In our study, we found that the expression level of MMP2, MMP9 and CCND1 were both increased in KLF12 transfected cells or tumor tissues. CCND1 is an important cell-cycle regulator which is essential for cell transition from G1 phase to S phase. Accumulation of CCND1 has been implicated in the development of breast cancer, ovarian cancer and EC through acceleration of the cell cycle, promoting cell growth and angiogenesis [30–32]. Activation of AKT could phosphorylate GSK3 which would cause accumulation of CCND1 and cyclin E in cells and promoted G1/S progression of the cell cycle [33]. Briefly, we found that KLF12 could induce the expression of pAKT and CCND1 which also proven that KLF12 was closely involved in the apoptotic and proliferation process of EC cells. MMP-2 and MMP-9 were chief members in the Matrix metalloproteinases (MMPs) family which regulated the processes of migration and invasion of cancer cells by affecting the degradation of extracellular matrix and basement membranes [34]. It has been verified that the increasing in MMP-2 and MMP-9 expression levels are associated with metastasis of various malignant tumors, including EC [35,36]. When we found the migration gaps were enhanced in KLF12-transfected cells, we detected the increase expression of MMP2 and MMP9, so we thought the migration capability change of EC cells might be related to increasing MMP2 and MMP9 expression due to up-regulation of KLF12.

In the present study, we demonstrated that KLF12 up-regulation is involved in EC development and progression. Overexpression of KLF12 could effectively affect cell proliferation, apoptosis and migration in EC cell lines and promote tumor growth in vivo. Combined with previous evidence, we argue that KLF12 may promote the malignant proliferation and migration of EC cell lines by activating AKT, upregulating CCND1 and MMP2, MMP9. In our near further study, we will try to study the target genes of KLF12 relating EC development by CHIP-sequencing to reveal the specific molecular mechanisms underlying KLF12 regulates the AKT and related signaling pathway. Moreover, overexpression of KLF12 is strongly associated with poor prognosis of EC patients. Since KLF12 plays an essential role in EC development and progression, KLF12 may be a novel therapeutic strategy for the treatment of EC patients and a promising marker for evaluating the prognosis. In addition, KLF5, a member of the KLFs family, is frequently abnormal in human cancers, and several small molecules targeting KLF5 as potential therapeutics have been developed [37]. It is believed that molecules targeting KLF12 will be developed in the future. Of particular note, KLF6 regulates EGFR signaling in metastatic lung cancer, the combination of the intervention of KLF6 and EGFR may represent a novel therapeutic approach [38]. In our KLF12 story, KLF12 positively regulates AKT signaling, MMPs expression and CCND1 molecule. Thus, the rational combination of the intervention of KLF12 and its downstream signaling/target genes may be an attractive therapeutic approach for EC treatment. Our study, along with studies targeting KLF5 and KLF6, provides proof of concept for therapies targeting specific KLFs in cancer.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygyno.2018.10.028>.

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Author contribution

L. D., G.Y. and H. Z. designed the experiments and prepared the manuscript; L. D., Y. D., X. K., J. W, and J.F. performed the experiments; all authors analyzed the data; H. Z. conceived and supervised the project.

Declaration of interest

The authors report no conflicts of interest.

Competing interests

The authors declare no competing financial interest.

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