Original paper

Leukaemia-related protein 16 is highly expressed in oestrogen-dependent endometrial carcinoma and potentially promotes Ishikawa human endometrial cancer cells growth – a histopathological study

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Leukaemia-related protein 16 (LRP16) has been found to be highly expressed in various tumours and to be related to poor prognosis. However, the role of LRP16 in endometrial carcinoma remains to be explored. We aimed to investigate the prognosis and role of LRP16 in endometrial carcinoma. Overall, 160 endometrial carcinoma (EC) tissues and 60 benign samples were collected. The expression of LRP16 protein in EC tissues was significantly increased compared with that in normal endometrial tissues, and high LRP16 expression was related to poor patient prognosis. Reduced LRP16 expression markedly inhibited cancer cell growth. The proliferation rates in the prophylactic bilateral salpingectomy (PBS) group and the shNon group were 0.727 ±0.015 and 0.743 ±0.009, respectively, while the proliferation rate in the shLRP16 group was only 0.373 ±0.012. The migration experiment showed that the number of cells passing through the basement membrane in the shLRP16 group was 34.2 ±5.1, which was significantly different to the shNon (161.6 ±7.8) and PBS groups (138.0 ±7.2). The results of the invasion experiment showed that the number of cells was 39.2 ±6.2 in the shLRP16 group, 146.7 ±8.2 in the shNon group, and 141.2 ±8.1 in the PBS group ($p < 0.05$). Leukaemia-related protein 16 is highly expressed in oestrogen-dependent EC and may promote cancer cell growth.

Key words: endometrial carcinoma, LRP16, proliferation, migration, invasion.

Introduction

Endometrial carcinoma (EC) is one of the most common malignant gynaecological tumours of the female reproductive system. The age-standardised incidence is 26.0 cases per 100,000 women and is increasing each year. Endometrial carcinoma is already one of the primary diseases that endangers women's health [1]. Endometrial carcinoma is divided into 2 types: type I EC are oestrogen-dependent and often occur in young and perimenopausal women; type II EC are non-oestrogen-dependent and often occur in women a decade older than those with type I carcinoma, and in contrast to type I carcinoma, these tumours usually arise in the setting of endometrial atrophy. Type I cancers are believed to be closely associated with long-term unopposed oestrogen exposure [2].

Leukaemia-related protein 16 belongs to the macro domain family and was initially found to be associated with leukaemia. This protein serves as an intermediate signalling factor for oestrogen to regulate cell function and morphological characteristics

[3]. Previous studies suggest that LRP16 expression is related to poor tumour outcomes in many malignancies, such as colorectal carcinoma, breast cancer, and pancreatic cancer [4–6]. As for EC, Meng *et al*. reported that the oestrogen receptor in oestrogen-dependent EC cell lines (ISK cells) directly activates LRP16 transcription in response to oestrogen signalling. Over-expressed LRP16, in turn, represses E-cadherin expression and promotes the invasive growth of ISK cells [7]. However, the role and effect of LRP16 expression have not been explored in EC patients. Short hairpin RNA (shRNA) is a DNA molecule that can be cloned into an expression vector where it expresses small interfering RNA (siRNA, double-stranded RNA 19–21 nucleotides in length) to achieve gene knockdown.

The aims of this study are therefore to clarify the relationship between LRP16 expression and the prognosis of type I EC patients. Moreover, using shLRP16 interference, we downregulated LRP16 expression in human EC cells to confirm the effect on the biological characteristics of oestrogen-dependent EC cells (ISK) and oestrogen-independent EC cells (HEC-1a) *in vitro*.

