LRP16 Promotes Proliferation and Migration of Esophageal Squamous Cell Carcinoma by Regulating Hippo Signaling Pathway

Cong Liu
First Affiliated Hospital of Zhengzhou University

Chao Xiong
The First Affiliated Hospital of Nanchang University

Xianzeng Wang
Linzhou People's Hospital of Henan Province

Ting Sun
First Affiliated Hospital of Zhengzhou University

Zhenzhen Ren
First Affiliated Hospital of Zhengzhou University

Bingxue Li
University of Traditional Chinese Medicine

Man Zhang
First Affiliated Hospital of Zhengzhou University

Zhaoyang Zheng
University of Traditional Chinese Medicine

Hongchun Liu (xingyunerliu@163.com)
First Affiliated Hospital of Zhengzhou University

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Abstract

The present study aimed to investigate the expression of LRP16 in the development of ESCC and the relationship between Hippo signaling pathway and LRP16. Immunohistochemistry was used to detect the expression of LRP16 in ESCC tissues. After transfection, the expression of LRP16 was detected by reverse transcription quantitative PCR (RT-qPCR) and western blot techniques. Cell counting kit (CCK-8), clone formation experiment, flow cytometry and wound healing were used to determine the proliferation, apoptosis, cell cycle and migration of ESCC cells. The changes of factors related to Hippo signaling pathway were determined via RT-qPCR and western blot experiments. The results showed that the LRP16 expression in ESCC tissues was higher than that in normal tissues. High expression of LRP16 was related to the depth of invasion, TNM stage and lymph node metastasis of ESCC. Furthermore, the knockdown of LRP16 inhibited proliferation, migration and promoted cell apoptosis and made cells arrested in G2/M phase. It also resulted in decreased expression of Yes-associated protein (YAP), and increased expression of mammalian STE20-like protein kinase (MST1/2), suggesting that LRP16 promoted the development of ESCC through Hippo signaling pathway. The results of this study suggest that LRP16 may be a carcinogenic gene of ESCC and promotes the progression of ESCC through the regulation of Hippo signaling pathway. Our study provides a new idea for the diagnosis and treatment of ESCC in the future.

Introduction

Esophageal cancer (EC) is one of the most common malignant tumors in the digestive tract, and also one of the top ten most common malignant tumors in the world\textsuperscript{[1, 2]}. According to the latest global cancer survey in 2018, the number of new cases of EC is about 570,000, accounting for 3.2% of all cancers at present and ranking the seventh among all malignant tumors\textsuperscript{[3, 4]}. The number of deaths from EC is about 510,000, accounting for 5.3% of the total global cancer incidence and sixth among all malignant tumors. Due to its poor prognosis and extremely high mortality rate, it is considered as one of the most malignant tumors\textsuperscript{[5]}. EC is the most common in China and southern Iran, and about 50% of EC occurs in China, especially in parts of Henan Province\textsuperscript{[6, 7]}. According to the histopathological types of EC, it can be divided into two types: esophageal adenocarcinoma and esophageal squamous cell carcinoma\textsuperscript{[8]}. At present, the research on the pathogenesis of EC has not been thoroughly studied, and the mechanisms of adenocarcinoma and squamous cell carcinoma are different. For EAC, the main causes are consistent with human obesity and gastroesophageal reflux. However, the mechanism of ESCC is still unclear, which is also the main reason for the delayed detection method of ESCC. Although about 90% of EC can be eradicated by surgery, the onset of EC is hidden, and early diagnosis is difficult, it has the potential of invasion and metastasis, once it is found that most of them are already late. Therefore, at present, the main treatment is still surgery, supplemented by radiation therapy and drug therapy, but after 5 years of survival rate is only 15-25\textsuperscript{[5, 9]}. Early diagnosis of ESCC reduces the likelihood of tumor cell metastasis and significantly improves patient prognosis. Therefore, there is an urgent need to explore new molecular mechanisms related to the development of ESCC, so as to improve the diagnosis and treatment of ESCC\textsuperscript{[10]}. 
As a recently developed macro domain protein\cite{11, 12}, Leukemia-associated protein 16 (LRP16) can interact with multiple nuclear receptors, belonging to the coactivator of the nuclear receptor family. It is also unique related to estrogen and is the coactivator of ERα. Through the analysis of gene expression sequence, it was found that the expression of LRP16 in most tumor tissues was higher than that in the corresponding normal tissues, and it was also closely related to sex hormone receptors. First, LRP16 expression was significantly increased in a variety of estrogen-dependent tumors\cite{13-15}. It has been confirmed that estrogen-dependent tumors, including insulinoma, breast cancer and ovarian cancer, have different clinicopathological changes, such as tumor invasion depth, TMN stage, lymph node metastasis and so on. Furthermore, in non-estrogen-dependent tumors such as liver cancer, gastric cancer, colorectal cancer, it has also been found that the expression of LRP16 protein was different from the corresponding normal tissues, and related to the clinicopathological features\cite{13, 16, 17}. It suggests that LRP16 is a tumor-related gene that participates in the occurrence and development of tumors. Based on the above research, we put forward the following hypothesis: whether LRP16 is involved in the development of ESCC. This study will investigate the biological function and molecular mechanism of LRP16 in ESCC, and provided new ideas for the diagnosis and treatment of ESCC in the future.