Material and methods

Patient information and tissue specimens

In this retrospective study, all tissues were obtained from patients who visited the Department of Pathology, the First Affiliated Hospital of Bengbu Medical University, from January 2015 to December 2016. Their ages ranged 18–88 years. The case group consisted of 160 cases of oestrogen-dependent EC confirmed by routine pathological examination after surgery; the control group consisted of cases of endometrial biopsy or other benign diseases including 60 normal endometrial tissues. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the institutional ethics board of The First Affiliated Hospital of Bengbu Medical University (No. BBMEC-2020-12); informed consent was obtained from all patients. The inclusion criterion was oestrogen-dependent EC, and the exclusion criterion was oestrogen-independent EC by routine pathological examination.

Cell lines and culture conditions

The human EC cell line (Ishikawa, ISK cells, HEC-1a cells, accession number, CVCL_2529, CVCL_2580) was purchased from the Cell Centre of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Gibco, Thermo Fisher Scientific) containing 10% heat inactivated FBS and 100 μg/ml penicillin/streptomycin in an incubator at 37°C and 5% CO_2. The experiment selected cells in the logarithmic growth phase.

RNA interference assay

shRNA-LRP16 was constructed by cloning the full-length cDNA into pCMV plasmid vectors. The shRNA sequences are described below: shRNA-LRP16:

5'-gatcccgcagcgggaggaacattacttcaagagagtaatgttcctcccgctgctttttggaaa-3'. The negative control, termed shRNA-Non, was a vector with a nonsense cDNA fragment; shRNA-non:

5'-agcttttccaaaaaagcagcgggaggaacattactcttgaagtaatgttcctcccgctgcGG-3'. Then, ISK cells were seeded in a 6-well plate at a density of 3×10^5 cells/well. When the cell density reached 70–80% after transfection with shRNA-LRP16 vectors, the ratio of shRNA plasmid vector to lipofectamine concentration was 6 : 1.

Western blotting

Harvested cultured cells were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS) with proteinase inhibitor. A BCA protein assay kit (Takara Bio Inc., Otsu, Shiga, Japan) was used to measure the protein concentrations. Cell lysates were electrophoresed by SDS-PAGE, after which the proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore Corp., Kenilworth, NJ, USA). The membranes were blocked with 5% non-fat milk for one hour at RT, followed by incubation with an anti-LRP16 primary antibody (ab122688, Abcam). After 3 washes in PBST for 5 minutes each time, the membranes were probed with HRP-conjugated secondary antibody (1:2000, SC-2004, Santa Cruz Biotechnology, Dallas, TX, USA), and proteins were visualised using the Odyssey Infrared Imaging System (Li-COR, USA). The experiment was repeated 3 times. β-actin served as the intern control and was detected using anti-β-actin (ab8229, Abcam).

RT-PCR detection

TRIzol reagent was used to extract total RNA from each group of cells, which was verified by measuring the optical density (OD) 260/OD 280 (ratio, R). A retroviral reverse transcriptase kit (Takara, Tokyo, Japan) was used to synthesise cDNA under the reaction conditions of 37°C for 60 min and 95°C for 3 min. *LRP16* gene forward primer: 5'-CCGCAGCGACATCACCAAGC-3', reverse primer: 5'-TCCGGCACTCGTCGGTAAGC-3'; β-actin forward primer: 5'-AAAGACCTGTACGCCAACAC-3', reverse primer: 5'-GTCATACTCCTGCTTGCTGAT-3'. Real-time PCR was performed using a 7500 real-time quantitative PCR instrument (Applied Biosystems,

CA, USA) with the following conditions: 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 40 cycles. Data were normalised using the β-*actin* gene and were expressed as 2-ΔCt.

Immunohistochemistry

Tissue specimens were fixed in 10% neutral-buffered formalin and then embedded in paraffin for immunohistochemical examination. Briefly, 4-μm-thick paraffin sections were deparaffinised in xylene 3 times and rehydrated in graded ethanol solutions. After the sections were subjected to heat-mediated antigen retrieval in 0.01 M citric acid buffer solution, the sections were incubated in 3% H_2O_2 to block endogenous *peroxidase* activity. Sections were then probed with an LRP16 antibody (ab122688, Abcam) at a 1 : 100 dilution overnight at 4°C. Before and after incubation with the secondary antibody for 20 minutes at room temperature, sections were washed 3 times in PBS. Coloured products were developed by an SP immunohistochemical kit and a DAB chromogenic reagent kit (Fuzhou Maixin Biotech, Fuzhou, China). After counterstaining in haematoxylin, dehydration, and clearing, the slides were finally mounted using coverslips. The negative control received PBS instead of the primary antibody. The mean percentage of tumour cells was calculated in 5 areas of a given sample at a magnification of 400× and scored 0–3. The staining intensity was scored 0 for negative, 1 for weak, 2 for moderate, and 3 for strong. The weighted score is the intensity score multiplied by the percentage of tumour cells. We defined a score ≥ 75 as positive expression. The staining intensity of the cells and the percentage of positive cells were comprehensively scored [8, 9]. Image-Pro Plus software was used for image analysis.

CCK-8 proliferation assay

Cells in the logarithmic growth phase were selected, adjusted to a density of 5×10^4 cells/ml, seeded in a 96-well plate with 150 μl of cell suspension per well, incubated for 8 hours to allow cells to adhere, and then transfected. After 24, 48, 72, and 96 hours, 20 μl of CCK-8 reagent (Beyotime Biotechnology) was added to each well followed by a 6-hour incubation. The optical density value of each well was measured with a microplate reader at 450 nm. The experiment was repeated 3 times.

Cell invasion and migration experiments

In the invasion experiment, we evenly spread 50 μl Matrigel per well on the membrane of a Transwell chamber. After 48 hours of cell transfection, the cells in each group were digested into a cell suspension. The cell density was adjusted to 4×10^5 cells/ml, after which the cells were seeded in a Transwell chamber. We then added 200 μl of the cell suspension to a 24-well plate containing medium; the Transwell chamber was removed after 24 hours, and the cells in the upper layer of the filter were wiped with a cotton swab, fixed in methanol for 20 min, and stained with 0.1% crystal violet solution for 20 minutes. Finally, the average number of cells that passed through the membrane was counted in 15 random fields per membrane under a microscope, with 3 chambers in each group. This experiment was repeated 3 times. In the migration experiment, the Transwell chamber cell membrane was not covered with Matrigel, but the other steps were the same as those in the invasion experiment.

Statistical analysis

The Statistical Package for Social Sciences, version 25.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The measurement data for normality were used in the Shapiro-Wilk test, and the value was expressed as the mean ±SEM. After comparing multiple groups with the homogeneity of variance test, one-way analysis of variance (F test) was used, while the LSD method was used for comparisons between groups. $P < 0.05$ defined a significant difference.

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Results

Leukaemia-related protein 16 is overexpressed in human endometrial carcinoma samples

To investigate the role of LRP16 in EC, we compared LRP16 expression levels in 160 EC specimens from EC patients and 60 normal endometrial biopsy specimens using immunohistochemistry and Western blot analysis. The results showed that LRP16 is primarily expressed in the cytoplasm or nucleus and that its expression was weakened or even absent in normal endometrial tissues but was highly positive in EC tissues (Figs. 1A, B, C). The positive expression rates of the 2 groups were 13.3% (normal, $n = 60$) and 73.8% (cancer, $n = 160$).

The relationship between leukaemia-related protein 16 expression and clinicopathological factors of endometrial carcinoma

We further determined the clinical significance of LRP16 expression. The results showed that the expression of LRP16 in EC was closely related to histological grade, International Federation of Gynaecolo-