Hippo signaling pathway regulates cell proliferation and promotes apoptosis\cite{18}, and is involved in regulating organ size and tissue regeneration\cite{19-21}. The key protein molecule in Hippo signaling pathway is Yes-associated protein (YAP)/transcriptional co-activator with PDZ binding motif (TAZ), which is the most important effector of Hippo signaling pathway\cite{19}. Other factors in this pathway, such as mammalian STE20-like protein kinase (MST1/2) and large tumor suppressor 1/2 (LATS1/2), can regulate YAP negatively through phosphorylation, resulting in decreased expression of downstream transcription factors, such as cyclin E and Death-associated inhibitor of apoptosis 1 (DIAP1), inhibiting cell proliferation and promoting cell apoptosis\cite{22-24}. Recent studies have shown that ERα is related to YAP, the enhanced binding of YAP1/TEAD4 to ERα can induce the growth of breast cancer\cite{25}. Therefore, we speculated that LRP16 protein may be related to the Hippo signaling pathway.

In this study, we detected the expression level of LRP16 in ESCC tissues and analyzed its relationship with the progression of ESCC. LRP16 was knocked down to study the changes of growth, proliferation, cell clustering, apoptosis and cell cycle of ESCC cells. The changes of Hippo signaling pathway were preliminarily investigated after LRP16 knocked down. This study determined the expression level of LRP16 in ESCC and its relationship with clinicopathological features, clarified the biological function of LRP16 in ESCC and the molecular mechanism, providing new research ideas for the treatment of ESCC in the future.

**Material And Methods**

**Ethics statement**
All studies involving human participants were approved by the Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University, including any relevant details (Permit No.2020-KY-318). All patients provided written informed consent. We confirm that all experiments were conducted in accordance with the relevant guidelines and regulations. All studies involving human participants were conducted in accordance with the Declaration of Helsinki.

**Tissue samples collection**

Paraffin sections of esophageal squamous cell carcinoma: 53 cases of cancerous tissues, 20 cases of atypical hyperplasia and 19 cases of normal tissues were donated by the Pathology Laboratory of the First Affiliated Hospital of Zhengzhou University. All patients were certified as ESCC and had not been treated with surgery, chemotherapy or radiotherapy before. The clinicopathological features of all patients, including age, sex, tumor grade, lymph node metastasis and TNM stage, were detailed in Table 1 below, Table 2 showed that the expression of cancer tissues and atypical hyperplasia tissues were different from that of normal tissues. The paraffin sections are made by the operator of the Pathology Department of the Third Affiliated Hospital of Henan University of Traditional Chinese Medicine. This study was approved by the Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All patients provided written informed consent.
| Clinicopathological parameters | LRP16                  | Total                  | LRP16 |
|-------------------------------|------------------------|------------------------|-------|
|                               | Low, n=20               | High, n=33             |       |
| Sex                           |                        |                        | 0.015 |
| Male                          | 13                     | 22                     | 35    |
| Female                        | 7                      | 11                     | 18    |
| Age                           |                        |                        | 0.034 |
| < 65 years                    | 9                      | 14                     | 23    |
| ≥ 65 years                    | 11                     | 19                     | 30    |
| Infiltration depth            |                        |                        | 5.461 |
| T1+T2                         | 7                      | 3                      | 10    |
| T3+T4                         | 13                     | 30                     | 43    |
| TNM stage                     |                        |                        | 7.272 |
| I-II                          | 9                      | 4                      | 13    |
| III-IV                        | 11                     | 29                     | 40    |
| Lymphatic invasion            |                        |                        |       |
| Yes                           | 8                      | 24                     | 32    |
| No                            | 12                     | 9                      | 21    |
| Pearson $\chi^2$             | 0.015                  | 0.034                  | 5.461 |
| $P$ Value                     | 0.901                  | 0.854                  | 0.019*|