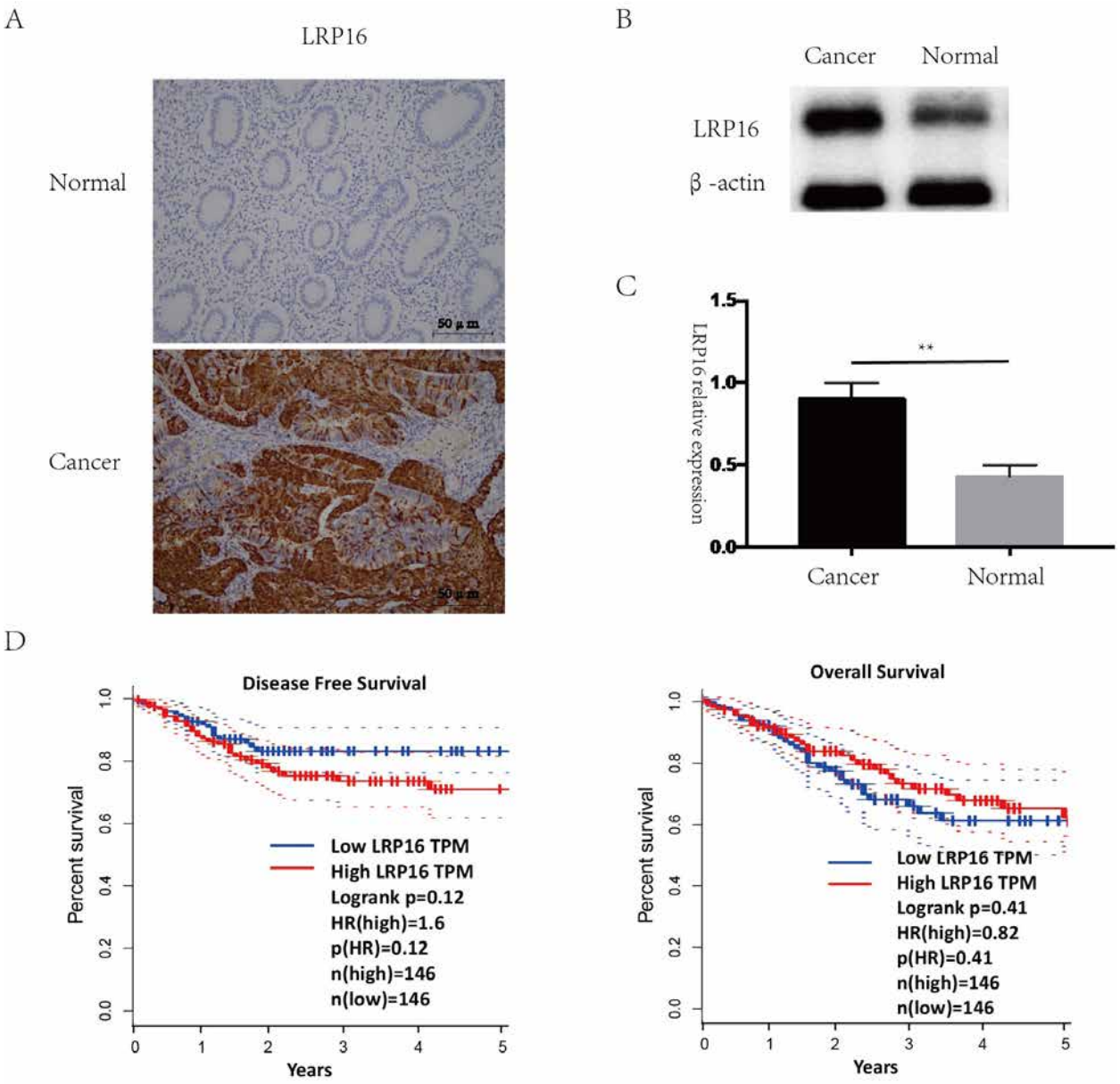

Fig. 1. LRP16 is overexpressed in human endometrial carcinoma samples. A) LRP16 expression in cancerous and normal endometrial tissues by immunohistochemistry staining. B, C) Western blotting was used to detect expression of the LRP16 gene in different endometrial tissues. D) The disease-free survival and overall survival of endometrial carcinoma patients with different LRP16 expression levels based on the data in the gene expression profiling interactive analysis system

*** $p < 0.01$*
The results are shown as the mean with SEM, unpaired Student's t-test.

gy and Obstetrics (FIGO) stage, myometrial invasion, and lymph node metastasis ($p < 0.05$) but was not significantly related to age ($p = 0.073$) (Table 1). In the control group, only 8 cases were LRP16 positive, including 6 cases of uterine leiomyoma and 2 normal endometrial biopsies. Moreover, we also found that EC patients with high LRP16 expression had a shorter disease-free survival and a high hazard ratio (HR) of 1.6 based on previous data from the gene expression profiling interactive analysis (GEPIA) system. The log-rank p-value was 0.12 (Fig. 1D). We hypothesised that this nonsignificant difference might have been caused by the small sample size and the lack of differentiation between type I and type II EC at the time of enrolment, which requires further verification.

The downregulation of leukaemia-related protein 16 in human endometrial carcinoma cells significantly inhibits tumour cell proliferation, migration, and invasion

These experimental data suggest that LRP16 may be related to the malignant growth of endometrial

Table 1. LRP16 expression was highly associated with endometrial carcinoma development

Parameters		LRP16		
Factor	Cases	Negative	Positive (%)	*p*-value
Histological type				
Normal endometrium	60	52	8 (13.3)	
EC	160	42	118 (73.8)	< 0.01
Age (years)				
< 50	44	16	28 (73.7)	
> 50	116	26	90 (73.8)	0.073
Histological grade				
G1	43	14	29 (67.4)	
G2	87	16	71 (86.6)	
G3	30	12	18 (51.4)	0.037
FIGO stage				
I + II	92	32	60 (65.2)	
III + IV	68	10	58 (85.3)	0.004
Muscular layer infiltration				
≤ 1/2	96	33	63 (65.2)	
> 1/2	64	9	55 (86.0)	0.004
Lymph node metastasis				
No	88	30	58 (66.0)	
Yes	72	12	60 (83.3)	0.013

EC – endometrial carcinoma, FIGO – International Federation of Gynaecology and Obstetrics

cancer cells. Next, we further evaluated the roles of LRP16 in cell proliferation and invasion in EC cells. By Western blot analysis, we found that the LRP16 protein was highly expressed in ISK cells. Specific shRNA for LRP16 was stably transfected into ISK cells to silence LRP16 expression. The efficiency of LRP16 knockdown was confirmed through qRT-PCR and Western blotting. The expression of LRP16 protein in the shLRP16 group was significantly decreased compared with that in the shNon group and the PBS group ($p < 0.05$) (Figs. 2A, 2B). Then, the proliferation and invasiveness of EC cells upon LRP16 downregulation were investigated. As shown in Figure 2C, reduced LRP16 expression markedly inhibited ISK cancer cell proliferation. After incubation for 96 hours, the proliferation rates in the PBS group and shNon group were 0.727 ±0.015 and 0.743 ±0.009, respectively, while the proliferation rate in the shLRP16 group was only 0.373 ±0.012.

Moreover, downregulation of LRP16 reduced cell invasion and migration in Transwell assays compared with the PBS and shNon groups. The results of the migration experiment showed that the number of cells that passed through the basement membrane in the shLRP16 group was 34.2 ±5.1, which was significantly different than the number of cells that passed through the membrane in the shNon group (161.6 ±7.8) and the PBS group (138.0 ±7.2). The invasion experiment showed that the number of cells that passed through the membrane was 39.2 ±6.2 in the shLRP16 group, 146.7 ±8.2 in the shNon group, and 141.2 ±8.1 in the PBS group ($p < 0.05$) (Fig. 2D). Similar results were obtained in HEC-1a EC cells. However, the inhibition efficiency of cell proliferation, invasion, and migration in HEC-1a cells were weaker than that of ISK cells (Fig. 3).