*Statistical significance ($P<0.05$). aContinuity correction: $1<n\leq5$. LRP16, Leukemia-associated protein 16.
### Table 2
The relationship among ESCC tissue, atypical hyperplasia tissue and normal tissue.

| Contrast group                                      | LRP16 | $\chi^2$ | $P$ Value* |
|-----------------------------------------------------|-------|----------|------------|
|                                                     | High  | Low      |            |
| Cancer tissue and atypical hyperplasia tissue        | 33    | 20       | 0.047      |
|                                                     | 13    | 7        | 0.829−     |
| Cancer tissue and normal tissue                      | 33    | 20       | 7.252      |
|                                                     | 5     | 14       | 0.007*     |
| Atypical hyperplastic tissue and normal tissue       | 13    | 7        | 5.867      |
|                                                     | 5     | 14       | 0.015*     |

# Bonferroni correction (#P<0.0167). *Statistical significance (P<0.05).

LRP16, Leukemia-associated protein 16.

### Immunohistochemical (IHC) staining

Tissue samples of patients and normal tissues (4µm paraffin section, 4% paraformaldehyde fixed at room temperature for 48 hours) were taken for IHC staining. The paraffin slices were placed in an oven at 60 °C for 1 hour, then dewaxed and hydrated, and rinsed with PBS for 3 minutes each time. To repair the antigen, the slices were slowly put into the EDTA antigen repair solution, treated in a pressure cooker for 3 minutes and rinsed with PBS for 3 minutes each time. Subsequently, the slides were treated with 3% hydrogen peroxide to inhibit the activity of endogenous peroxidase, and 10min was incubated at room temperature. 10% goat serum (Beijing Kangwei Century Co., Ltd.) was used to block the non-specific binding site 10min at room temperature. The slices were incubated overnight with anti-LRP16 (Abcam) first antibody at 4 °C, and then incubated with biotin-labeled sheep anti-rabbit second antibody (Beijing Kangwei Century Co., Ltd.) at room temperature for 11 minutes. The DAB chromogenic solution was dripped slowly on the slice to develop color, and then carefully observed under the microscope. Re-dyeing with hematoxylin at room temperature for 3 minutes, dehydration (70, 75, 90, 95 and 100% alcohol reduction series 1 min, xylene 15 min), dried with gum seal, and observed by two pathologists in the pathology laboratory of the first affiliated Hospital of Zhengzhou University under light microscope (magnification, X100). The experimental results are calculated according to different staining degrees and the proportion of chromogenic cells. The staining degree ranges from 0 to 3, and the proportion of chromogenic cells ranges from 0 to 100%. Define the dyeing degree score: 0 (no coloring), 1 (light yellow), 2 (yellow-brown), 3 (brown). Positive staining areas: 1 (1-10%), 2 (11-50%), 3 (51- 80%), 4 (> 80%). The final score was calculated by multiplying the staining degree and the percentage of chromogenic cells. 0: negative, 1-4: weak positive, > 4: strong positive.

### Cell Culture and Cell Lines
The cell lines involved in this experiment, including TE-1, EC9706, EC109, KYSE510 and KYSE30. TE-1 and EC9706 are donated by Henan University of Traditional Chinese Medicine, EC109, KYSE510 and KYSE30 are kept by our laboratory. These cells were cultured in RPMI 1640 medium (Beijing Solaibao Technology Co., Ltd.) containing 10% foetal bovine serum (Zhejiang Tianhang Biological Technology Co., Ltd.) with culture conditions of 37.0°C with 5% CO₂.

**Western blotting**

Total proteins were extracted from the cells using RIPA lysis buffer (Beijing Kangwei Century Biotechnology Co., Ltd.) and quantified using a bicinchoninic acid protein detection kit (Sangon Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Then, the protein was separated by 10% SDS-PAGE and transferred to the PVDF membrane. After sealing with skimmed milk powder at room temperature for 1 hour, washed the gel with TBST, added diluted primary antibody, and incubated at 4°C overnight. The membrane was then washed with TBST and incubated with the corresponding secondary antibody at room temperature for 2 hours. Protein strip images were displayed with an ECL kit (Sangon Biotechnology Co., Ltd.), captured by chemiluminescence imaging system (EMD Millipore), and then analyzed by ImageJ (Version 1.8.0; The National Institutes of Health). GAPDH is used as an internal reference for standardization.