Discussion

Endometrial carcinoma is one of the most common and lethal malignancies of the female reproductive system. For type I EC in postmenopausal women treated for menopausal symptoms, long-term unopposed oestrogen stimulation is one of the main pathogenic factors [10, 11]. In our study, we analysed the LRP16 expression level in EC and found that high LRP16 expression was closely related to tumour histological grade, FIGO stage, muscular layer infiltration, and lymph node metastasis. Our *in vitro* experiments further verified that the proliferation

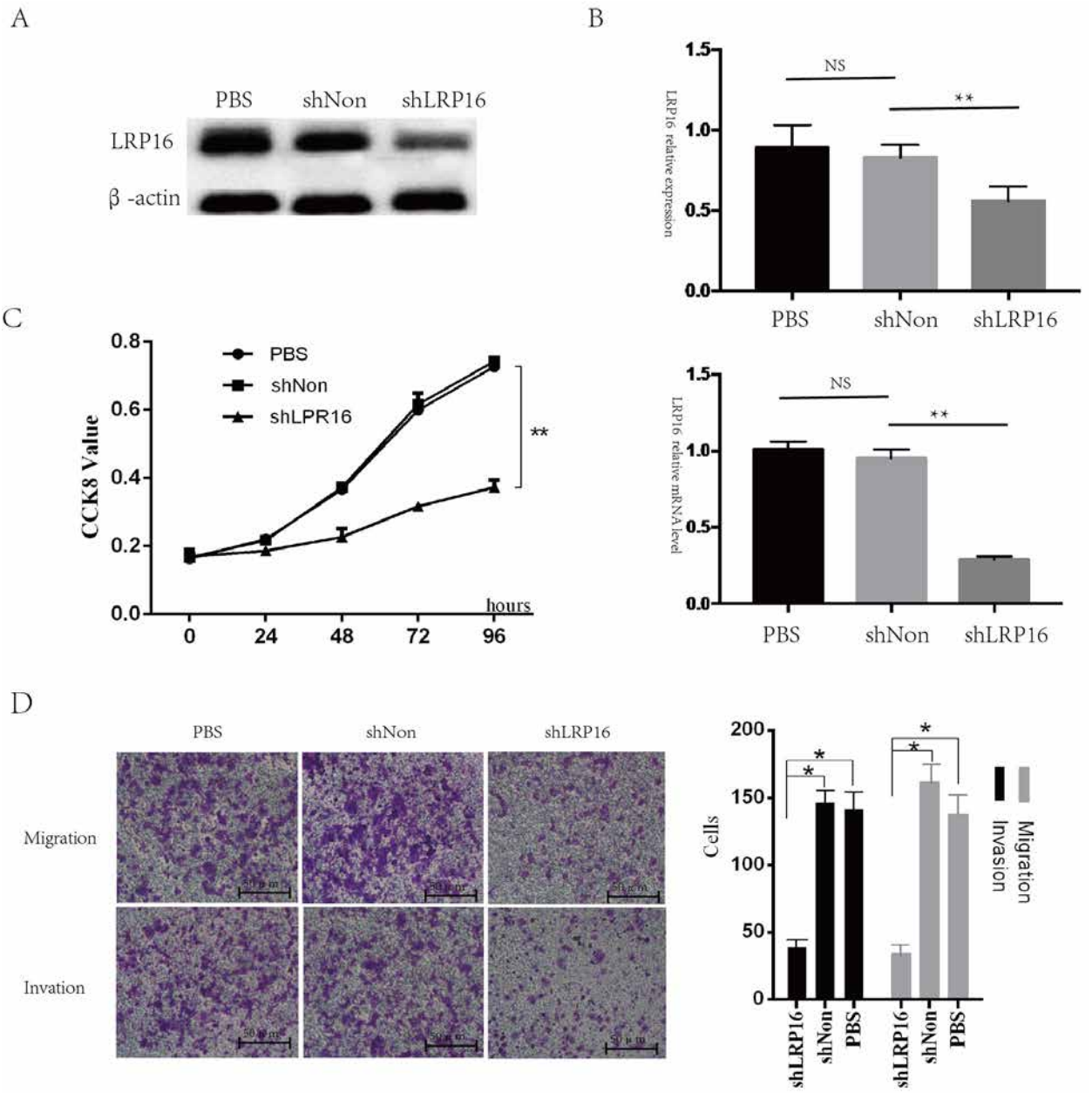

Fig. 2. The effects of LRP16 knockdown on ISK cells. A) Western blotting was used to detect the shRNA-mediated downregulation of LRP16 in ISK cells. B) The efficiency of LRP16 knockdown was confirmed through qRT-PCR and Western blotting analysis. C) Reduced LRP16 expression markedly inhibited ISK cell proliferation. D) Reduced LRP16 expression markedly inhibited ISK cell migration and invasion

*$p < 0.05$
**$p < 0.01$
The results are shown as the mean with SEM, unpaired Student's t-test.

time of EC cells was significantly prolonged and that the invasion and migration ability of ISK cells was significantly inhibited after LRP16 downregulation.

The downstream targets of the oestrogen receptor may be involved in the initiation and progression of type I EC. Therefore, the search for new oestrogen-related target genes and their biological functions has essential diagnostic and therapeutic value for the study of EC at the molecular level. The *LRP16* gene was first discovered in 1999 [12]. Serial analysis of gene expression revealed that the expression level of this gene in a variety of human tumour cells is significantly higher, especially in oestrogen-dependent tumours, than in normal tissues [13, 14]. Researchers found that LRP16 selectively interacts and activates double-stranded RNA-dependent kinase (PKR) and assists in the formation of a ternary complex of PKR and IKKβ, prolonging the polymers of nuclear factor kappa B (NF-κB) transactivation caused by DNA-damaging agents, and it confers acquired chemoresistance in colorectal cancer [15, 16]. Studies have found that