**RNA extraction and reverse transcription quantitative PCR (RT-qPCR)**

According to the manufacturer’s scheme, the total RNA was extracted from Trizol reagent (INVITROGEN). According to the manufacturer’s protocol (Takara Biotechnology Co., Ltd.), total RNA was reverse transcribed into cDNA using PrimeScript Reverse Transcription kit. Then, according to the manufacturer’s scheme (Takara Biotechnology Co., Ltd.), TB Green PreMix Taq II kit was used for qPCR, to quantitatively detect LRP16 mRNA in cells on LightCycler 480II Real-Time fluorescence PCR system (Roche Diagnostics). The thermal cycle conditions are as follows: pre-denaturation at 95 °C for 30 sec for 1 cycle, then 95 °C for 5 sec, 60 °C for 20 sec for 40 cycle and followed by the melting curve and cooling stage at 65 °C for 20 sec. The RNA levels were calculated by $2^{-\Delta\Delta ct}$ method. The following primers were applied to qPCR:

LRP16 forward: 5’-ACTTTGTCAGGCTGAAGAAGAT-3’

LRP16 reverse: 5’-AGCAGGGAGATTTTCTCATTGA-3’;

GAPDH forward: 5’-GTGGACCTGACCTGCCGTCTAG-3’

GAPDH reverse: 5’-GGAGTGGGTGTCGCTGTTGAAG-3’.

GAPDH was used as an internal reference for standardization.

**In Vitro Transfection**
The following shRNA plasmids were used in this study for in vitro transfection: shRNA-pGPU6/GFP/Neo-LRP16-homo-1019: 5'-GCTGATCATCTGCGTGTTCCT-3'; shRNA-pGPU6/GFP/Neo-LRP16-homo-407: 5'-GGAGGCGAAATCCTTTCTGAA-3'; shRNA-pGPU6/GFP/Neo-shNC: 5'-TTCTCCGAACGTGTCACGT-3'.

The plasmid was purchased in Shanghai Jima Pharmaceutical Technology Co., Ltd. The cells were transfected with liposomes. The plasmid was mixed with the transfection reagent at a ratio of 1:1-1:5, and let the mixture stand for 30 minutes. The above mixture was then added to the medium with the cells. The medium was replaced with new medium after 24-48 hours. The transfected cells were screened using G418. A stably transfected cell line was finally obtained. RT-qPCR and Western blotting were used to verify the expression of LRP16 mRNA and protein in ESCC after knockdown.

**CCK-8 assay**

An enhanced Cell Counting Kit-8 (CCK-8, BIOSS) was used to measure the cell proliferative ability. Transfected cells were digested to 5.0 × 10^4 cell/ml, and 100µl cell suspension was added to the 96-well plate, which was divided into negative control group and experimental group. Five compound holes were made in each control group and monitored continuously for 4 days. The aseptic PBS solution was dropped around the 96-well plate to prevent the evaporation of the culture medium in the plate. Add 10µl of CCK-8 to every 100µl medium. The OD value of TE-1 cells at 450nm was determined after being cultured for 2h in an incubator with 5% CO₂ at 37°C.

**Clone formation experiment**

Transfected cells were inoculated on a 6-well plate and cultured in an incubator containing 5% CO₂ at 37°C. The fluid was changed every 2-3 days, and 3 controls were made in each group. The culture can be terminated when the cloned cells are clearly visible to the naked eye. Discard the supernatant and wash it twice carefully with 4°C precooled PBS solution. The cells were fixed at room temperature for about 15 min by dropping 1ml 4% paraformaldehyde. Suck out the fixed solution from the straw, dye the crystal violet solution for 10-30 minutes, wash the crystal violet dye slightly with running water, and dry it upside down. Count under the microscope. Clone formation rate = (amount of clones / number of inoculated cells) × 100%.

**Wound healing assay**

Transfected cells in logarithmic growth phase were placed in a 6-well plate with a cell density of 6.0×10^5/well and cultured in an incubator at 37°C and 5% CO₂. When the cell monolayer adhered to the wall, it was scraped with 200µl pipette tube. The cells were washed by PBS, cultured with 1% low serum medium and placed in an incubator with 5% CO₂ at 37°C. The wound area was observed under the inverted microscope at 0, 24, 48 and 72h, recorded and analyzed the results carefully.

**Flow cytometry**

1-5×10^5 resuspend cells were washed once with PBS, and 500µL diluted 1×Annexin V Binding Buffer was added to resuspend the cells. 5µL Annexin V-APC and 5µL 17-AAD were gently mixed into the cell
suspension, AnnexinV-APC/7-AAD (KeyGen Biotech) double staining and dark culture of 15min. The apoptosis rate was detected by flow cytometry (FACScan;BD Biosciences). The cells were collected in the same way, under the same conditions. The cells were resuspended with precooled 75% ethanol and then was fixed at 4°C for 2h. The cells were collected by centrifugation and washed once by PBS. The cells were supplemented with 2µl RNase A (2.5mg/ml) and 15µl propidium iodide (PI, 15X) (KeyGen Biotech), and placed at 37 °C to avoid light 30min. The cell DNA content was detected by flow cytometry PI staining. Cell cycle phases were divided into G1/G0 phase, S phase and G2/ M phase, and the proportion of each phase was calculated by special software.

Statistical analysis

Statistical analysis was performed using SPSS 21.0. Data for normally distributed is presented as the mean ± standard deviation. The expression level of LRP16 in the matched tumor and adjacent normal tissues was compared using the paired Student's t-test. Data containing two independent groups were compared using an unpaired Student's t-test. Multiple groups were compared using one-way ANOVA followed by the LSD post hoc test. Pearson's χ2 test was used to analyze the correlation between LRP16 expression and clinicopathological features. P<0.05 was considered to indicate a statistically significant difference.

Results

LRP16 was highly expressed in ESCC tissues and cell lines

In this study, the expression of LRP16 protein in ESCC tissues, corresponding paracancerous tissues and normal esophageal epithelial tissues was studied by IHC. The results showed that the expression of LRP16 was negative in normal tissues (Fig. 1A), but positive in atypical hyperplasia and cancer tissues, and most of them were cytoplasmic positive and few were nuclear positive (Fig. 1B and C). The expression of LRP16 in cancer and atypical hyperplasia tissues was significantly higher than that in normal tissues (P< 0.0167) (Table 2). At the same time, the relationship between LRP16 expression level and ESCC clinicopathological features was discussed. The expression level of LRP16 was not related to sex and age, but related to the depth of invasion, TNM stage and lymphatic metastasis, indicating that LRP16 was involved in the metastasis and spread of ESCC (Table 1).

The relative expression of LRP16 in five ESCC cell lines was studied by RT-qPCR and western blot. TE-1 cell line was used as the reference, and the method was used to analyze and calculate the LRP16 expression. The results showed that LRP16 mRNA expression in TE-1 cell line was relatively high and abundant, which was consistent with the western blot results (Fig.1D and E). Therefore, TE-1 cells with the highest LRP16 expression were selected as the target cell of the later experiment.

Establishment and validation of knockdown LRP16 esophageal squamous cell carcinoma cell line
To investigate the biological function of LRP16 in ESCC, we transfected TE-1 cells with sh-LRP16 plasmid to obtain stable LRP16 knockdown cell lines. The fluorescence expression efficiency of TE-1 cells was very low, about 30% at 48 hours after transfection (Fig. 2A). After G418 screening for four weeks, the fluorescence expression efficiency increased to 85% (Fig. 2B). The morphology of TE-1 cells after transfection was clearly observed under 200x microscope (Fig. 2C, D), and normal growth of TE-1 cells, which proved that G418 was successful in screening stable transformants. It can be used in the following experiment. The knockdown efficiency was detected by RT-qPCR and western blot. The mRNA and protein expressions of the stable transfected LRP16 strain after screening were significantly lower than those of the empty plasmid transfected group and the blank control group, and the LRP16 expression in sh-LRP16#1 and sh-LRP16#2 cells in TE-1 cells were much lower than that in the normal control group and negative control group (Fig. 2E and F). The stable LRP16 knockdown cell line was proved successful and could be used in subsequent experiments.