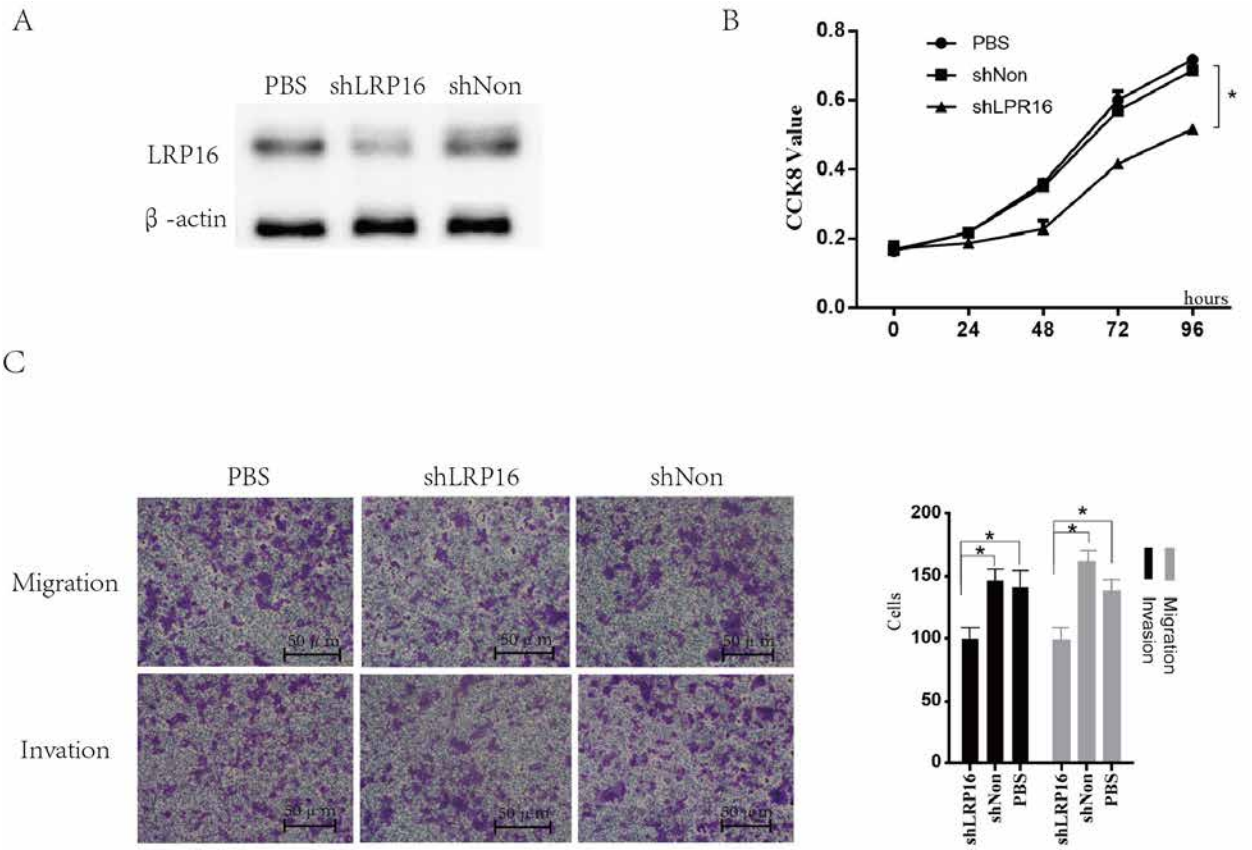

Fig. 3. The effects of LRP16 knockdown on HEC-1a cells. A) Western blotting was used to detect the shRNA-mediated downregulation of LRP16. B) Reduced LRP16 expression inhibited HEC-1a cell proliferation. C) Reduced LRP16 expression inhibited HEC-1a cell migration and invasion

** $p < 0.05$*
The results are shown as the mean with SEM, unpaired Student's t-test.

the *LRP16* gene is a nucleoprotein factor response gene in the oestrogen signalling pathway, which is related to the genesis and development of oestrogen-dependent tumour cells [5, 17, 18]. Meng *et al*. found that the oestrogen receptor in ISK cells (ERα-positive EC cells) directly activates LRP16 transcription, then the LRP16 represses E-cadherin expression, increasing the probability of invasion and metastasis [18]. We also confirmed these results by downregulating LRP16 expression and found that downregulation of LRP16 in HEC-1a cells (ERα-negative EC cells) also inhibits tumour cell proliferation, migration, and invasion. However, the inhibition efficiency in HEC-1a cells was weaker than that of ISK cells. We supposed that this may be related to the lack of oestrogen receptor activation.

However, most studies are merely based on bioinformatic analyses and cell experiments. Our large-scale research study demonstrated that LRP16 expression was increased in EC patients and that the positive rates of LRP16 expression (73.8%) were higher in EC than in normal tissues. The expression of the *LRP16* gene in EC was closely related to tumour histological grade, clinical stage, myometrial invasion, and lymph node metastasis ($p < 0.05$). Endometrial carcinoma patients with high LRP16 expression had a shorter disease-free survival (HR = 1.6) based on previous data in the GEPIA database, but the overall survival was similar, which indicates that patients with high LRP16 expression are more likely to experience tumour recurrence.

This study also has some limitations. First, a sample size calculation was not performed, and second, the relationship between the abnormal expression of the *LRP16* gene and the prognosis of patients with type I EC is still under investigation.

Conclusions

Our results suggested that LRP16 may promote human EC progression. The exact mechanism by which LRP16 mediates its target genes and signalling pathways, and the effects on the occurrence and development of EC remain to be further studied. *In vivo* experiments are needed to further support the finding that the expression levels of LRP16 are positively correlated with EC progression.

The authors declare no conflict of interest.

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