**LRP16 knockdown inhibits the proliferation and promotes apoptosis of esophageal carcinoma cells**

In order to determine the effect of LRP16 on the proliferation of ESCC cells, CCK-8 was used to detect the proliferation of ESCC cells after knocking down LRP16 gene. The results showed that compared with the control group, the proliferation rate of cells transfected with sh-LRP16#1 and sh-LRP16#2 decreased (Fig. 3A), suggesting that LRP16 knockdown could significantly inhibit the growth of TE-1 cells. Moreover, the results of colony formation assay showed that, compared with the control group, silencing LRP16 could significantly inhibit the proliferation of TE-1 cells and decrease the clustering ability of TE-1 cells (Fig. 3B and C). The cell cycle distribution and apoptosis were detected by flow cytometry. The results showed that the apoptosis of TE-1 cells with LRP16 knockdown increased significantly (**P < 0.01**) (Fig. 3D), the number of cells in G1 phase decreased and the number of cells in S phase increased (**P < 0.01**) (Fig. 3E). The results showed that after LRP16 knockdown, cell apoptosis increased and cells were blocked in S phase, which indicated that LRP16 knockdown promotes cell apoptosis, inhibited DNA replication activity and cell proliferation.

**Knockdown of LRP16 inhibits cell migration**

In order to evaluate the effect of LRP16 on the migratory ability of ESCC cells, we used scratch test to detect the changes of TE-1 cells after silencing LRP16. The results showed that the wound closure rate of negative control cells was faster than that of the experimental group (Fig. 4A and B), indicating that silencing LRP16 decreases the migratory repair ability of TE-1 cells significantly. These results suggested that LRP16 knockdown can significantly inhibited the migration of ESCC cells.

**Knockdown of LRP16 increases the expression of MST1/2 and decrease the expression of YAP1 in Hippo signaling pathway**
LRP16 is a co-activator of ERα. First of all, we detected ERα and ERβ expression in estrogen receptors. The results showed that LRP16 knockdown could significantly increase the mRNA expression of ERα, while the protein expression of ERα was decreased. Silencing the LRP16 gene also reduced the expression of ERβ (Fig. 5A and B). Then, we detected the expression of YAP1 and MST1/2 in Hippo signaling pathway after silencing LRP16 in TE-1 cells. The results showed that compared with the negative control group, silencing LRP16 significantly increased the expression of MST1/2 and decreased the expression of YAP1, suggesting that LRP16 activated the Hippo signaling pathway (Fig. 5C and D).

Discussion

Early detection, early diagnosis and early treatment are important ways to improve the survival rate of ESCC patients. However, as ESCC patients at early stage do not show obvious clinical manifestations, most ESCC patients have entered the progressive stage when first diagnosed, and the therapeutic effect is very poor. Protein is the ultimate functional unit, and it shows that protein molecules can be used as tumor markers for early diagnosis and prognosis evaluation of tumor[26]. For example, alpha-fetoprotein (AFP) can be used as a diagnostic marker of primary liver cancer and prostate specific antigen (PSA) is widely used in the diagnosis of prostate cancer.

LRP16 has a simple structure compared with other members, because its C-terminal region has only an independent macro domain and the protein module is highly conserved[11]. LRP16 has a unique function. It acts as a coactivator of ERα and androgen receptor (AR), and enhances their transcriptional activity in a ligand-dependent manner, thus establishing a positive feedback regulatory loop between LRP16 and ERα / AR[27–29], and is also their target gene. The results showed that the chemical modification sites of LRP16 (such as phosphorylation, glycosylation[30], etc.) involve a variety of biological functions, and its sequence is very similar to the end of human histone H2A1C (a member of the H2AX family). H2AX participates in DNA damage repair and stabilizes chromosome structure. If there is a gene mutation in LRP16, it is bound to induce tumorigenesis[31,32]. Therefore, the researchers inferred that LRP16 protein may be closely related to cell cycle regulation, activation and inhibition of gene transcription, DNA damage repair and tumorigenesis. Studies have shown that in breast cancer, LRP16 overexpression promotes the increase of cyclinE expression, which leads to the transition of cell cycle from G0/G1 phase to S phase, and promotes breast cancer cell proliferation[28]. Testosterone can also activate the response of LRP16 gene promoter in COS-7 cells through AR[33]. In insulinoma, LRP16 overexpression protects MIN6 cells from fatty acid-induced apoptosis by partially restoring Akt phosphorylation and inhibiting nuclear redistribution of FOXO1 (forkhead box O1)[34,35]. Studies have shown that LRP16 is a tumor suppressor gene with low expression in non-estrogen-dependent tumors such as hepatocellular carcinoma. LRP16 can negatively regulate Wnt/β-catenin signal transduction in the progression of hepatocellular carcinoma[17]. Blocking the LRP16-PKR-NF–κB signal transduction axis makes colorectal cancer cells sensitive to cytotoxic therapy for DNA damage[36]. In summary, these results show that LRP16 has varying degrees of abnormal expression in a variety of tumors, and participates in the regulation of a variety of signaling pathways.
The immunohistochemical results showed that LRP16 was related to the depth of ESCC invasion, TNM stage and lymphatic metastasis, suggesting that LRP16 may be involved in the proliferation and metastasis of ESCC. In ESCC and atypical hyperplasia tissues, the expression of LRP16 protein is higher than that in normal squamous epithelium, suggesting that LRP16 is abnormally high expression in the early stage of ESCC, which may be an early tumor marker of ESCC. At the same time, we clarified the basic functional mechanism of LRP16. After shLRP16 transfected into ESCC cells, the expression of LRP16 gene was inhibited, the proliferation of ESCC cells slowed down, cell apoptosis was accelerated, and the cell repair ability was weakened, and the cell cycle changed from G1 phase to S phase. It is speculated that the reason for the change of cell cycle may be DNA double strand break, which prevents the synthesis of DNA. These results suggest that LRP16 can inhibit the cell growth of ESCC. LRP16 gene is also shown to be involved in tumor proliferation and apoptosis in hepatocellular carcinoma, insulinoma and breast cancer. It can be inferred that LRP16 may be a tumor-related gene that regulates a variety of tumors, which is consistent with previous reports[17, 28].

In the exploration of tumor suppressor genes in Drosophila, researchers discovered the Hippo signaling pathway for the first time. Once the Hippo gene is mutated and inactivated, it will lead to the phenotype of overgrowth of Drosophila[19, 37]. The core of this pathway can show a highly conservative posture in different species, and the Hippo signaling pathway also has a similar ability in mammalian tumors[38, 39]. Current studies have shown that the biological functions of Hippo signals are as follows: first, organ size control, by stabilizing the state between cell proliferation and apoptosis. For example, reduce the number of cardiomyocytes by inhibiting Wnt signals, and control the size of the heart[19–21]. Studies have shown that in the state of extremely high density, an increasing number of tight junctions and adhesion junctions between cells will activate LATS1/2, which phosphorylates YAP, and the phosphorylated YAP will enter and remain in the cytoplasm, thus inhibiting the transcriptional expression of downstream genes of YAP, and cell proliferation will be inhibited[18]. Second, it participates in the regulation of cell contact inhibition, which is a mechanism to avoid excessive tissue growth and can maintain the normal tissue of the body. Third, participate in tumorigenesis. So far, the signal pathway mechanism of LRP16 in ESCC is not clear. However, some studies have confirmed that ERα is involved in the Hippo signal pathway[40]. At the same time, studies showed that ERα was also abnormally expressed in ESCC[41]. According to the above theoretical conjecture, we inferred that LRP16, as a co-activator of ERα, may have a specific relationship with ERα and Hippo signal pathway. Our study also confirmed this inference. This study confirmed for the first time that LRP16 can affect the Hippo signal pathway. When LRP16 was knockdown, the mRNA expression of ERα increased, but its protein level did decrease. However, the ultimate unit of executive function was protein, so when the expression of LRP16 protein was inhibited, the expression of ERα protein was still decreased. The possible reasons for the increase in mRNA level are the participation of ncRNA, the post-transcriptional modification of mRNA or the low expression of ERα in ESCC, which leads to a great change in RNA level[41, 42]. Similarly, the expression of ERβ decreased accordingly. The expression of YAP also decreased, while the expression of MST1/2 increased accordingly. It can be inferred that the decreased expression of LRP16 not only affects ERα but also inhibits the activity of downstream key factors YAP, and the inhibited YAP will be phosphorylated and
inhibit the whole Hippo signal pathway. Phosphorylated YAP can’t enter the nucleus, where it participates in apoptosis, inhibites tissue growth and cell proliferation. Moreover, when the expression of MST1/2 is activated, phosphorylated MST1/2 binding regulatory protein SAV1, and then phosphorylated LATS1/2, activated LATS1/2 can also inhibit YAP expression, resulting in cell apoptosis.

In conclusion, LRP16 inhibits the growth and differentiation of ESCC cells, and promotes ESCC cells apoptosis. Moreover, LRP16 is involved in the regulation of Hippo signaling pathway by affecting the expression of key factors in this pathway. However, the specific mechanism of how LRP16 affects the expression of YAP is still unknown, which will be further studied in the future.

Declarations

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Disclosure Statement:

The authors declare no competing interests.

Authors’ contributions

CL and CX designed the study, and CL wrote a manuscript and contributed to the analysis or interpretation of the data. ZR, XL and ZZ participated in the experimental performance. XW provides design for research and data analysis. TS revised the manuscript. As a newsletter author, HL was responsible for the design of the experiment, the revision of the manuscript and the final decision to submit the article for publication, and was responsible for all aspects of the work. All the authors have read and approved the final manuscript.

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**Figures**

**Figure 1**

Expression of LRP16 in tissues and cell lines. (A) Negative staining of LRP16 in normal esophageal tissues (magnification, x200) (negative). (B and C) High expression of LRP16 in adjacent normal tissues and ESCC tissues (magnification, x200). (D) The mRNA expression of LRP16 in five ESCC cell lines was analyzed by RT-qPCR. ***P<0.001. (E) The protein expression of LRP16 in five ESCC cell lines was analyzed by western blotting. LRP16, Leukemia-associated protein 16; ESCC, esophageal squamous cell carcinoma.
Figure 2

Cell fluorescence transfection screening and transfection efficiency detection. (A) Fluorescence expression was observed 48h after transfection (magnification, x40). (B) Fluorescence expression after screening (magnification, x40). (C) Cells under the background of white light (magnification, x200). (D) Cells under the background of fluorescence (magnification, x200). (E) Expression of LRP16 in TE-1 cell lines between blank group and cells transfected with sh-LRP16 or shLRP16 negative control, as
determined via RT-qPCR. (F) Expression of LRP16 in TE-1 cell lines between cells with nothing treatment and cells transfected with sh-LRP16 or sh-LRP16 negative control, as determined via western blotting. Compared with the NC group. ***P < 0.001. LRP16, Leukemia-associated protein 16; ESCC, esophageal squamous cell carcinoma.

**Figure 3**

A

![Graph showing CD value over time](image)

B

![Bar graph showing colony number](image)

C

![Images of petri dishes with different treatments](image)

D

![Histograms of cell cycle distribution](image)

E

![Flow cytometry plots for apoptosis](image)
Downregulation of LRP16 inhibites the proliferation and promotes cell apoptosis in ESCC cells. (A) Proliferation of TE-1 cells treated with sh-LRP16 was decreased as indicated by the CCK-8 assay. (B and C) Cell clone formation assay showed that silencing LRP16 expression significantly inhibited the clustering ability of ESCC cells. Number of TE-1 cells decreased in the transfected groups in which TE-1 cells were transfected with sh-LRP16. ***P < 0.001. (D) Cells were arrested at the S phase after TE-1 cells were transfected with sh-LRP16. Compared with Vector, **, ##P < 0.01. (E) Number of apoptotic cells increased after TE-1 cells were transfected with sh-LRP16, as determined by flow cytometry. LRP16, Leukemia-associated protein 16; ESCC, esophageal squamous cell carcinoma.

Figure 4

Downregulation of LRP16 suppresses the migration of ESCC cells. (A) Cell migratory ability was inhibited when LRP16 was silenced in TE-1 cell lines, as evaluated by wound-healing assays. (B) Bar chart of percentage wound closure. *P<0.05,**P<0.01. LRP16, Leukemia-associated protein 16; ESCC, esophageal squamous cell carcinoma.
Figure 5

Regulation of Hippo signaling pathway by silencing LRP16. (A) The mRNA expression of ERα and ERβ in estrogen receptor after LRP16 silencing of TE-1 cells was analyzed by RT-qPCR. (B) The protein expression of ERα and ERβ in estrogen receptor in LRP16 silenced TE-1 cells was analyzed by western blotting. (C) The expression of YAP1 and MST1/2 in Hippo signaling pathway after silencing LRP16 in TE-1 cells was detected by RT-qPCR. (D) Western blotting analysis of YAP1 and MST1/2 in Hippo signaling pathway after silencing LRP16 in TE-1 cells. ***P<0.001. LRP16, Leukemia-associated protein 16; ESCC, esophageal squamous cell carcinoma.

Supplementary Files

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